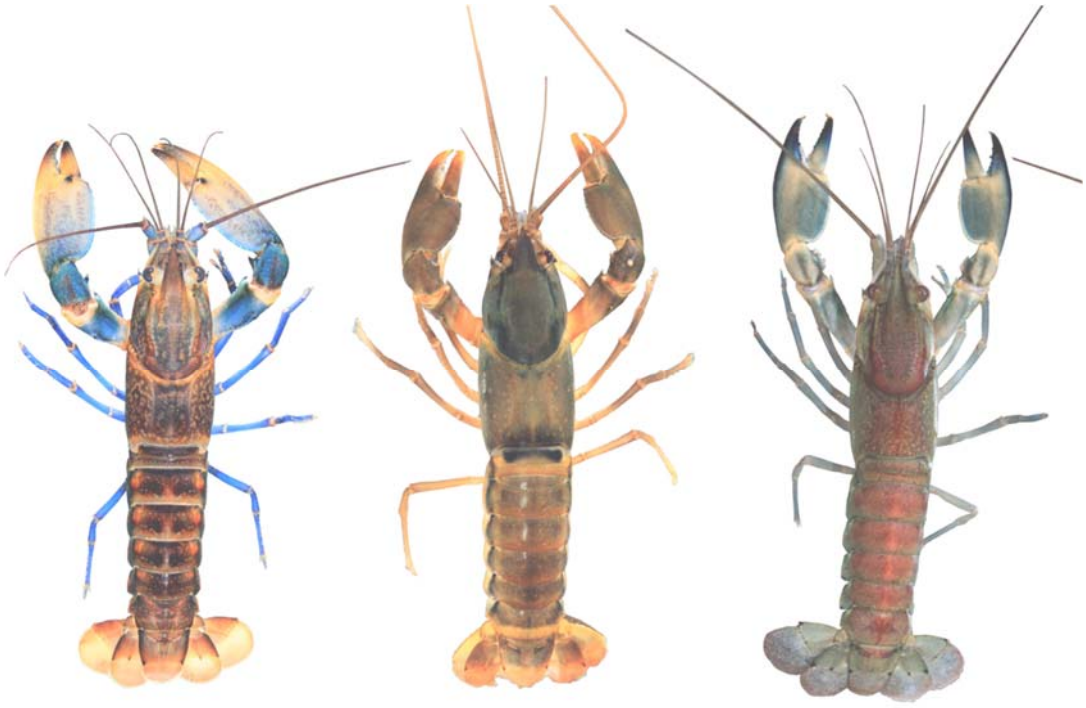
Faculty of Fisheries and Protection of Waters
University of South Bohemia
České Budějovice

The diversity of crayfish and major threats they face

Habilitation thesis



Martin Bláha

Vodňany, 2021

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Appendixes

Supplement 1: Grandjean, F., Tan, M.H., Gan, H.N., Lee, Y.P., Kawai, T., Distefano, R.J., **Bláha, M.**, Roles, A.J., Austin, C.M., 2017. Rapid recovery of nuclear and mitochondrial genes by genome skimming from Northern Hemisphere freshwater crayfish. Zoologica Scripta 46(6): 718-728.

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Supplement 3: Bláha, M., Patoka, J., Japoshvili, B., Let, M., Buřič., M., Kouba, A., Mumladze, L., 2020. Genetic diversity, phylogenetic position and morphometric analysis of *Astacus colchicus* (Decapoda, Astacidae): a new insight into Eastern European crayfish fauna. Integrative Zoology *accepted* doi.org/10.1111/1749-4877.12493

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Supplement 14: Lipták, B., Mojžišová, M., Gruľa, D., Christophoryová, J., Jablonski, D., **Bláha, M.**, Petrusek, A., Kouba, A., 2017. Slovak section of the Danube has its well-established breeding ground of marbled crayfish *Procambarus fallax* f. *virginalis*. Knowledge and Management of Aquatic Ecosystems 418: 40.

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Introduction

The freshwater crayfish are a diverse group of decapod crustaceans. Decapods – including crabs, shrimps, lobsters and crayfish – are among the most species-rich groups of crustaceans, representing approximately 175 families and 15,000 described taxa (extant and extinct). The economic importance of this group, together with their distinctive morphology and ecological diversity, makes decapod crustaceans popular research subjects in all fields of biology (Bracken et al., 2009). The freshwater crayfish are the largest freshwater invertebrates, comprising almost 700 described species (Crandall and De Grave, 2017). Therefore the crayfish compose an important group in the meaning of their ecological role in aquatic ecosystems, being called keystone species in stream communities (Momot, 1995; Parkyn et al., 1997) and flagship species for conservation efforts in highly endangered freshwater habitats (Richman et al., 2015). As large-bodied omnivorous macroinvertebrates, crayfish often represent an important proportion of the biomass of the benthos, feeding on it and also serving as a prey for a range of predators (Hein et al., 2007; Tablado et al., 2010). Crayfish are benthic organisms, they mix and aerate deeper layers of sediments, and increase rates of recycling of macronutrients and micronutrients by bioturbation and faecal production (Covich et al., 1999). Generally, they mediate nutrient and energy flows within (Lipták et al., 2019; Ruokonen et al., 2012) and even between ecosystems as they also feed in riparian and terrestrial habitats (Grey and Jackson, 2012). Beside their ecological role, crayfish have been significant in the social and cultural society of Europe since the Middle ages. They became important in the diet of common people as well as aristocrats and even today people are capturing and consuming them worldwide (Harlıoğlu and Deniz, 2012; Saoud et al., 2013; Taugbøl, 2004).

Freshwater crayfish are represented by two monophyletic superfamilies, Parastacoidea and Astacoidea, inhabiting the Southern and Northern hemisphere, respectively. While superfamily Parastacoidea contains one monophyletic family Parastacidae, superfamily Astacoidea contains three existing monophyletic families (Crandall and De Grave, 2017; Stern et al., 2017). As historically defined, family Astacidae has a disjunct distribution ranging across Europe and west of the Rocky Mountains in North America; the range of family Cambaridae includes North America east of the Rocky Mountains, Central America, and the Caribbean, and the last, recently restored (Crandall and De Grave, 2017; Grandjean et al., 2017) family Cambaroididae has the centre of its distribution in East Asia and Japan (Kawai et al., 2015; Fig. 1). There are two known hotspots for crayfish diversity: one in North America with more than 450 taxa and one in Australia and surrounding islands with more than 150 taxa (Crandall and Buhay, 2007). While Southern Hemisphere crayfish taxonomy, radiation and colonization routes have been subjected to numerous studies and mostly solved (Toon et al., 2010), regarding their Northern Hemisphere counterparts, there the situation is still not fully resolved with several unknowns. Advanced molecular methods, however, have helped greatly during the last decade to shed light on many taxonomically problematic issues in freshwater crayfish and to reveal a lot of new species (e.g. Helms et al., 2015; Mathews et al., 2008; Pârvulescu, 2019). On the other hand, many of them are still waiting to be resolved and discovered (Lovrenčić et al., 2020; Maguire et al., 2014).

European species belong to three genera *Astacus* Fabricius, 1775 *Austropotamobius* Skorikov, 1908 and *Pontastacus* Bott, 1950. According to the most recent updated classification (Crandall and De Grave, 2017), there is a rich taxonomic nomenclature of European crayfish as suggested by earlier studies (Albrecht, 1982; Karaman, 1962; Starobogatov, 1995) with respect to species and subspecies within the genera *Astacus* (three species and two subspecies), *Austropotamobius* (two species and three subspecies) and *Pontastacus* (nine species and one subspecies), respectively. On the other hand,

some of these species and subspecies are defined solely based on morphological traits and/or zoogeography, but have not been tested with modern molecular or morphometric tools.

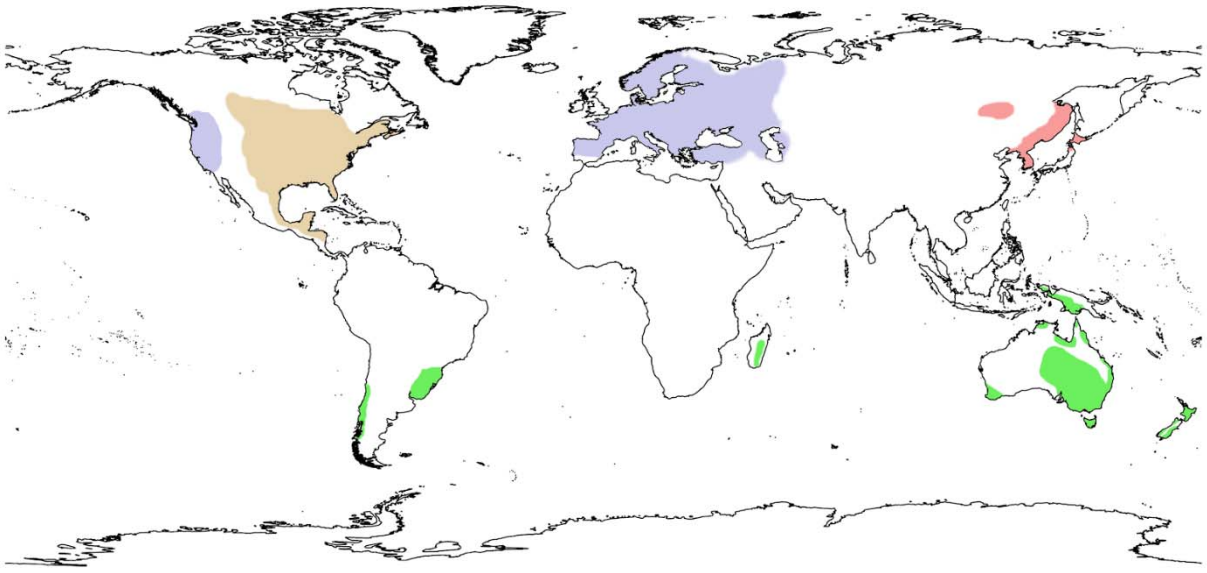

Figure 1. Geographical distribution of freshwater crayfish on the Earth representing two superfamilies Parastacoidea (Southern Hemisphere) and Astacoidea (Northern Hemisphere). Parastacoidea is represented by a single family Parastacidae (green), while Astacoidea is represented by three families. Astacidae (blue), with disjunct distribution of the genus *Pacifastacus* in North America and European species, Cambaroididae (red) and Cambaridae (pale brown).

Knowledge of the phylogeography of the main native European crayfish species has recently made significant progress due to advanced molecular tools, with most of the studies dedicated to *Austropotamobius* species (Jelić et al., 2016; Klobučar et al., 2013; Pârvulescu, 2019; Pârvulescu et al., 2019; Trontelj et al., 2005; Zaccara et al., 2004) and some to the noble crayfish *A. astacus* (Gross et al., 2013; Laggis et al., 2017; Schrimpf et al., 2017; Schrimpf et al., 2014). Although the original studies determined two cryptic species within the white-clawed crayfish *Austropotamobius pallipes* complex: *A. pallipes* and *A. italicus* with three (Grandjean et al., 2002; Grandjean et al., 2000) or four subspecies in the latter (Fratini et al., 2005), respectively, further research resulted in the conclusion that there is only one species *A. pallipes* (Chiesa et al., 2011; Scalici and Bravi, 2012), however with several distinct mitochondrial lineages and strong discordance between mitochondrial and nuclear diversity throughout the species distribution (Jelić et al., 2016). A similar pattern of several distinct lineages was also found in stone crayfish *A. torrentium* (Klobučar et al., 2013; Lovrenčić et al., 2020; Trontelj et al., 2005), however since the phylogroups were recovered on mitochondrial and nuclear DNA, with a lack of any morphological character conserved within lineages, these are most likely cryptic subspecies (Lovrenčić et al., 2020). On the other hand, recently, Parvulescu (2019) has coupled molecular differences with distinct morphological traits and has described a new species of the Idle crayfish *Austropotamobius bihariensis* from the Apuseni Mountains in Romania (Fig. 2), adding another species to the European crayfish species list. The situation with the widespread European species *Astacus astacus* is complicated by the fact that this species was exploited from medieval times and therefore frequently translocated to distant areas (Souty-Grosset et al., 2006), moreover most of its populations became extinct during crayfish plague outbreaks and alternated from populations of different origin.

This fact is underlined by the low haplotype diversity throughout the Europe (Schrimpf et al., 2011), on the other hand, there are areas, especially in Balkan countries, where the diversity is still much higher compared to the rest of Europe (Gross et al., 2013; Laggis et al., 2017; Schrimpf et al., 2017). Obviously, South-eastern Europe served as a glacial refugium also for crayfish.

The other species, namely *Astacus colchicus* and the Thick-clawed crayfish *Pontastacus pachypus* have been just recently subject of this thesis author's molecular and/or morphological study to reveal further information about their relationships and possible history. As such, the last big unknown is the Narrow-clawed crayfish *P. leptodactylus*. In spite of several attempts to reveal its phylogeography and genetic diversity (Akhan et al., 2014; Maguire et al., 2014), the results are limited by the sampling effort to gather most of the area of species distribution seems to be the biggest obstacle. There is an extent morphological variability and also species diversity described earlier (Starobogatov, 1995). In spite of that, the author's preliminary results suggest only one species, i.e. *P. leptodactylus*, with distinct diversity at mitochondrial DNA while nuclear markers express only low variability (unpublished data).

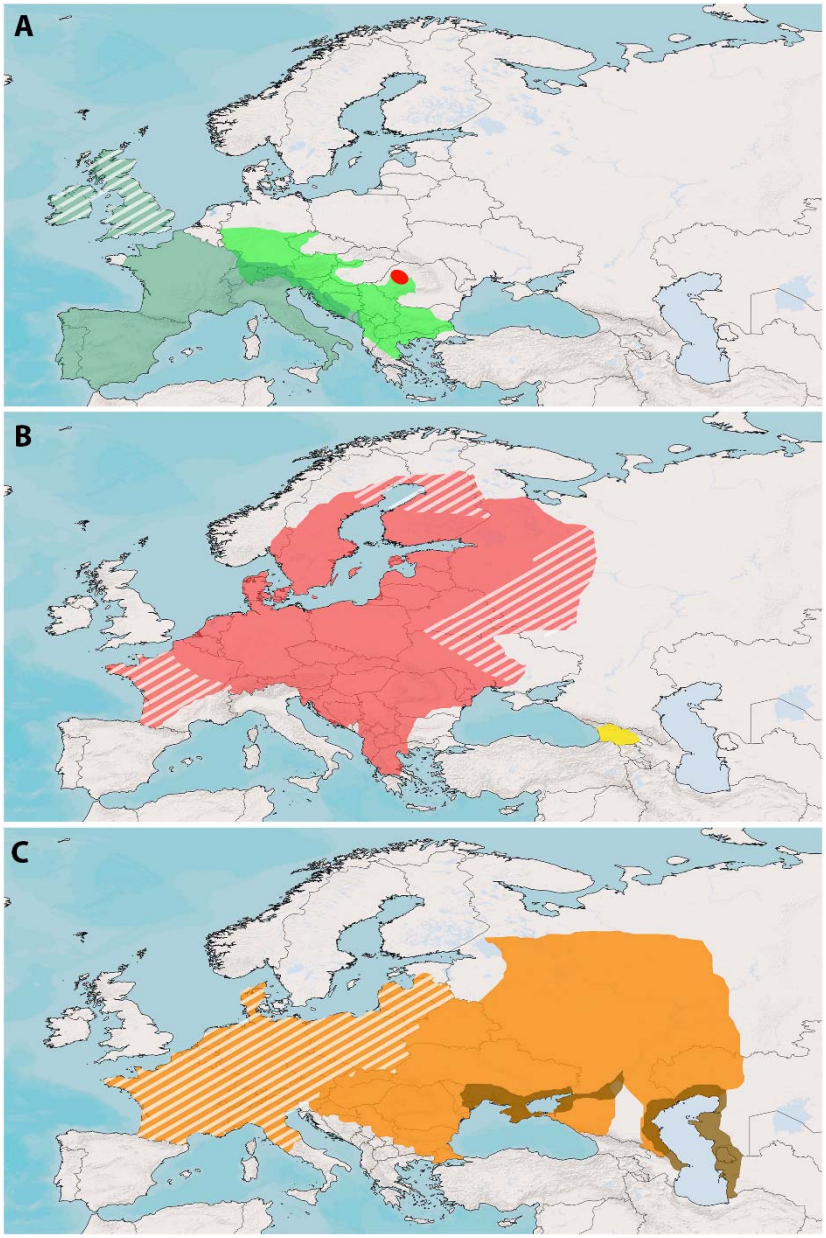

Figure 2. Distribution of European crayfish species. Hatched colour refers to area where species were introduced out of their native range. A) *Austro-potamobius pallipes* species complex (dark green), *A. torrentium* (pale green), *A. bihariensis* (red oval) B) *Astacus astacus* (red), *A. colchicus* (yellow); C) *Pontastacus leptodactylus* species complex (orange) and *P. pachypus* (brown). Based on maps from Kouba et al. (2014).

Throughout the world, crayfish are found in a variety of habitats from lotic to lentic, including cave pools and rivers as well as temporary ponds and estuaries (Holdich et al., 2009). A rapidly growing human population, however, has increased the demand on freshwater resources leading to a freshwater biodiversity crisis (Vörösmarty et al., 2010). Although freshwater ecosystems occupy less than 1% of the earth's surface, they support approximately 10% of the world's species (Strayer and Dudgeon, 2010). Obviously, the value and ecosystem services provided by these systems are enormous. During the last century, the species composition and distribution of freshwater crayfish species in Europe have been drastically changed. The most obvious reasons are alteration and degradation of natural habitats by humans (especially in regions with long traditions in land-use) and also introduction of non-native crayfish species. Several life history traits mainly associated with growth and reproduction (Buřič et al., 2011; Kozák et al., 2007), dominance in direct interactions, and competition for resources including food and shelter (Lele and Pârvulescu, 2017; Vorburger and Ribi, 1999), as well as environmental tolerance, have been identified as the causes of the native species replacements (Lodge et al., 2012).

The most drastic decline of native European species populations has however been caused by an unwanted gift, the crayfish plague pathogen, the oomycete *Aphanomyces astaci*, a pathogen classified among the world's 100 worst invasive alien species (Lowe et al., 2000). This pathogen has become established out of its natural range due to the introduction of the non-native North American crayfish species, following pathways of their spread. North American species are chronic carriers of this pathogen due to long time coevolution in North America, on the other hand crayfish species not originating in North America are highly susceptible to this pathogen and die quickly from crayfish plague, with all populations in contact becoming extinct. During the last decades, as the number of introductions and introduced new species increased, the diversity of this pathogen was represented by five different genotype groups linked to four different non-indigenous crayfish species - NICS (Diéguez-Uribeondo et al., 1995; Grandjean et al., 2014; Kozubíková et al., 2011) and the first genotype group to invade Europe isolated from infected crayfish of the genus *Astacus*, the original host of which remains unknown (Huang et al., 1994). There are still traces of evidence about chronic presence of the crayfish plague pathogen from older outbreaks in the late 19th century in certain populations of native crayfish (*A. astacus* and *P. leptodactylus*) which may have persisted as a chronic infection for several decades in crayfish populations (Makkonen et al., 2012; Panteleit et al., 2018).

The first accidental introduction of the crayfish plague pathogen took place in southern Europe in 1859 (Souty-Grosset et al., 2006). However, the first documented non-native species, the spiny cheek crayfish *Faxonius limosus* (FL) was introduced in the 1890s involving 90 specimens into a 0.1 ha fish farm pond near Barnówko (Berneuchen) in Pomerania, currently in western Poland (Kossakowski, 1966). This seems to be the only introduction from which all spiny cheek crayfish populations in Europe had originated, which also reflects the species' very low genetic diversity throughout European populations, represented by a dominant single mitochondrial haplotype (Filipová et al., 2011). Two other widespread non-native species, the signal crayfish *Pacifastacus leniusculus* (PL), and the red swamp crayfish *Procambarus clarkii* (PC), were brought to Europe several times and in large numbers. More than 100,000 *P. leniusculus* were introduced in the 1960s to Swedish and Finnish lakes as replacements for extinct *A. astacus* populations and around 40,000 *P. clarkii* were introduced in 1973 to south-western Spain, region Extremadura (Henttonen and Huner, 1999; Skurdal et al., 1999; Souty-Grosset et al., 2006). Commercial success of both species led also to illegal introductions into other European countries (Holdich et al., 2009). Ironically, in the time of NICS introductions, the fact that North American species are chronic carriers of crayfish plague pathogen was not known or taken into

consideration. Therefore, despite the original idea to support the disappearing native crayfish species population these introductions added fuel to the fire. Nowadays, all three species, also called 'old NICS', are relatively widespread especially in western and southern Europe (FL, PL, PC) or in Scandinavia (PL) (Kouba et al., 2014) and besides extinction of thousands of native crayfish populations also caused massive ecosystem devastation (e.g. Freeman et al., 2010; Souty-Grosset et al., 2016).

Although old NICS were introduced for aquaculture purposes, the new NICS, introduced after the 1980s, have been introduced mostly for ornamental purposes. From that time, the number of pet traded organisms, including crayfish species, increased rapidly. In fact, the pet industry is one of the crucial pathways for introduction of non-native invasive species globally (Magalhães and Vitule, 2013; Perrings et al., 2000; Putra et al., 2018). On the other hand, it is also a multi-billion-dollar global business bringing some positives such as economic profit for producers and vendors, education of hobbyists, and popularization of the species (Lockwood et al., 2019). Besides North American species of *Procambarus* (*P. acutus*, *P. alleni*) or *Faxonius* (*F. immunis*, *F. juvenilis*, *F. virilis*), even exotic *Cherax* species from New Guinea or *Cambarellus* from Mexico have been found in European freshwaters. The species of *Procambarus* or *Faxonius* originating in temperate zones have acclimatized to European conditions quite well including successful overwintering. However, *Cherax* species survive in conditions of Europe mostly in thermally polluted waters or hot springs (Jaklič and Vrezec, 2011; Weiperth et al., 2019) or even naturally in warmer areas of Southern Europe. Additionally, species of *P. clarkii* and *C. quadricarinatus* present acute threats to Indonesian aquatic biotal diversity, including decapod crustaceans. Among all of these, one species is remarkable by its way of reproduction and ability to survive. The marbled crayfish *Procambarus virginalis* is a triploid reproducing by apomictic parthenogenesis, an unique species among all decapod crustaceans (Lyko, 2017). Therefore, only females are known and just one individual could establish a new population.

This thesis is divided into two parts. The first part summarizes my research focused on phylogenetic relationships and genetic diversity of crayfish, while the second part is focused on threats resulting from non-native crayfish and other aquatic organism introductions and spread. That covers my research focused on phylogenetic relationships and genetic diversity of European and New Guinean crayfish species (summarized in the first part), and possible threats to native species from non-native species introduction and spread of diseases (summarized in the second part). I discuss the effect of translocation on genetic diversity of *A. astacus* populations, and the phylogenetic position and diversity of two native European crayfish species (*P. pachypus* and *A. colchicus*), in which molecular methods were used for the first time to describe them. I also point out the hidden diversity within New Guinean *Cherax* species and, following inferences, and underline the unprecedented threat to hotspot decapod diversity in Indonesian part of New Guinea caused by tardy attitude of authorities to effectively control trade with non-native species of decapods in Indonesia.

Chapter I - Phylogenetic relationships and diversity of freshwater crayfish

Northern Hemisphere crayfish

Although freshwater crayfish have been subjected to a number of molecular genetic studies, there are still issues concerning phylogenetic relationships among major groups within each superfamily. In the case of Northern Hemisphere crayfish, the position of the genera *Cambaroides* and *Pacifastacus* was for a long time somehow defying the general division of higher crayfish taxa, i.e. family Astacidae representing mostly European crayfish and Cambaridae involving North American crayfish (Ďuriš,

2015). Various studies resulted in different positions of these two crayfish genera depending on the number of markers and species used in analysis (Ahn et al., 2006; Braband et al., 2006; Breinholt et al., 2009; Crandal et al., 2000; Sinclair et al., 2004) sometimes producing conflicting results.

Although the genus *Cambaroides* was taxonomically placed in the family Cambaridae, it has often been recovered as sister to species of Astacidae or else in a basal position as sister to other members of Cambaridae from North America. In our study, to clarify phylogenetic relationships within Northern Hemisphere crayfish, we assembled the complete mitochondrial genomes from ten species of European, North American and Asian crayfish. We also used genome skimming to recover complete or near-complete sequences of nuclear 18S and 28S RNA genes and the histone H3 gene from these samples plus data from 11 additional species of crayfish and lobsters were used (**Supplement 1**). The nucleotide-based phylogenetic tree was generated from the robust alignment (＞16 kbp) (Fig. 3). Both Maximum likelihood (ML) and Bayesian inference (BI) trees inferred from this data set implied a monophyletic Astacidae and a polyphyletic Cambaridae, with the split occurring between the North American cambarids (*Procambarus*, *Cambarus*, *Faxonius*) and the Asian cambarids (*Cambaroides*). Further topology testing supported North American cambarids as sister taxa to astacids as most likely. At the time of our study, all results achieved contributed to growing evidence suggesting that the family Cambaridae is non-monophyletic, but contradicted suggestions that the genus *Cambaroides* should be included within the family Astacidae. Several studies using a variety of morphological and molecular data sets from a range of genes and varying taxonomic sampling concur that North American cambarid species and Asian cambarid species (genus *Cambaroides*) do not share a common ancestor (Ahn et al., 2006; Bracken-Grissom et al., 2014; Bracken et al., 2009; Breinholt et al., 2009; Crandal et al., 2000; Porter et al., 2005; Rode and Babcock, 2003). Based on our suggestion the Asian crayfish of genus *Cambaroides* were elevated in a new family Cambaroididae in the revision of the taxonomic classification of freshwater crayfish (Crandall and De Grave, 2017). The most important thing is a basal position for the Cambaroididae which conflicted with the earlier taxonomic classification based on morphological and reproduction-related characters. It will require a reappraisal or reinterpretation of morphological and reproduction-related characters as either ancestral or convergent within the lineages as recovered in our study. It still somehow puzzles the astacologists and to explain and suggest possible scenarios about historical biogeography of Northern Hemisphere crayfish will be a task for future studies combining fossils and molecules to calibrate molecular clocks.

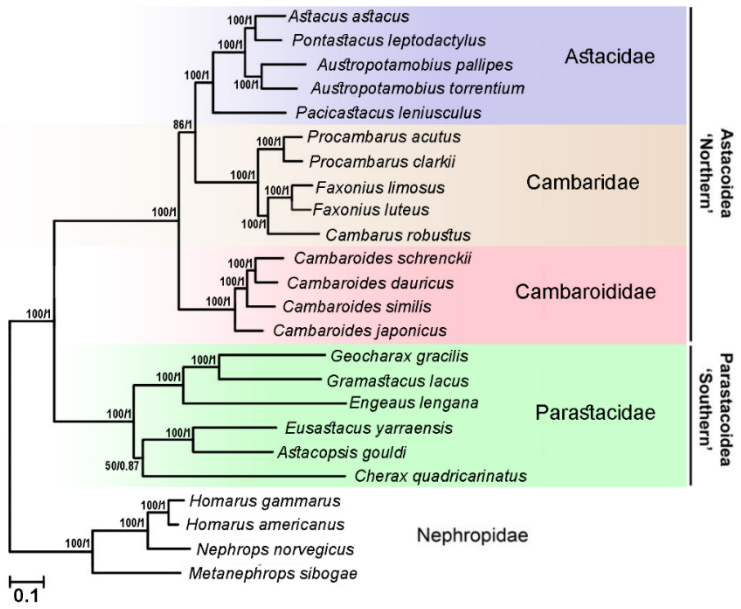

Figure 3. Phylogenetic relationships among Northern Hemisphere freshwater crayfish inferred based on nucleotide alignment consisting of 16 211 sites. Some species scientific names from the original version of figure were adjusted according to current taxonomic nomenclature with also naming Cambaroides species as family Cambaroididae.

After a long time, the currently updated classification of the freshwater crayfishes (Crandall and De Grave, 2017) was an essential work in understanding European species and subspecies which were not mentioned in scientific literature for decades, despite the fact that their morphology and/or zoogeography was distinct enough for individual species or subspecies status (Albrecht, 1982; Brodski, 1981; Karaman, 1962; Starobogatov, 1995). Nevertheless, for some unknown reason, the western astacologists ignored or overlooked the rich nomenclature of mostly Eastern European species for a long time rather than thoroughly testing it. Thus, one might get an impression that there are just five native European crayfish species, *Austropotamobius pallipes* sensu lato (including *A. italicus* with several subspecies), *A. torrentium*, *Astacus astacus*, *Pontastacus leptodactylus* and *P. pachypus*.

Undoubtedly, the less studied European crayfish species are members of the genus *Pontastacus*, due to several reasons. The most obvious one is the long-time limitation in sample access for western astacologists to Russian speaking countries (both species *P. leptodactylus* and *P. pachypus* have their distribution area centred in the Ponto-Caspian region), and also language barriers might play an important role. Even today, there is an evident reluctance in sharing the information, and differences between techniques applied in these studies making them non-comparable with most currently published ones. In fact, all recent east European studies use karyotyping and/or morphology as methods to evaluate diversity and species status of *Pontastacus* (Kostyuk et al., 2013; Mezhzherin et al., 2015). Moreover, *P. pachypus* is the only species known from both freshwater and brackish localities in Ukraine and Russia (Cherkashina, 1999) and is thought to be also the least widespread native crayfish in Europe today (Policar et al., 2018). These facts supported our team in their effort to gather relevant information and signs of positive cooperation from our colleagues from Eastern Europe. The first break-through was a successful research survey across Ukraine in order to find out and confirm selected localities with presumed occurrence of *P. pachypus* (Policar et al., 2018). During this field trip 94 localities with potential occurrence of *P. pachypus* in eight southern and central regions of Ukraine were surveyed. Despite enormous effort, time and money, only four populations of this species were found. In all of them, the species was co-occurring with *P. leptodactylus*, although exploiting different habitats (Policar et al., 2018). Our team had a chance to analyse individuals from two populations in the Dnieper river (**Supplement 2**). Results, based on mitochondrial and nuclear DNA analyses, for the first time suggested that *P. pachypus* and *P. leptodactylus* are related evolutionary lineages. Unfortunately, because of the limited number of populations analysed we could not say much about genetic diversity in general. We recorded only four and three haplotypes at mitochondrial genes (COI and 16S rRNA, respectively), which is a relatively low number compared to the *P. leptodactylus* haplotypes described by Akhan et al. (2014) from Turkey (56 at COI), however, their number varies between one and five among particular sampling sites. Additionally, genetic diversity of *A. astacus* across Europe is even lower, with 30 haplotypes determined (Schrimpf et al., 2014). Low haplotype number might be a consequence of overfishing and very limited use of Ukrainian fishery legislation and regulation in practice. The fishermen were not taking care whether they sold *P. leptodactylus* or *P. pachypus*, especially when the latter composed less than 13% in daily catch (Policar et al., 2018). Moreover, habitat degradation and introduction of the non-native species *Procambarus virginalis,* already recorded in the Dnieper River catchment (Vodovsky et al., 2017), currently lose high risk to all native crayfish species.

The other successful cooperation, with our colleagues from Georgia, resulted in the description of species morphometry, genetic diversity and phylogenetic position of *Astacus colchicus* (**Supplement 3**), the species overlooked for a long time. Up to now, there was almost no available relevant information about this species except for the classical works of Karaman (1962) and Albrecht (1982).

No molecular genetic methods have so far been applied to *A. colchicus*, and no relevant data existed about its genetic diversity, phylogenetic position, and morphometry. Moreover, this species was also assigned as a subspecies of *A. astacus*. Therefore, we carried out the molecular and morphological analysis of this species with two main aims. The first aim was to provide morphological and genetic data for this species, and the second one to describe its phylogenetic position and reveal whether it represents a separate lineage to *A. astacus* or is clustered within *A. astacus* species and thus any morphological differences should be accounted as high intraspecific variability only. Based on morphology, we corroborated that this species belongs to the genus *Astacus*, despite its Ponto-Caspian origin and known occurrence only in Georgia. In addition, the individuals of *A. colchicus* demonstrated also more rounded abdominal somites in comparison to *A. astacus*, which has abdominal somites wedge-shaped. *Astacus colchicus* have well-developed posterior postorbital ridges, approximately 2 times longer than the anterior ones and posteriorly curved inward. Despite a high morphological similarity with *A. astacus*, RDA analysis based on morphometric indices resulted in differences between these analysed species that were highly significant, and several characteristics appeared to be useful for the differentiation from this species, namely abdomen height to the total length (ABH/TL), head length to the total length (HEL/TL), carapace length (rostrum length, head length, areolar length are included) to the total length (CPX/TL), width of the carapace at the hind edges to the total length (CEW/TL), and rostrum length to the rostrum width (ROL/ROW) showing the most obvious differences.

All the combined mtDNA and nDNA phylogenetic analyses recovered sequences of *A. colchicus* comprising a monophyletic clade with high statistical support being a sister clade to *P. leptodactylus* and *P. pachypus*. It is not surprising with regards to the area of species occurrence. Moreover, results also clearly indicated a deep molecular divergence with relatively high molecular distance for particular genes. The mean model-corrected sequence distances among *A. colchicus* and *A. astacus* were at a very similar level to those recorded for *P. leptodactylus* or *P. pachypus.* All of our findings raise a lot of questions, for instance about the species status of another valid species *A. balcanicus*. The species is also morphologically very similar to *A. astacus* and its occurrence is restricted to only a limited area, the Vardar river system in Greece and Macedonia, and Ohrid Lake in Macedonia (Albrecht, 1982, 1983). Although the area of this species has been recently sampled, two new phylogroups were identified belonging to *A. astacus*. However, molecular distances recorded were much lower compared to those recorded between *A. colchicus* and *A. astacus* in our recent study, so not suggesting species level status (Laggis et al., 2017). Moreover, no morphometric study has been carried out on these individuals to tell more about the relationship to *A. astacus*. Thus, there are still gaps in our knowledge about European crayfish species diversity and phylogeography, which hopefully will be filled in the near future.

After the first crayfish plague outbreaks in Europe in the 19th century, drastic declines of native crayfish populations took place, with many of them becoming extinct. Most of the documented lost populations were of *A. pallipes* and *A. astacus*, however no relevant records are available from these times and most likely also *A. torrentium* was affected. Later on, after NICS introductions and further pressures on native crayfish populations either from a NICS itself, from crayfish plague or habitat degradation, so-called ark sites were established to safeguard and maintain genetic diversity of native crayfish species (Kozák et al., 2011; Nightingale et al., 2017; Souty-Grosset and Reynolds, 2009). 'Ark sites' means safe areas protected from NICS and with suitable conditions for long term survival of native crayfish to where part of a wild population is translocated. Most of the documented effort was spent in England regarding *A. pallipes*, while in central Europe and eastern Europe, a more common species is *A. astacus*. Although not always well documented, there is a long tradition in rescuing

threatened populations of *A. astacus* intentionally or even unintentionally. The most striking question is, however, what effect the translocation might have on maintaining genetic diversity of the whole population, with the well-known bottleneck effect (Nei et al., 1975). Without a known genetic structure of source populations and influence of a limited number of transferred specimens, these activities should be considered carefully with respect to the genetic diversity of crayfish in the area of interest. The use of genetically diverse populations for reintroduction to new localities has already been proposed, as well as the repatriation of native crayfish species suggested as an essential part of management and conservation strategies in Europe (Kozák et al., 2011; Schulz et al., 2002; Souty-Grosset and Reynolds, 2009).

Therefore, a pilot project was carried out during 2000 when several promising ponds and brooks were selected as donor and acceptor sites in Czech Republic. Later, crayfish specimens from donor populations were transferred into the ponds. The ponds were stocked with either adult females and males, or with 0+ juveniles. We added into our analysis one more newly established population in a small pool, where the originally stocked individuals were only four berried females in 1988. Moreover, this population underwent restriction to ca. 50 % of adults in 2002, resulting in 170 adult specimens present in the pool. The aims of our study, carried out more than ten years after initial stocking, were to evaluate the successfulness of translocation of particular noble crayfish populations, and to measure the genetic diversity among individuals in translocated populations and make a comparison with the genetic diversity and structure in source populations of noble crayfish (**Supplement 4**). All populations were found stable and surviving, thus from this point of view the translocations were successful. We documented no significant decline in genetic diversity and differences between pairs of source and translocated populations, however significant genetic structure was found among populations that originated from Central compared to Southern Bohemia populations. This is also true for overall observed heterozygosity, the analysed populations from Southern Bohemia showing lower values than usual for central and western Europe, while the source population from central Bohemia including a translocated population originating from there displayed higher levels of heterozygosity. Similarly, very low heterozygosity was found in *A. pallipes* including also high rates of inbreeding (Matallanas et al., 2012), which were documented in all our populations and that strongly contrasted with other noble crayfish European populations (Gross et al., 2013). Most likely the small size of localities and mating of close relatives cause an increase of inbreeding. Reduced effective population size and bottlenecks could affect the number of alleles rather than heterozygote deficiency and could be disguised by genetic drift, caused by the rapid growth of a population after its establishment. Unfortunately, there are no relevant historical records about management in most of the sites with *A. astacus* in the Czech Republic. Therefore, genetic screening should be accomplished in advance when considering any population for conservation purposes in the area of interest. The clear genetic differentiation among populations then suggests a distinct management unit for conservation purposes.

Southern Hemisphere crayfish

Southern Hemisphere crayfish of the family Parastacidae have their biodiversity hotspot in Australia and neighbouring islands, including New Zealand, Tasmania, and New Guinea, where 11 out of 15 genera occurred. The genus *Cherax* comprises a group of moderately burrowing crayfish species that is most widespread across Australia and southern New Guinea, accounting for 54 species (Crandall and De Grave, 2017; Lukhaup et al., 2018). Currently, 26 crayfish species have been scientifically described from New Guinea, the vast majority of them from the Indonesian part of the island. Just two of them,

C. quadricarinatus and *C. rhynchotus,* also occur in Australia, and the others are truly endemic to New Guinea (Patoka, 2020). When the pet trade with crayfish started in the 1990s (Chucholl, 2013), certain *Cherax* crayfish native to New Guinea, were exploited for ornamental purposes in those years (Chucholl, 2013; Papavlasopoulou et al., 2014; Patoka et al., 2014). The vast majority of these crayfish were field captured and exported by Indonesian wholesalers into the European, US and Japanese pet markets (Lukhaup and Herbert, 2008; Patoka et al., 2015). Unfortunately, some of these species were scientifically undescribed and advertised under misnomers or trade names only (Chucholl, 2013; Patoka et al., 2014). Moreover, the population status and trends of New Guinean crayfish species are not known, so a potential decline of abundance because of intensive capture can be easily overlooked.

The pet trade was therefore the way through which our team gathered the first suspicious *Cherax* specimens, which were not matching currently known and scientifically described species. At that time, it was only through descriptions by the German crayfish hunter and photographer Chris Lukhaup, who described species from New Guinea partly found in old museum samples, but mainly from the pet trade (Lukhaup, 2015; Lukhaup and Herbert, 2008; Lukhaup et al., 2015; Lukhaup and Pekny, 2006, 2008). However, all these descriptions are based solely on morphology and not using analysis of at least the most common and widely used COI gene to barcode new species. Thus, we decided to also include identification using molecular methods. After a detailed morphological survey, we identified at least three undescribed species, which resulted in description of two new species *C. gherardii* and *C. subterigneus* (**Supplement 5 and 6**). These descriptions were subjected to strong discussions with Chris Lukhaup due to an unknown type locality, only roughly estimated based on communication with local wholesalers. He was not reluctant to contact the editor of the journal we submitted our description of *C. subterigneus* to and insisted on stopping the review process for the reason mentioned above. In the meantime, he published his own description of this new species under the name *C. snowden*.

Our motivation for publishing species description without precise knowledge of type locality was clearly practical, to have scientifically described species, easily identifiable by wholesalers and local hunters, to prevent wrong identification and using misnomers in pet trade markets. In fact, all possible species protection activities could be applied only when the species is clearly named, identified, and scientifically described. *Cherax subterigneus* was found to be a junior synonym of *C. snowden* because published concurrently. This species is the most similar to *C. holthuisi* and can be distinguished using sequence divergence, and morphologically by the body and chelae colour (in live individuals), narrow gap between the fingers when closed, and rows of setose hairs present on dactyl and fixed finger of the chela. *Cherax gherardii*, the second newly described species was found to be most similar to *C. boesemani*, and can be distinguished using sequence divergence or by coloration; chelae shape; position and colour of the uncalcified patch on the outer margin of chelae of adult males; rostral reaching; and large teeth on propodal cutting edges. Nevertheless, it has been shown recently that *C. boesemani* sequences from the type locality (Lukhaup et al., 2017) are different from many sequences assigned as *C. boesemani* stored at NCBI databases GenBank (Supplement 8). This dismal situation is however quite common regarding not only *Cherax* species, when molecular techniques reveal very often the existence of more species being morphologically hardly indistinguishable. In this case, the fact that numerous species descriptions in the past were performed before the molecular definition of type specimens was possible further complicates species assignment. The morphological characteristics mentioned were not powerful enough to enable distinguishing between the *C. boesemani* and other very similar specimens, genetically completely different as shown later by another of our studies (Supplement 8).

Considering all the above, we decided to carry out phylogenetic analyses of recently described *Cherax* species including also accessible sequences from GenBank database (**Supplement 7**). We used the advantage of access to pet traded *Cherax* specimens. Our aim was simple, to assign sequences to recently described species determined based on morphological description and obtain a basic frame for relationships within New Guinean crayfish species. There was already information about the existence and relationship of three main phylogenetic groups of *Cherax* species referring to their geographical area of distribution (Munasinghe et al., 2004), however with very little of New Guinean species included. We corroborated the existence of these three groups and found two highly supported lineages with the Northern group of *Cherax* species, one containing species occurring also in Northern Australia and the other with species strictly occurring in New Guinea (Fig. 4). For quite a long time, two subgenera were recognized within *Cherax*: *Astaconephrops* and *Cherax* (Holthuis, 1996; Holthuis, 1949). The distinguishing characteristics were well-developed rostral and sometimes also median carinae and shape of scaphocerite, but the most apparent was the presence of an uncalcified patch on the male chelae (Holthuis, 1949; Lukhaup and Pekny, 2008). We pointed out non-monophyly of species placed within the subgenus *Astaconephrops*, which resulted, together with subjective assessment of particular morphological characteristics, in omitting usage of the subgenera. This was already suggested by Davie (2002) and Ahyong (2014) and later on reflected by Crandall and De Grave (2017) taking into account molecular results of our study in an updated classification of freshwater crayfish. Our study, together with the most recent one (**Supplement 8**) documented unrevealed diversity in New Guinean crayfish. In spite of the increasing number of newly described *Cherax* species (summarized in Patoka, 2020), there are still lots of sequences in the GenBank database without appropriate species assignment and obviously belonging to as yet scientifically undescribed species. When looking for more information, most of them originated from specimens found in the street market in Sorong, the biggest town in the west part of New Guinea, called Bird's head (Eprilurahman, 2014). These markets are a hub for local hunters and fishermen to sell their catches from much larger areas. Moreover, the crayfish in these markets are only a part of the amount of crayfish traded by wholesalers into European countries, US, and Japan as well (Chucholl, 2013; Patoka et al., 2015).

In recent years, various monitoring programs including astacological surveys have yielded valuable distributional data on non-native crayfish species all around Europe (e.g. Jaklič and Vrezec, 2011; Maguire et al., 2018; Weiperth et al., 2019; Zorić et al., 2020). Regarding the *Cherax* species, the most interesting is Hungary, where these crayfish are found in open waters. Indeed, Hungary has plenty of thermal springs and waters to host warm water crayfish and other decapod species (Gál et al., 2018; Seprős et al., 2018; Weiperth et al., 2017). During routine monitoring in 2019 and 2020, five *Cherax* species were recorded in Varosliget pond in Budapest. The most numerous was *C. quadricarinatus* (almost 30 individuals caught), while only several individuals of *C. holthuisi* and *C. snowden* were found. These species have distinctive morphology and species were confirmed by molecular analysis as well. On the other hand, the remaining individuals recorded there shared the habitus of species resembling *C. boesemani* and *C. pulcher*, however none of them matched the sequences of *C. boesemani* from the type locality recently published by Lukhaup et al. (2017), moreover, according to species delimitation analysis and molecular divergences it is likely that neither of these crayfish belongs to *C. pulcher*. Thus, each belongs to a different, as yet scientifically undescribed species. It only underlines the high unrevealed diversity of *Cherax* species in New Guinea and the need of revision and definition of new suitable morphological characteristics that could be helpful for species identification (Patoka, 2020).

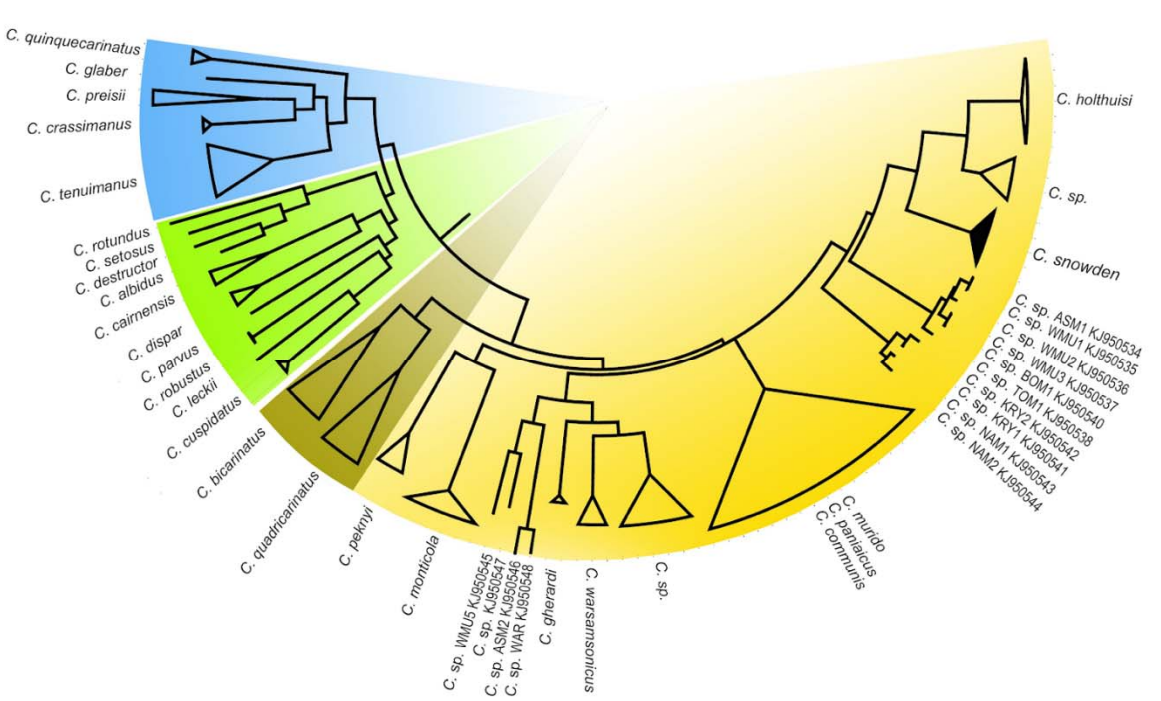

Figure 4. Phylogenetic tree depicting the relationship of *Cherax* species based on COI sequences. The background colour refers to different geographical areas corresponding to main phylogenetic lineages. South-Western group (blue), Eastern group (green), Northern group with species occurring in New Guinea and Northern Australia (pale brown), and with species occurring only in New Guinea (yellow).

Our previous species descriptions were based on specimens found in the pet trade, which clearly limit their potential. Therefore, after almost two years of preparation and careful planning, we carried out our first expedition to New Guinea. At the beginning of this story was a routine google search for *Cherax* pictures by my colleague Jiri Patoka which that day resulted in a picture of "weird" white crayfish from a habitat resembling a cave and a reference to a local guide offering tours to a small village near a cave in the vicinity of Wamena town. At the end of the story, two years later came the discovery and description of the first cave-dwelling crayfish *C. acherontis* found in the Southern Hemisphere, in the submerged river Yumugima in Hagepma/Jugurama cave in the New Guinea Highlands (**Supplement 9**). To date, truly cave-dwelling (troglobitic) crayfish species were found only in North America and Cuba, all belonging to the family Cambaridae, with around 45 described species (Crandall and De Grave, 2017; Stern et al., 2017). Regarding specific habitat, the troglobitic crayfish are characterised by morphological traits such as long and slender claws, reduced eyes, loss of body pigmentation, and long antennae (Hobbs Jr et al., 1977). The same characteristics were also found in *C. acherontis*, especially long and thickened third maxillipeds provided with dense and usually filtrating setae and long and slender claws. On the other hand, eye pigmentation was still somehow retained, however the size of the cornea was reduced in comparison to other *Cherax* species. It could mean that either the expansion to the cave habitat could be relatively recent or that there are places in the underground river where light still enters the cave system through some holes or even the river flows overground. We found plastic garbage in the stream bottom in the cave suggesting the latter option. In comparison to all New Guinean *Cherax* species, the new species is most similar to *C. monticola* and differs from this species especially by body and chelae colouration of live individuals, length and width of rostrum, longer and narrower chelae, absence of soft uncalcified margin of chela in adult males, and the longer third maxilliped and second pereiopod. In spite of our huge effort to find more crayfish species in the area of highlands around Wamena town, we identified only *C. monticola* here, and based

on literature there should also be another species inhabiting small creeks, *C. minor* (Holthuis, 1996). It should be highlighted that the very high diversity of *Cherax* species in New Guinea is centred mostly in western parts of the island called Bird's head. On the other hand, the habitats in the south of the islands, hardly accessible and covered by tropical forest, might harbour another yet scientifically undescribed species.

Chapter II - Diversity of crayfish under threats

After the rapid expansion of the pet trade in the 1990s, at present about 30 crayfish species are sold relatively frequently and kept in aquaria in various countries (e.g. Faulkes, 2015a; Chucholl and Wendler, 2017; Vodovsky et al., 2017). The presently listed pet traded crayfish species include those harvested from the wild, such as *Cherax* crayfish from New Guinea (Lukhaup et al., 2017; Lukhaup et al., 2015; Patoka et al., 2015), as well as species cultured exclusively for aquaria, such as *Procambarus virginalis* (Faulkes, 2015b). On the other hand, certain crayfish exploited for human consumption are also rapidly growing in popularity as ornamental species, especially *C. destructor*, *C. quadricarinatus* and *P. clarkii* (Patoka et al., 2016; Souty-Grosset et al., 2016). Indonesia has already been identified as the leading supplier of ornamental crayfish (Faulkes, 2015a; Patoka et al., 2015), which are exported mainly to Europe, East Asia, and North America. Although the importation of *P. clarkii* to Indonesia is banned by national regulation, which prohibits the import of hazardous fish species into the territory of the Republic of Indonesia, its culture and transport within the country are legal. Moreover, the likelihood of escape or release of *P. clarkii* into the wild increases because of the general ignorance of the threat of biological invasions among Indonesian policymakers: at present, there is no regulation of the breeding, handling, or release of *P. clarkii* by farmers, hobbyists, or the general public. It is also worth mentioning that Indonesia provides generally favourable climatic conditions across the entire country, which may facilitate the establishment of many alien crayfish species, including *P. clarkii*.

The aim of our study (**Supplement 10**) was therefore obvious, to highlight the potential threat that *P. clarkii* may pose to Indonesian freshwater diversity. We assessed the availability of *P. clarkii* in Indonesian pet shops and aquaculture, and additionally individuals obtained during the survey were tested for the presence of the crayfish plague pathogen. The main pet shops in Jakarta, which can be considered the hub of the ornamental pet trade in Java (and Indonesia in general), and in the nearby large city of Bogor, were surveyed for the sale of North American crayfish, and for *P. clarkii* in particular. We also inspected several ponds used for fish and crayfish farming situated near Pasir Angin village, Cisaat Subdistrict, Java. The area harbours many interconnected water bodies such as paddy fields, drainages, and brooks, and the potential threat became very real. *Procambarus clarkii* was found in all pet shops visited and in the public aquarium, as well as in one of the three surveyed street markets. Based on answers from the shop owners, all crayfish were produced locally. The semi-intensive culture of *P. clarkii* in ponds near Pasir Angin village started in 2007, and can be considered as well developed, with an estimated yield of approximately 1 t/ha. Many captured adult females were ovigerous. Moreover, we found the invasive shrimp *Macrobrachium lanchesteri* and the native crab *Parathelphusa convexa* occuring together with ornamental fish in ponds where *P. clarkii* were also cultured. The most alarming was that the pathogen causing crayfish plague *Aphanomyces astaci* was recorded in one of the pet shops in Jakarta, and in the outdoor population in ponds connected with Cilegok brook. In addition, both decapod species occurring there with *P. clarkii* were also positive for *A. astaci* presence. It is most likely that this crayfish will be released by hobbyists and farmers

intentionally for further exploitation at new localities. Taking into account that *P. clarkii* can be legally transported within Indonesia, it could be easily introduced into neighbouring islands and in the worst case also to the Indonesian part of New Guinea. It would be a disaster for the biodiversity hotspot of *Cherax* crayfish there. Nevertheless, other decapod species, which in this case served as crayfish plague pathogen carriers, could spread the disease into much broader areas where it could cause economic losses if they meet the aquaculture of *C. quadricarinatus*. Moreover, the sensibility of other decapod crustaceans to crayfish plague is not known, only being tested on limited taxa (Svoboda et al., 2017). The local authorities should consider a total ban of *P. clarkii* and other crayfish species of North American origin in Indonesia. This should be a feasible way of protecting the rich Indonesian biota, particularly its indigenous freshwater crustaceans.

The main target species of freshwater crayfish culture in Indonesia is *C. quadricarinatus*, which is farmed and harvested both in natural lakes and rivers, and in artificial ponds and reservoirs (Patoka et al., 2018). This crayfish is produced both for human consumption and for ornamental purposes (Patoka et al., 2016), but as pointed out above *C. quadricarinatus* is susceptible to crayfish plague (Svoboda et al., 2017), and therefore its culture could be dramatically affected by the spread of the pathogen. Although *C. quadricarinatus* has been previously introduced for aquaculture into numerous countries, especially in tropical or subtropical regions (Ahyong and Yeo, 2007; Kouba et al., 2014; Lodge et al., 2012), it has its native range in the Southern part of New Guinea and the Northern part of Australia (Bláha et al., 2016; Munasinghe et al., 2004). Therefore, the species distribution west of the Wallace line make it non-native in areas out of New Guinea, although still in Indonesia. In fact, there were two established populations in Indonesia west of the Wallace line recorded in 2016 (Patoka et al., 2016). Therefore one of our aims during the expedition to Indonesia and New Guinea in 2017 was to investigate the current distribution of *C. quadricarinatus* in several Indonesian islands, with the associated goal of providing a starting point for future management actions to be implemented (**Supplement 11**).

Populations of *C. quadricarinatus* were found in all 35 surveyed waterbodies in Batam and Bintan Islands (Riau Archipelago), Java and Kalimantan (Borneo) inhabiting various habitats including natural lakes and rivers, and also artificial ponds and reservoirs usually in very high densities above hundreds of adults including ovigerous females. *Cherax quadricarinatus* seems to be popular for exploitation in Indonesia and, because release of this crayfish species into the wild is not illegal in the country, without effective legislative measures against non-native crayfish introductions in Indonesia, more and more waterbodies will probably be used for its culture (Fig. 5). As a consequence, more unintentional escapes can be expected. Unfortunately, local people have very poor knowledge of the risks of invasive species. This is a highly alarming scenario, given that the region contains prominent global biodiversity hotspots, such as Sundaland and Wallacea (Myers et al., 2000). As eradication of established crayfish populations is practically impossible, further education of the general public seems crucial for prevention of new introductions of *C. quadricarinatus* and other exotic crayfish species in the area. Therefore, active measures implemented by wildlife managers and national policymakers are urgently and strongly recommended to address this crayfish invasion.

Figure 5. Net cages used for the culture of ornamental fish and crayfish in Kemang Lake, Java, Indonesia.

The keeping of non-native species for ornamental purposes is a world-wide issue (Duggan, 2010; Maceda-Veiga et al., 2016; Padilla and Williams, 2004). The trend has been for this hobby to accelerate in recent decades within Europe, including Czech Republic (Patoka et al., 2015; Peay, 2009; van der Velde et al., 2002). The parallel increases in species quantity and availability on the market lead to high propagule pressure (Duggan et al., 2006). Therefore, in our survey (**Supplement 12**), we focused on garden pond vendors, and especially on their awareness and responsible behaviour, investigating the hypothesis that these vendors constitute a risk associated with the introduction of non-native invasive species. Almost one quarter of commercial garden pond architects and builders in the Czech Republic were surveyed. Among the animal species offered by vendors for keeping in garden ponds, 26 were vertebrates (24 fishes, two frogs) and nine were invertebrates (three crayfishes, three bivalves, and three gastropods). Among them, for instance, two species of bivalves (*Anodonta anatina* and *A. cygnea*) as well as two species of frogs *Rana temporaria* and *Bombina bombina* were sold illegally and two latter even caught illegally according to our laws (Act No. 114/1992 Coll, n.d.). Three crayfish species were of North American origin, i.e. the potential crayfish plague carriers, *Faxonius limosus*, *P. virginalis* and *P. clarkii*. At the time of the study, just two of these species had established populations in the wild, able to survive winter conditions of Central Europe. The problem described in that case study could even be worse, taking into account that many vendors do not always publicize offerings of "problematic" species. Additionally, some of the species could be easily misidentified by the pet retailers due to lack of experience. All sales to garden ponds should be accompanied by educational material and warnings to hobbyists about the dangers of releasing non-native species. Given that escape from garden ponds is considered one of the main pathways for introducing freshwater organisms globally (Copp et al., 2007; Leuven et al., 2009; Lodge et al., 2000), banning the stocking of potential invaders in outdoor reservoirs, including garden ponds, and strict enforcement of laws prohibiting the illegal capture and sale of native species, are essential for the conservation of native aquatic biota.

The intentional or unintentional releases are most likely behind the occurrence and further spread of several crayfish species through Europe. This is especially true for *P. virginalis*. This species, with a unique reproduction strategy among decapod crustaceans, obligatory parthenogenesis, together with low intraspecific aggressiveness, a very short generation time of less than 6 months, and high fecundity, frequently shows population explosions in a very short time (Scholtz et al., 2003; Vogt et al., 2004). The crayfish has been reported in the wild in several European countries (Germany, Slovakia, Austria, Ukraine and others) and it could be expected that most of them are still unrecognized. This species is very popular among aquarium keepers due to its unique way of reproduction, however it is also the sticking point of intentional releases when the crayfish literally fill the aquarium with its clones. We expect such a scenario and surveyed several promising places in conurbation areas and their surroundings to finally confirm *P. virginalis* population in Prague and in the vicinity of Bilina (**Supplement 13**) as well as in Bratislava, Slovakia (**Supplement 14**). Although the sites in Czech Republic were almost isolated, the side arm in Bratislava is connected with the Danube river. Given the role of crayfish in ecosystems in general and characteristics of marbled crayfish in particular, the spread of marbled crayfish has the potential for significant consequences for a much broader range of taxa. This is a serious issue since the Danube possesses habitats for diverse biota, being a unique ecosystem of European importance. To illustrate the seriousness of this threat, out of 39 adult females caught, 27 were carrying eggs or juveniles of 1st or 2nd instar. Altogether, these berried females carried 11348 offspring. Any attempts to eradicate this marbled crayfish population are likely to be ineffective because of its obligate parthenogenetic reproduction mode, when even a single survivor may re-establish a whole population. Its remarkable characteristics mentioned above suggest that the marbled crayfish will become a permanent part of the Danube ecosystem, with great potential for an extension of its range, with largely unknown consequences so far. Besides this species we also identified the first established population of the North American species *Cambarellus patzcuarensis* in Europe, in Hungary, Budapest (**Supplement 15**), most likely intentionally released from aquaria. In contrast to *P. virginalis*, *C. patzcuarensis* is a warm water species, and was found in one of many thermal ponds in Budapest. Although several adult individuals were also caught in the Danube river close to the outflow from this thermal pond, its presence here was most likely enabled by the still suitable temperature similar to that in the thermal pond. Its spread downstream Danube river is questionable since its tolerance to low winter temperature is most likely minimal, but we propose this species to the attention of conservationists, wildlife managers and policymakers of European countries. Moreover, the abundance of *C. patzcuarensis* in aquaria may increase in the future because it is usually offered by pet shop owners to replace the recently banned, and previously the most traded and kept crayfish, *P. clarkii* and *P. virginalis* in the European Union (Regulation No. 1143/2014). Occurrence of some NICS in European waters, including *P. virginalis*, is entirely driven by propagule pressure in relation to the pet trade, and crayfish are usually released into the nearest ponds or streams in the vicinity of conurbations. Our observations support this view. In the light of this finding, the indigenous crayfish species in Europe are now outnumbered more than two-fold by non-indigenous species.

Conclusions and future perspectives

- Our research contributed to reappraisal of the taxonomic system of freshwater crayfish to elevate the representative of the genus *Cambaroides* into a new family Cambaroididae as well as to omit the usage of the subgenera *Astaconephrops* and *Cherax* within the genus *Cherax*. Moreover, in regard to the former, we assembled the complete mitochondrial genomes and used genome skimming to recover nuclear genes from almost 20 species of crayfish and lobsters.

- For the first time, we described the phylogenetic position of *Pontastacus pachypus* and *Astacus colchicus*, suggesting that *P. pachypus* and *P. leptodactylus* are closely related evolutionary lineages, and providing detailed morphometric studies to show *A. colchicus* as a species related to *Astacus* in spite of its Ponto-Caspian area of distribution.

- Successful repatriation procedures with *Astacus astacus* were documented after one decade from the original attempt. Although populations were established using a very limited number of individuals, microsatellite markers and comparison with donor populations showed that genetic diversity was not significantly affected. On the other hand, a small level of heterozygosity was found in all tested populations in comparison to central and western European populations of *A. astacus* resulting most likely from shrinking size of populations of this native species.

- We brought to science description of three new *Cherax* species, including the first documented cave dwelling crayfish species in the Southern Hemisphere. Furthermore, we pointed out unrevealed diversity in New Guinea *Cherax* species and the necessity of proper usage of morphological characteristics in combination with molecular methods to clearly identify all possible species.

- The threats resulting from the international pet trade and crayfish aquaculture were highlighted especially in connection with native decapod fauna in Indonesia and Europe. In particular, *Procambarus clarkii* and *Cherax quadricarinatus*, being out of their native range of occurrence, negatively affect local biota, moreover the former could transfer the crayfish plague pathogen which is fatal for all crayfish not originating in North America.

- We showed that intentional or unintentional releases are most likely behind the occurrence and further spread of several crayfish species through Europe, *Procambarus virginalis* in particular. Established populations of this species were found near to conurbation areas as well as other species originating in the ornamental trade, further corroborating our hypothesis. We identified five *Cherax* species together with *P. clarkii* and *P. virginalis* in one thermal pond system in Budapest, while *Cambarellus patzcuarensis* was found to have established a population in another thermal pond system there. In the case of the latter one, it was the first established population outside North America documented. The indigenous crayfish species in Europe are now outnumbered more than two-fold by non-indigenous species.

We will continue our researches of *Cherax* species occurring in New Guinea; the situation of some species described solely based on morphology is puzzling and a revision of the species and their morphology is essential. Furthermore, there is still a certain amount of undescribed species in the pet trade calling for scientific description. We would like to realize more expeditions to Indonesia and New Guinea to study the remarkable crayfish fauna in situ. There are still gaps in understanding historical

biogeography of Northern Hemisphere crayfish. With the help of molecular methods and fossil records we would like to disentangle and suggest a possible scenario of historical crayfish pathways. Regarding European crayfish species, there is one crayfish species which despite a huge distribution area still hasn't been properly analysed despite the number of populations from much larger areas covering the whole species distribution range. We are finalizing the molecular analyses of *Pontastacus leptodactylus* to be able to soon publish these interesting results. Nevertheless, it might sound like a challenge to describe the fine population structure of *Astacus astacus* across Europe with the gradient from Southern Balkan countries to Scandinavia in the north; this could help to identify high diversity spots for further management and protection activities of this disappearing native species. In cooperation with other colleagues from Croatia, France, Germany, Estonia and Sweden, I am confident we could carry out this study.

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Supplement 1

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Rapid recovery of nuclear and mitochondrial genes by genome skimming from Northern Hemisphere freshwater crayfish

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Molecular phylogenetics has benefited tremendously from the advent of next-generation sequencing, enabling quick and cost-effective recovery of whole mitogenomes via an approach referred to as 'genome skimming'. Recently, genome skimming has been utilised to recover highly repetitive nuclear genes such as 18S and 28S ribosomal RNA genes that are useful for inferring deeper evolutionary relationships. To address some outstanding issues in the relationships among Northern Hemisphere freshwater crayfish (Astacoidea), we sequenced the partial genome of crayfish species from Asian, North American and European genera and report the successful recovery of whole mitogenome sequences in addition to three highly repetitive nuclear genes, namely histone H3, 18S and 28S ribosomal RNA. Consistent with some previous studies using short mtDNA and nuclear gene fragments, phylogenetic analyses based on the concatenation of recovered mitochondrial and/or nuclear sequences recovered the Asian cambarid lineage as basal to all astacids and North American cambarids, which conflicts with the current taxonomic classification based on morphological and reproduction-related characters. Lastly, we show that complete H3, 18S and 28S ribosomal RNA genes can also be consistently recovered from a diverse range of animal taxa, demonstrating the potential wide utility of genome skimming for nuclear markers.

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Introduction

Estimation of evolutionary relationships among organisms using DNA sequence information is now an established part of comparative biology due to the PCR and automated Sanger sequencing revolution since the late 1990s (Hillis *et al.* 1996; Avise *et al.* 2000). Early in this phylogenetic revolution, most studies utilised sequences of single mitochondrial genes such as the 16S ribosomal RNA, cytochrome b, cytochrome oxidase I or nuclear genes especially the 18S ribosomal RNA. Recent studies have increasingly used nucleotide data from multiple mitochondrial genes and several nuclear genes, resulting in larger data sets commonly in the order of 5000–10 000 bp. The development of next-generation sequencing (NGS) technology has led to rapidly declining costs for DNA sequencing and shows promise in producing data sets comprising of hundreds, if not thousands of loci or characters.

Assembling data sets using PCR-based methods presents several challenges especially with the increasing expectations that multiple loci are required for robust phylogenies. This amounts to thousands of base pairs and is often coupled with the need for adequate taxon samples, requiring up to and even in excess of 100 samples. These challenges become amplified with the use of museum-held specimens, which have many advantages in relation to supporting biodiversity-related and phylogenetic studies (Thomas *et al.* 1990; Graham *et al.* 2004; Suarez & Tsutsui 2004; McCormack *et al.* 2015). Tissue samples from museum specimens are often limited in volume and are usually characterised by highly degraded DNA requiring multiple rounds of short amplicon sequencing often with low success rate (Andersen & Mills 2012; Tin *et al.* 2014; Aznar-Cormano *et al.* 2015; Li *et al.* 2015; McCormack *et al.* 2015). The use of next-generation sequencing rather than Sanger sequencing can reduce the cost of this approach, often referred to as Targeted Amplicon Sequencing (Bybee *et al.* 2011). Nevertheless, this does not overcome the time and cost of multiple and failed PCR reactions which will be common for degraded samples, and also the bioinformatics workload of assembling and aligning data from multiple short fragments (Meimberg *et al.* 2016).

With high-throughput sequencing, it is now possible to sequence the genome of eukaryotic organisms for a few thousand dollars in a matter of weeks (Goodwin *et al.* 2016). However, it is still costly and time-consuming to generate sufficient sequences for robust phylogenetic reconstruction, while also maximising taxon sampling. Currently, the two most popular methods for generating sizable phylogenomic data sets are (i) the anchored hybrid enrichment approach (Lemmon *et al.* 2012; Ruane *et al.* 2015) and (ii) the ultra-conserved element procedure (Faircloth *et al.* 2012; McCormack *et al.* 2015). Other methods are being developed to exploit museum samples with highly degraded DNA but require whole-genome resources for read mapping (Tin *et al.* 2014).

Alternatively, a simple, rapid and low-cost method of rapidly assembling data sets of approximately 10–15 kbp is to use an NGS-based approach involving partial genome scans of samples, also referred to as genome skimming (Straub *et al.* 2012; Gan *et al.* 2014; Malé *et al.* 2014; Tan *et al.* 2015). Most animal genome skimming studies have focused on mitochondrial sequences that are present in many copies in the eukaryotic cell. Mitochondrial genomes have reduced intergenic elements making them straightforward to recover, assemble and annotate using a suite of bioinformatics methods and pipelines (Bernt *et al.* 2013; Hahn *et al.* 2013; Malé *et al.* 2014), some of which also facilitate phylogenetic analysis (Tamura *et al.* 2013; Tan *et al.* 2015). A major drawback of this approach is that phylogenies based on mitochondrial sequences may not be reflective of the full evolutionary history of the organisms under study as represented by their nuclear genomes (Timm & Bracken- Grissom 2015).

In this regard, an important recent development is the discovery that repetitive nuclear genetic elements, predominately from the nuclear ribosomal cluster, can also be recovered by genome skimming. The data from these partial genome scans, often representing less than 1% of the genome, contain sufficient reads from repetitive nuclear genes to allow them to be routinely recovered for phylogenetic studies (Straub *et al.* 2012; Kocher *et al.* 2014, 2015; Malé *et al.* 2014; Dodsworth *et al.* 2015; Richter *et al.* 2015; Besnard *et al.* 2016). In this study, we demonstrate the wide utility of genome skimming to 22 species of Northern and Southern Hemisphere freshwater crayfish. We show that, in addition to extracting the full

mitogenomes for each species, it is also possible to recover the complete 18S rRNA, 28S rRNA and histone (H3) nuclear gene sequences from a fraction of a MiSeq run (approximately 800 Mbp output). All three of these nuclear genes are considered especially useful for establishing deeper level relationships as demonstrated by a number of studies on crustaceans, including freshwater crayfish (Toon *et al.* 2010; Bybee *et al.* 2011; Bracken- Grissom *et al.* 2014), that utilised information from these genes using PCR-based methods.

While freshwater crayfish have been subject to a number of molecular genetic studies that use conventional PCR-based approaches, there are still outstanding issues concerning phylogenetic relationships among major groups within each superfamily (Braband *et al.* 2006; Toon *et al.* 2010; Bracken- Grissom *et al.* 2014). One of the persistent issues in freshwater crayfish systematics is the unresolved phylogenetic placement of the Asian freshwater genus *Cambaroides*. Although this genus is taxonomically placed in the family Cambaridae, it is often recovered as sister to species of Astacidae or in a basal position rather as sister to other members of Cambaridae from North America. While several studies have used molecular data to study relationships among Northern Hemisphere crayfish species, these often have limitations with respect to taxon sampling (either limited or unbalanced) and number of molecular characters (Ahn *et al.* 2006; Braband *et al.* 2006; Bracken-Grissom *et al.* 2014), sometimes producing conflicting results. In this study, we assemble the complete mitochondrial genomes from ten species of European, North American and Asian crayfish and one lobster species. We also use genome skimming to recover complete or near-complete sequences of nuclear 18S and 28S RNA genes and the histone H3 gene from these samples plus data from 11 additional species of crayfish and lobsters. Our phylogenetic analyses show that the mitochondrial and nuclear trees are fully congruent and the combined data set produces trees with consistently high nodal support that indicates the polyphyly of the Cambaridae. We also demonstrate that our genome skimming approach recovers the same three nuclear genes from samples of a number of major animal groups (e.g. Mammalia, Teleostei, Aves, Mollusca, Arthropoda), suggesting this approach has wide utility for animal molecular systematics.

Material and methods

Sampling and sequencing

For this study, ten Northern Hemisphere freshwater crayfish samples belonging to the superfamily Astacoidea and one lobster sample in the superfamily Nephropoidea that do not have mitogenome representative sequences on NCBI were acquired from various geographical locations (marked with '*' in Data S1), identified based on morphology and further validated with nucleotide similarity searches against publicly available COI, 16S and 12S rRNA gene fragments for the corresponding species (Data S2). For *Cambaroides similis*, whose mitogenome is already available on NCBI, a new additional sample of the same species was collected from Korea (94% identity from a 810-bp alignment to the *C. similis cox1* gene from NC_016925.1) to further scan for nuclear genes. For the *Astacus* species, due to low mitogenome content in the muscle tissue, additional isolates of each species were further enriched for mitochondria (Grandjean *et al.* 1997) and sequenced to recover the complete mitogenome. For all samples, purification of ethanol-preserved tissues and partial whole-genome sequencing on the Illumina MiSeq (2 × 250 bp or 2 × 150 bp) was carried out as described in Gan *et al.* (2014) at the Monash University Malaysia Genomics Facility.

Several species from other superfamilies, Parastacoidea (Southern Hemisphere crayfish) and Nephropoidea (lobsters), were also included in this study for comparative purposes or as out-group species to the Northern Hemisphere crayfish group. For these taxa, nuclear gene sequences or raw sequence read data sets were recovered from various sources – existing mitogenome and nuclear sequences for some species were obtained from NCBI (accession numbers cited in Data S1), whereas raw sequence reads for other species were available from previous mitogenome studies by our group (studies also cited in Data S1).

Genome skimming for mitochondrial and nuclear sequences

Genome skimming was performed according to the workflow illustrated in Fig. 1. Sequences generated from the partial genome sequencing of each sample were initially preprocessed using Trimmomatic (Bolger *et al.* 2014) to remove adapters and low-quality sequences (Illumina clip 2:30:10, sliding window 4:20, leading: 3, trailing 3, min length 100). The resulting quality-filtered reads were then assembled using: (i) IDBA-UD (Peng *et al.* 2012), an iterative *de novo* assembler for data with uneven sequencing coverage; or (ii) MITObim (Hahn *et al.* 2013) for challenging assemblies through the provision of bait sequences to recruit reads for more localised assemblies of the mitogenome or specific nuclear genes.

Target sequences were identified from these assemblies through sequence type specific methods (e.g. mitochondrial, nuclear protein-coding genes (PCGs), nuclear ribosomal RNA genes). Complete mitochondrial sequences were recovered for most samples from either *de novo* (IDBA-UD) or baited (MITObim) assemblies and annotated with MITOS (Bernt *et al.* 2013). Any recalcitrant gaps (i.e. more than one contig) were gap-closed through PCR using

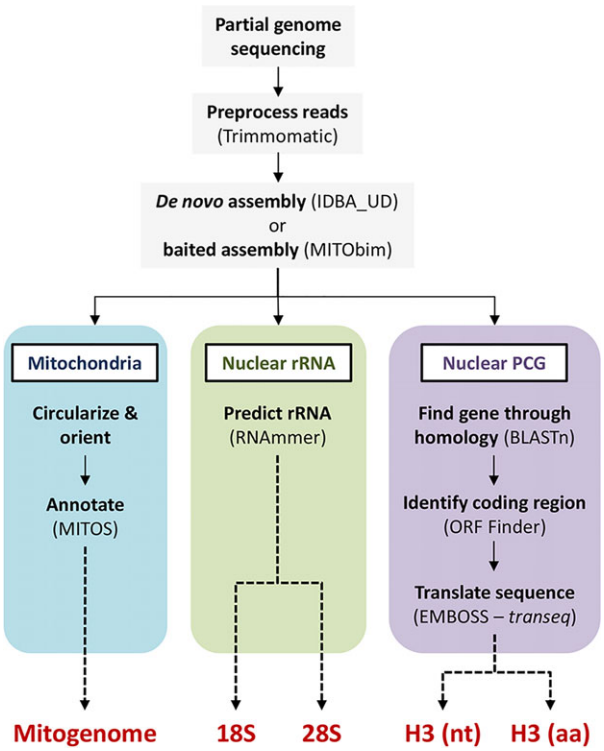


Fig. 1 Genome skimming workflow used to recover the mitogenome and high copy number nuclear genes from partial genome scans.

gap-bridging primers and Sanger sequencing. Nuclear ribosomal RNAs were predicted with RNAmmer (Lagesen *et al.* 2007), and the nuclear protein-coding gene (histone H3) was recovered through a BLASTn search (Altschul *et al.* 1990) against existing H3 sequences of related species. For histone H3, the start and stop coordinates were further refined with ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and translated with the *transeq* component provided by EMBOSS (Rice *et al.* 2000) to obtain the amino acid sequence.

The same genome skimming workflow was tested on reads sequenced from species representing a diversity of animal phyla, including representatives of the Mammalia, Arthropoda, Aves, Teleostei and Mollusca to evaluate the general applicability of our methods across different animal groups and tissue types. Specifically, sequence reads were obtained from nine other sequencing projects in our laboratory and three projects on NCBI's SRA database for species from a variety of animal phyla and classes and tissue sources (e.g. fin clips, liver, muscle, whole organism). These sequence data sets were inspected for the presence of reads for the same three nuclear genes (18S, 28S, H3) recovered from this study of crayfish and lobster species.

Phylogenetic analyses

The construction of phylogenetic trees was carried out on seven different alignments (data sets A–G, Table 1), consisting of various combinations of genes, sequence types (amino acid, aa, vs nucleotide, nt) and lengths. In analyses that utilise only mitochondrial gene sequences (13 protein-coding genes, two rRNAs), a total of 33 samples from the families Astacidae (seven), Cambaridae (16), Parastacidae (six) and Nephropidae (four) were included. Data sets that included the nuclear genes (18S rRNA, 28S rRNA and histone H3) sampled fewer taxa (24), subject to the availability of these gene sequences on NCBI for species that were not sequenced in our laboratory (e.g. *Procambarus alleni*, *Procambarus fallax*).

Amino acid sequences of protein-coding genes (mitochondrial, H3) as well as nucleotide sequences of non-coding rRNAs (12S, 16S, 18S, 28S) were aligned with MAFFT (*mafft-linsi*) (Katoh & Standley 2013) and trimmed with trimAl (*automated1*) (Capella-Gutiérrez *et al.* 2009). Nucleotide sequences of protein-coding genes were aligned with TranslatorX (Abascal *et al.* 2010), which carries out nucleotide sequence alignment guided by amino acid translations followed by alignment trimming with Gblocks (Castresana 2000) implemented internally by the same program.

For phylogenetic analyses, alignments were concatenated for each of the seven data sets (Table 1) and supplied as partitioned alignments to IQ-TREE (Nguyen *et al.* 2014) for model testing and maximum-likelihood (ML) analysis, with node supports obtained with the ultrafast bootstrap option (Minh *et al.* 2013). The same partitioned alignments were used for Bayesian inference (BI) using ExaBayes (Aberer *et al.* 2014). Four independent chains were run for a minimum of 5 million generations each, with 25% of initial samples as burn-in, and convergence of chains was determined when the average standard deviation of split

Table 1 Data sets used to construct alignments used in phylogenetic analyses

Data set	# Taxa	# Genes	Genes included	Alignment length (sites)
A	33	13	mt-pcg (aa)	3657
B	33	15	mt-pcg (aa) + 12S + 16S	5254
C	24	18	mt-pcg (aa) + 12S + 16S + 18S + 28S + H3	9459
D	33	13	mt-pcg (nt)	10 449
E	33	15	mt-pcg (nt) + 12S + 16S	12 006
F	24	18	mt-pcg (nt) + 12S + 16S + 18S + 28S + H3	16 211
G	24	3	18S + 28S + H3	4205

Trees inferred from these data sets are available in Data S5.

frequencies (*asdsf*) fell below 1% indicating good convergence.

Topology testing

Topology testing was carried out using IQ-TREE (Nguyen *et al.* 2014) to evaluate the likelihood of alternate topologies (e.g. a monophyletic Cambaridae). The following tree topology tests were performed using Data set F (Table 1), comprised of 18 genes (13 mitochondrial PCGs, 12S, 16S, 18S, 28S rRNA, H3):

I((Astacidae, Cambaridae-NA), Cambaridae-Asia), out-groups).
II(Astacidae, (Cambaridae-NA, Cambaridae-Asia)), out-groups).
III((Astacidae, Cambaridae-Asia), Cambaridae-NA, out-groups).

The tree topology tests include the Shimodaira-Hasegawa test (Shimodaira & Hasegawa 1999) carried out using the RELL approximation (Kishino *et al.* 1990) based on 1000 replicates and the approximately unbiased (AU) test (Shimodaira 2002).

Results

Genome skimming effectively recovers the mitogenome sequence and high copy number nuclear genes

An average of approximately 739.4 Mbp of raw sequence data per sample was generated from the freshwater crayfish and lobster libraries (Data S1). Mitogenome sequences assembled for these species vary in size from 14 895 bp to 20 677 bp with AT content ranging from 67.9% to 73.1%. The typical 13 mitochondrial protein-coding genes, 22 transfer RNA genes and two ribosomal RNA genes (12S, 16S) are found in all Northern Hemisphere crayfish and lobster mitogenomes recovered in this study (marked with '*' in Data S1). The organisation of these genes in the mitogenomes of the ten Northern Hemisphere crayfish taxa we assembled is identical to the first sequenced species, *Procambarus clarkii*, which itself shows a large departure from the ground pancrustacean pattern (as represented by *Drosophila*, *Penaeus monodon* and the out-group species, *Homarus americanus*). The gene order for the lobster *Metanephrops sibogae* is also aberrant compared to *H. americanus*, a result of multiple translocated protein-coding and tRNA genes. Most notably, *M. sibogae* possesses two control regions, each approximately 2 kbp in length, resulting in a much longer mitochondrial genome size of 20 677 bp. The lengths of the 12S and 16S rRNA genes are generally shorter in the Northern Hemisphere crayfish compared to the Southern Hemisphere crayfish. Details such as coding regions, AT content and intergenic lengths for each mitogenome are available in Data S3.

Complete or near-complete sequences were recovered for the three nuclear genes 28S rRNA (4144–5391 bp), 18S rRNA (1869–1885 bp) and H3 (all 411 bp) from the same partial genome scan. Their degrees of similarity to available sequences on NCBI for the same species are detailed in Data S4. Out of the total nuclear sequences contributed through this study, 38 gene sequences from 13 species are 'novel' (i.e. do not have any representation on NCBI). The remaining 28 gene sequences are highly similar to sequences held on NCBI based on local alignment, with average per cent identities of 98.7% (28S), 99.4% (18S) and 98.9% (H3) for matching species. The lengths of the 18S rRNA sequences recovered in this study are comparable to those already available on public databases through PCR-based methods. However, the other nuclear sequences (28S, H3) obtained from genome skimming are much longer in length than those deposited on NCBI for crayfish and lobster species. Notably, the 28S rRNA gene sequences contributed in this study are almost double the length of their same-species counterpart available on NCBI. Also, the full length amino acid sequence of the histone H3 gene (411 bp) complete with start and stop codons was recovered, as opposed to the currently available partial H3 sequences that are mostly 333 bp or shorter. All recovered mitochondrial and nuclear sequences are available on NCBI at accession numbers listed in Data S1.

Phylogenetic analyses and topology tests point to a polyphyletic Cambaridae

The nucleotide-based phylogenetic tree was generated from the longest alignment (16 211 bp, Data set F; Fig. 2) with representative species from Parastacoidea (Southern Hemisphere crayfish) and Nephropoidea (lobsters) as out-groups. The focus of this study, the superfamily Astacoidea, is represented by species from two families, Astacidae (five species) and Cambaridae (nine species). Maximal support is observed for most nodes in this clade of interest, except for the weaker ML support for the sister relationship between astacids and North American cambarids (ultrafast bootstrap: 86, PP: 1.00). Both ML and BI trees inferred from this data set imply a monophyletic Astacidae and a polyphyletic Cambaridae, with the split occurring between the North American cambarids (*Procambarus*, *Cambarus*, *Orconectes*) and the Asian cambarids (*Cambaroides*). Further topology testing (Fig. 3) shows Topology I (North American cambarids as sister taxa to astacids) as most likely, followed by Topology III (Asian cambarids as sister taxa to astacids). Both Topology I and Topology III support a polyphyletic Cambaridae. Topology II, containing a monophyletic Cambaridae, is rejected (P-value <0.05 for tree tests in Fig. 3).

Nevertheless, tree topologies are variable depending on the data set and the method used to infer phylogeny

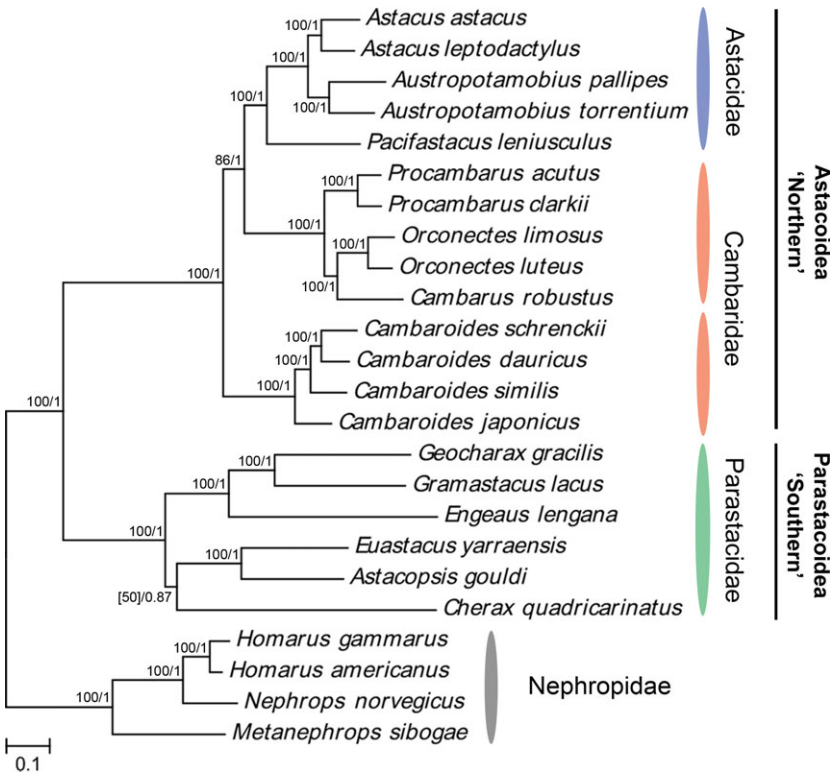
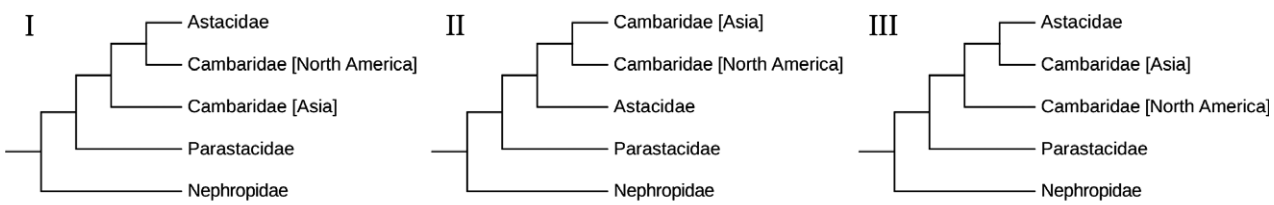


Fig. 2 Phylogenetic relationships among Northern Hemisphere freshwater crayfish inferred based on nucleotide alignment of Data set F (Table 1) comprising of 13 mitochondrial (protein-coding gene PCG), 12S, 16S, 18S, 28S and H3 (16 211 sites). Topology shown was obtained from Bayesian inference, with Ultrafast bootstrap values (from maximum-likelihood analysis) and posterior probabilities indicated as support values at each node. Square brackets '[]' indicate conflict in topology inferred by the two phylogenetic methods.



Tree	logL[1]	p-SH[2]	p-AU[3]
I	−155 582.703	1.0000 (+)	0.5227 (+)
II	−155 610.006	0.0350 (−)	0.0176 (−)
III	−155 593.224	0.3300 (+)	0.2577 (+)

Plus signs (+) denote the 95% confidence sets
Minus signs (−) denote significant exclusion

[1] Tree log-likelihood
[2] *P*-value of Shimodaira-Hasegawa test (2000)
[3] *P*-value of approximately unbiased test (Shimodaira, 2002)

Fig. 3 Evaluation of alternate tree topologies through topology testing based on Data set F (13 mt-pcg (nt) + 12S + 16S + 18S + 28S + H3).

(Fig. 4A–G). While the most common topology is consistent with the tree in Fig. 2, other observed topologies mostly differ in the relationships among groups of the North American cambarids (data sets B and C). The tree generated from Data set G, which consists of only the nuclear 18S, 28S and H3 gene sequences (4205 aligned sites), deviates from the other topologies. Its Bayesian tree does show a monophyletic Cambaridae but with only weak support (PP: 0.61) and is incongruent with the ML tree generated from the same alignment, which is similar to the other analyses and also fails to recover a monophyletic Cambaridae. Detailed ML and BI phylogenetic trees inferred from all data sets are available as Data S5.

Generality of genome skimming for nuclear genes for animals

Of the twelve tested animals, the 18S gene sequence was recovered from all species, whereas sequences from both 28S (partial or complete) and H3 were recovered from ten of twelve species. Similarly, genome skimming successfully recovered substantially longer 28S (approximately 4 kbp)

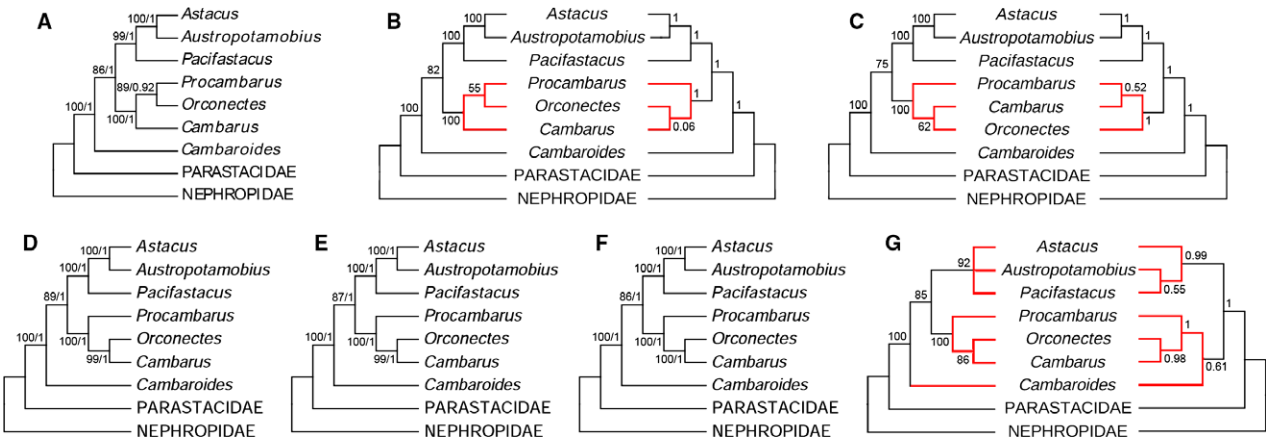


Fig. 4 An overview of evolutionary relationships within Astacoidea (out-groups: Parastacoidea and Nephropoidea). Tree topologies were constructed from each of the seven data sets (Table 1) and numbers at the upper left corner of each tree indicate data set used for phylogenetic inference. Ultrafast bootstrap and/or posterior probability values are used to show support at each node while coloured branches highlight differences in topology between ML (left) and BI (right) trees.

and H3 (411 bp) gene sequences in most cases compared to sequences available on NCBI (28S: 1.5–4 kbp, H3: 333–411 bp; Data S4). The 18S, 28S and H3 sequences recovered for these species are available as Data S6.

Discussion

Crayfish mitogenomes

This study increases the number of sequenced Northern Hemisphere crayfish mitogenomes from six to sixteen, substantially expanding the available resources for the family Cambaridae (*Procambarus*, *Cambaroides*, *Cambarus*, *Orconectes*) and Astacidae (*Astacus*, *Pacifastacus*, *Austropotamobius*). In addition, a new mitogenome for the lobster, *M. sibogae*, reveals an aberrant gene order for this group, but one identical to that recently described for *Metanephrops thomsoni* (Ahn *et al.* 2016). This is a surprising finding given that previous studies indicated that marine lobsters (*H. americanus* and *Enoplometopus*) possess a conserved mitogenome order that is common across the arthropods and is considered reflective of the primitive pancrustacean pattern (Boore *et al.* 1995; Shen *et al.* 2013).

Another equally surprising finding is the lack of mitogenome variation among Northern Hemisphere species, given the high frequency of novel mitogenome gene orders among Southern Hemisphere crayfish. No mitogenome gene rearrangements are apparent for the ten new mitogenomes provided from this study and the six from previous studies, all of which contributes to taxonomic sampling covering all families and the full geographical range of the superfamily. This is in stark contrast to the number and scale of mitogenome gene order rearrangements among Southern Hemisphere crayfish with most genera studied having distinct gene orders, including interspecific differences within the genus *Engaeus* (Tan *et al.* 2015; Lee *et al.* 2016).

The frequency of mitogenome rearrangements is not simply a function of divergence times. Based on the dated phylogeny of Bracken- Grissom *et al.* (2014), the *Engaeus* group of crayfish and its close relatives, containing significant rearrangements, diverged more recently (145.4 mya) than the Northern Hemisphere crayfish as a group (161.2 mya), which have none. Conversely, *Euastacus* and *Cherax*, which last shared a common ancestor approximately 200 mya, have identical mitogenome gene orders. Thus, crayfish exhibit both extreme conservation and extreme lability of mitochondrial gene order, that is not a simple function of divergence time, an observation that invites further investigation on the dynamics and evolutionary drivers of mitogenome evolution in this group (Okajima & Kumazawa 2010; Kilpert *et al.* 2012; Poulsen *et al.* 2013).

Phylogenetic results and the status of the family Cambaridae

This study contributes to growing evidence suggesting that the family Cambaridae is non-monophyletic, but contradicts suggestions that the genus *Cambaroides* should be included within the family Astacidae. Several studies using a variety of morphological and molecular data sets from a range of genes and varying taxonomic sampling concur that North American cambarid species and Asian cambarid species (genus *Cambaroides*) do not share a common ancestor (Crandall *et al.* 2000; Rode & Babcock 2003; Porter *et al.* 2005; Ahn *et al.* 2006; Braband *et al.* 2006; Bracken *et al.* 2009; Breinholt *et al.* 2009; Bracken- Grissom *et al.* 2014). The phylogenetic position of Asian cambarid species and their taxonomic treatment within the superfamily Astacoidea remains controversial.

While most studies have supported the Asian cambarid lineage as the most basal within the Astacoidea, Bracken-Grissom *et al.* (2014) found the Asian cambarids and the astacids to be monophyletic (using a combination of morphological characters, three mitochondrial and three nuclear gene fragments and based on two samples of *Cambaroides japonicus*, single samples of *Astacus astacus* and *Austropotamobius torrentium* and four samples of *Pacifastacus*). They suggested that the concept of the Astacidae should be expanded to include *Cambaroides*. Instead, our data set strongly supports the *Cambaroides* lineage to be basal, based on our data from five astacid species, two *Procambarus*, two *Orconectes*, one *Cambarus* and four *Cambaroides* species consisting of both nuclear and mitochondrial genes.

A basal position for the Asian cambarid lineage requires a re-evaluation or re-interpretation of morphological and reproduction-related characters as either ancestral or convergent within the lineages as recovered in this study (Ahn *et al.* 2006; Braband *et al.* 2006). We suggest a family level revision of the taxonomic classification of Northern Hemisphere crayfish that might consider placing the Asian cambarid crayfish in a new family, or placing all Northern Hemisphere crayfish in a single family, similar to the treatment of all Southern Hemisphere crayfish as members of the Parastacidae.

The utility of genome skimming for animal phylogenetics

This study demonstrates the utility of partial genome sequencing, also known as genome skimming, using the MiSeq NGS platform as a rapid and inexpensive approach to assemble substantial data sets to support phylogenetic studies. We used our crayfish data set to construct an alignment of 12 006 nucleotides from the mitochondrial genomes, which is now becoming a routine procedure for animal phylogenetic studies using NGS (Shen *et al.* 2013; Gan *et al.* 2014; Tan *et al.* 2015).

Less common is the use of sequences from nuclear genes that can also be recovered from the same partial genome scan used to assemble whole mitogenome sequences or to locate microsatellite markers for population genetic applications (Gan *et al.* 2014; Thai *et al.* 2016). Data S7 summarises the only four recent studies we could find on animals that have reported nuclear genes recovered from NGS-based genome scans (Kocher *et al.* 2014, 2015; Richter *et al.* 2015; Besnard *et al.* 2016). Genes and regions associated with the nuclear ribosomal cluster are the most common target, and these studies together with our data indicate that complete or almost complete gene sequences can be routinely recovered for the 18S and 28S genes from various animal groups including annelids, crustaceans, molluscs (Bivalvia and Gastropoda) and chordates (Aves, Chondrichthyes, Actinopterygii, Mammalia). Further, high copy number protein-coding genes can also be recovered. Our study is the first to report recovery of the histone H3 gene, for which the full amino acid sequence was retrieved for all our lobster and crayfish samples and ten of twelve species in our supplementary non-crustacean data sets (Table 2). It was also encouraging that other protein-coding genes can potentially be recovered from shotgun sequencing data sets (Besnard *et al.* 2016), especially as the phylogenetic utility of ribosomal nuclear genes has been called into question by some authors (Tsang *et al.* 2008).

We foresee exciting times ahead for the discovery and recovery of an increasing number of nuclear genes for

Table 2 Demonstration of the recovery of 28S rRNA, 18S rRNA and histone H3 sequences from performing genome skimming on sequence reads of animals from various taxonomic groups and tissue isolation sources

					Recovered gene length (bp)		
Phylum	Class	Species	Tissue source	Sequence data	28S	18S	H3
Chordata	Actinopterygii	*Gadopsis marmoratus*[a]	Fin clip	459 Mb	4492	1840	411
		Oryzias latipes[b]	SRA	1 Gb	4720	1842	411
	Aves	*Corvus splendens*[c]	Liver	813 Mb	–	1822	411
	Chondrichthyes	*Pastinachus atrus*[d]	Muscle	3.45 Gb	2699	1796	411
	Mammalia	*Gallus gallus*[e]	SRA	2 Gb	2065	1822	411
		Rattus norvegicus[f]	SRA	2 Gb	4803	1871	411
Mollusca	Bivalvia	*Lutraria rhynchaena*[g]	Muscle	623 Mb	4201	1839	411
		Tridacna squamosa[h]	Muscle	203 Mb	4314	1870	411
	Gastropoda	*Babylonia areolata*	Muscle	61 Mb	4394	1828	411
Arthropoda	Branchiopoda	*Triops australiensis*[i]	Whole	920 Mb	3988	1810	–
	Maxillopoda	*Lepas anserifera*	Whole	425 Mb	4125	1870	411
		Pandarus rhincodonicus[j]	Whole	480 Mb	–	1814	–

Gene sequences recovered for these animals are available in Data S6.

Raw reads were obtained from various internal projects and databases: [a]Gan *et al.* (2016c); [b]ERR110365 (SRA); [c]Krzeminska *et al.* (2016); [d]Austin *et al.* (2016a); [e]SRR2131206 (SRA); [f]ERR316506 (SRA); [g]Gan *et al.* (2016d); [h]Gan *et al.* (2016a); [i]Gan *et al.* (2016b); [j]Austin *et al.* (2016b).

phylogenetic analyses, given increasing use of NGS for partial genome sequencing for many animal samples plus the increasing number of whole-genome sequences becoming available for a diversity of animal species. Further, we anticipate that animal systematics is entering a new era in which even more robust data sets can be assembled, maximising both taxon and gene sampling while minimising expense to an extent hitherto impossible (Straub *et al.* 2012; Richter *et al.* 2015).

Declaration of interest

The authors report no conflict of interests, and the authors alone are responsible for the content and the writing of the article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Accession numbers for mitogenome, 18S, 28S and H3 sequences generated or used in this study.

Data S2. Sequence similarity of COI, 16S and 12S rRNA to genes on NCBI.

Data S3. Characteristics of mitogenomes.

Data S4. Sequence similarity of 28S, 18S and H3 to genes on NCBI.

Data S5. Phylogenetic trees constructed from all datasets in this study.

Data S6. 28S, 18S and H3 sequences generated for other animal species.

Data S7. List of genome skimming studies previously demonstrated in other animal species.

Supplement 2

Bláha, M., Uzhytchak, M., Bondarenko, V., Policar, T., 2017. The least known European native crayfish *Astacus pachypus* (Rathke, 1837) revealed its phylogenetic position. Zoologischer Anzeiger, 267: 151-154.

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Short communication

The least known European native crayfish *Astacus pachypus* (Rathke, 1837) revealed its phylogenetic position

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ABSTRACT

The thick-clawed crayfish *Astacus pachypus* (Rathke, 1837) is the least known species within the Astacidae, mostly due to limited access to samples and declining populations in recent decades. In the present study, for the first time, we report the phylogenetic position of this vulnerable native European freshwater crayfish within the genus *Astacus*, based on mitochondrial (COI and 16S rRNA) and nuclear (ITS2) molecular markers. Genetic results suggest its closest relationship is to *A. leptodactylus* (Eschscholtz, 1823), as previously suggested by morphology and common area of occurrence.

The thick-clawed crayfish *Astacus pachypus* (Rathke, 1837) is an endangered European native species with only restricted amount of information about its distribution (Füreder, 2015; Holdich, 2002; Kouba et al., 2014). The species is indigenous to the Ponto-Caspian Basin with its center of distribution in rivers and coastlines of northern parts of Black, Azov and Caspian Sea areas (Brodski, 1983; Cherkashina, 1999; Souty-Grosset et al., 2006), however, the number of population appears to be decreasing (Holdich, 2002; Cherkashina, 1999; Mezhzherin et al., 2015). Moreover, the thick-clawed crayfish is the only species from Astacidae able to colonize brackish waters (Cherkashina, 1999).

The systematics of European freshwater crayfish has improved significantly during the last decade due to advanced molecular methods. Most of the studies however were dedicated to *Austropotamobius pallipes* sensu lato, suggesting the existence of two species *A. pallipes* (Lereboullet, 1858) and *A. italicus* (Faxon, 1914) (Fratini et al., 2005; Grandjean et al., 2000; Trontelj et al., 2005). On the contrary, the eastern European species *A. leptodactylus* and *A. pachypus* have been on the fringes of scientific interest mainly due to poorly accessible samples and limited territory of distribution in the case of the latter species. However Maguire et al. (2014) and Akhan et al. (2014) studied the *A. leptodactylus* species complex, revealing two or three distinct evolutionary lineages, respectively.

Although the morphology of *A. pachypus* unambiguously assumed its relation to the genus *Astacus* (Brodski, 1983; Cherkashina, 1999; Starobogatov, 1995), distributional and some morphological characteristics suggested a closer relationship to *A. leptodactylus*. This led some scientists to place both species into the genus *Pontastacus* (Brodski, 1983; Śmietana et al., 2006; Starobogatov, 1995). However, no genetic methods were realized, and no relevant data exists about *A. pachypus* genetic diversity, or its phylogenetic position. Therefore, the aim of this study was to clarify and describe the phylogenetic position of the thick-clawed crayfish within the Astacidae, based on the analysis of mitochondrial and nuclear DNA.

Astacus pachypus tissue samples were collected from two sites of the main stream of the Dnieper River: the first near Nova Kakhovka town (46°46.452′N 33°22.090′E) and the second near Sadove village (46°41.893′N 32°49.660′E) in the Kherson region (for more details see Policar et al., 2017, accepted). For comparative purposes, individuals of *A. astacus* were sampled in two ponds, U Včelníku and U Sudu, in Czech Republic (for details see Bláha et al., 2016b). Except of *A. pallipes* sampled in Lough Lea Lake, Northern Ireland (54°16.28.773′N 7°22.54.322′W), remaining crayfish specimens originated from the Czech Republic – *Astacus leptodactylus* were caught in the stone quarry Kozárovice (49°55.122′N 14°10.624′E), *A. torrentium* originated in the brook Zubřina near to Nová Pasečnice (49°39.684′N 12°88.931′E), *Orconectes limosus* and *Pacifastacus leniusculus* were obtained from the river Blanice in Protivín (49°11.939′N 14°13.144′E) and Vodňany (49°15.641′N

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Table 1
List of species and gene fragments used in this study, including GenBank accession numbers and countries of particular haplotype (h).

Species	Locality	N	COI	16S	ITS	Acc. No. COI	Acc. No. 16S	Acc. No. ITS2
			h	h	h			
A. pachypus	Ukraine	41	4	3	1	KX018606-609	KX018610-612	KX029465
A. leptodactylus	Turkey	7	3	3	1	JQ421518	KF181958	KX029468
	Croatia					JQ421516	KF181957	
	Russia					JQ421515	KF181956	
	Czech Republic							
A. astacus	Czech Republic	3	3	2	1	KX029462-464	KX018613-614	KX029467
A. pallipes	Italy	7	4	2	1	AB443448	AF237603	KX029470
	France					AB443447	AF237604	
	USA					AB443450		
	Northern Ireland					AB443451		
A. torrentium	Croatia	9	4	4	1	JN683352	JF293403	KX029471
	Czech Republic					JN683353	JF293404	
						JF293458	JN683357	
						JF293467	JN683358	
P. leniusculus	USA	4	1	1	1	JF438000	JX077955	KX029466
	Czech Republic							
P. clarkii	Japan	4	4	4	1	JX120103	KJ645830	AF198596
	USA					JX120104	KJ645831	
	Italy					JX120105	KJ645832	
						JX120106	KJ645833	
O. limosus	Romania	3	2	2	1	JQ435818	JF293366	KX029472
	Czech Republic					JF437993	EU442690	
	Croatia							

14°169435′E), respectively. Sequences of the other European freshwater crayfish species used in this study, were downloaded from GenBank (Table 1) to create 27 combined haplotypes and show variability within the particular species.

Genomic DNA extraction and PCR amplification was done according to Bláha et al. (2016a). Two mitochondrial genes, COI, 16S rRNA, were amplified with primers LCO-1490 (Folmer et al., 1994) and COI 703r (CCRCCMGCAGGRTCAAAGAA, this study) and 16S ar and 16S br (Simon et al., 1994), respectively. Additionally the internal transcribed spacer 2 (ITS2) was amplified using the primers CAS5p8sFc and CAS28sB1d, and PCR protocol published in Ji et al. (2003). Product purification and sequencing was performed by Macrogen Inc., Korea. All newly obtained sequences were deposited in GenBank under accession numbers listed in Table 1.

Phylogenetic relationships were reconstructed using the concatenated dataset of 27 nucleotide sequences from 8 crayfish species from Astacidae and Cambaridae families. The final length of particular sequences used for alignment was 520 bp for COI, 379 bp for 16S and 510–694 bp for ITS2 sequences or 1664 bp in concatenated alignment. The optimal HKY + I + G model was found, based on BIC (Bayesian information criterion) in jModel Test 2.1.7 (Darriba et al., 2012) for the combined dataset and for COI, while HKY + G was the best substitution model for 16S rRNA, and K80 + G for ITS2. Nucleotide sequences were aligned using MAFFT v7.017 (Katoh et al., 2002) implemented in GENEIOUS 8.0.5 (Kearse et al., 2012).

The number of parsimony informative sites was calculated in MEGA 6 (Tamura et al., 2013). Haplotype relationships were determined using Bayesian inference and maximum likelihood (ML) algorithms. A ML tree was constructed in RAxML (Stamatakis et al., 2005), implemented in GENEIOUS 8.0.5. Bayesian analyses were conducted in MrBayes 3.2.4. (Ronquist et al., 2012) applying specific nucleotide substitution model for particular gene sequence set. For interspecies relations, pairwise model corrected genetic distances were calculated in PAUP v.4.0 (Swofford, 2003).

In total, 41 individuals of *A. pachypus* were analyzed from both sites and four, three and one haplotype were obtained for all analyzed genes (COI, 16S and ITS2, respectively). These haplotypes were deposited in GenBank (Table 1). The combined dataset

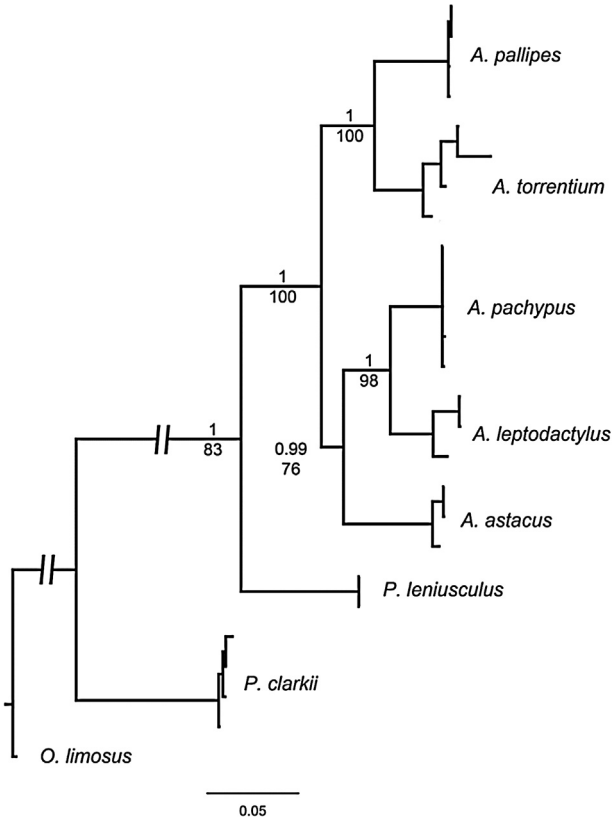

Fig. 1. Bayesian phylogram inferred from concatenated dataset, showing the phylogenetic relationship within the Astacidae family. Maximum likelihood bootstrap and Bayesian inference are displayed above and under each node.

(including outgroup) consisted of 1664 bp, containing 582 variable sites of which 568 are parsimony informative. All implemented criteria of phylogenetic reconstruction using ML and BI showed congruent topologies (Fig. 1), characterized by the six well supported phylogroups (bootstrap support 76–100%) representing different species, outgroup included. *Astacus pachypus* composes

a sister clade with *A. leptodactylus*. The mean model-corrected sequence distances between *A. leptodactylus* and *A. pachypus* ranged from 11 to 18% for mitochondrial genes, and 1–5% for nuclear ITS2. Gene divergence excluding outgroups (*O. limosus* and *P. clarkii*) indicated that COI (28%) is the most variable followed by 16S (17%). Nuclear ITS2 had the lowest divergence (4%). However, distance between *A. pachypus* and *A. leptodactylus* ranged from 1 (ITS2) to 21% (COI), and between *A. pachypus* and *A. astacus* ranged from 5 (ITS2) to 27% (COI).

The present results based on mitochondrial and nuclear DNA analyses, for the first time suggest that *A. pachypus* and *A. leptodactylus* are related evolutionary lineages. According to morphological features, these two species are more similar to each other than to *A. astacus*, the last member of the genus (Albrecht, 1982; Karaman, 1962; Śmietana et al., 2006; Starobogatov, 1995). Starobogatov (1995) and Śmietana et al. (2006) summarized common morphological traits of *A. pachypus* and *A. leptodactylus* such as the pleura of the abdominal somites 2–4 with one or two acute spines. Contrariwise, *A. astacus* has the pleura of its abdominal somites 2–4 wedge-shaped or rounded without spines at the ventral part. Additionally, the base of the exopodite of the second gonopod has no ventral process in *A. astacus* male, while *A. leptodactylus* and *A. pachypus* males have a ventral process on the second gonopod (Holdich, 2002; Souty-Grosset et al., 2006). The morphological features alone could not explain the exact taxonomy of the species, as for example has been shown in the species-rich crayfish genus *Cambarus* (Breinholt et al., 2012; Helms et al., 2015), however in combination with molecular data, assignment to species/subspecies is possible and appropriate. Although, this is not exactly the same situation considering *A. pachypus* and *A. leptodactylus*, Starobogatov (1995) and Śmietana et al. (2006) suggested assigning these two species into one individual genus, *Pontastacus* or *Caspiastacus* (Bott, 1950), according to their occurrence in the Ponto-Caspian Basin and distinct morphological characteristics. In spite of these attempts, most western European astacologists are still using the genus denomination *Astacus* for all three species (e.g. Holdich, 2002; Kouba et al., 2014; Kozák et al., 2015; Schrimpf et al., 2014). Only future studies may disentangle the status of several species based on morphology described by Starobogatov (1995) within the *A. leptodactylus* species complex and potentially consider them as a member of *Pontastacus* genus.

The number of haplotypes determined in our study (four at COI) is relatively low compared to the *A. leptodactylus* haplotypes described by Akhan et al. (2014) from Turkey (56 at COI), however their number varies between 1 and 5 among particular sampling sites. Genetic diversity of *A. astacus* across Europe is even lower, with 30 haplotypes determined (Schrimpf et al., 2014). Although this number is mainly affected by human impact and numerous translocations in the past, nevertheless two catchments in Germany and the Balkan countries represent centers with higher genetic diversity. We analyzed crayfish from only two sites on the river Dnieper, a rather low number to estimate or suggest some conclusions about *A. pachypus* genetic diversity. Moreover, the number of populations was found to be decreasing in recent decades (Holdich, 2002; Cherkashina, 1999; Policar et al., 2017, accepted). In addition, the recently determined occurrence of parthenogenetic marbled crayfish *Procambarus fallax* (Hagen, 1870) f. *virginalis* in Ukraine close to Dnieper River (Novitsky and Son, 2016) and availability of at least 14 further non-indigenous crayfish species in Ukrainian pet trade (Kotovska et al., 2016) are considered a strong threat for all native crayfish species in this area, including *A. pachypus*. Furthermore, the ability of this established non-native crayfish species to withstand winter conditions of the temperate zone and to establish viable population have recently been proven (Lipták et al., 2016; Patoka et al., 2016; Veselý et al., 2015). In spite of this, based on the species distribution centered around the Black, Azov and Caspian Seas, we can presume the existence of at least two different evolutionary lineages, similarly to that found in other invertebrate species distributed in Ponto-Caspian area (e.g. Cristescu et al., 2003; Nahavandi et al., 2013).

To conclude, analyses of mitochondrial and nuclear genes corroborate the phylogenetic position of *A. pachypus* as the closest relative to *A. leptodactylus*, which was previously suggested based only on morphological criteria. This study is a first step in discovering genetic diversity and phylogenetic patterns of other *A. pachypus* populations in the Ponto-Caspian area to finally bring more information about the last native European crayfish species still shrouded by mystery.

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Supplement 3

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ORIGINAL ARTICLE

Genetic diversity, phylogenetic position and morphometric analysis of *Astacus colchicus* (Decapoda, Astacidae): a new insight into Eastern European crayfish fauna

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Abstract

The phylogeny of European crayfish fauna, especially with respect to Eastern European species, is still far from being completely resolved. To fill this gap, we analyzed most of the European crayfish species focusing on the phylogenetic position of the endemic crayfish *Astacus colchicus*, inhabiting Georgia. Three mitochondrial and one nuclear marker were used to study evolutionary relationships among European crayfish species, resulting in the unique phylogenetic position of *A. colchicus* indicating independent species status to *A. astacus*. Phylogenetic analyses revealed a deep molecular divergence of *A. colchicus* in comparison to *A. astacus* (6.5–10.9% in mtDNA and 1.1% in nDNA) as well as to *Pontastacus leptodactylus* and *P. pachypus* (5.5–10.0% in mtDNA and 1.4–2.4% in nDNA). Absent ventral process on second male pleopod and abdominal somites II and III with pleura rounded lacking prominent spines clearly indicate taxonomic assignment to the genus *Astacus*; however, the species is distributed almost in the middle of Ponto-Caspian area typical by occurrence of the genus *Pontastacus*. Several morphological indices linked to head length, carapace, and total body length and width were found to demonstrate apparent differences between *A. colchicus* and *A. astacus*. Although this study provides a novel insight into European crayfish phylogeography, we also point out the gaps in comprehensive study of the *P. leptodactylus* species complex, which could reveal details about the potential species status of particular species and subspecies within this genus.

Key words: 12S rRNA, 16S rRNA, cytochrome c oxidase, histone H3, Ponto-Caspian crayfish

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INTRODUCTION

The Caucasus Mountains on the border between Asia and Europe, the Black and Caspian Seas, have played an important role in the formation of the current appearance of the Eurasian continent. In spite of a limited knowledge

of the region's biodiversity, the Caucasus are well known due to their high numbers of endemic species including plants, invertebrates, and vertebrate species (Mumladze *et al*. 2019). For instance, these include Caucasian rhododendron *Rhododendron caucasicum* Pallas, 1784; West Caucasian cave shrimp *Troglocharis kutaissiana* (Sadovskij, 1930); Buch's snail *Helix buchii* (Dubois de Montpéreux, 1840); Caucasian parsley frog *Pelodytes caucasicus* Boulenger, 1896; and Caucasian salamander *Mertensiella caucasica* (Waga, 1876) *sensu lato*. Some of these organisms are limited to only a small part of the Caucasus, while others have much wider ranges (Myers *et al*. 2000; Tarkhnishvili 2014). Georgia covers less than 20% of the Caucasus, but it lies in the central parts of the ecoregion, encompassing all the landscapes stretching from the peat bogs in the west through the semi-deserts to the east and high mountains to the north. The only native crayfish species in Georgia is *Astacus colchicus* Kessler, 1876. It has not often been mentioned in the literature, and the species description was based on specimens gathered in the upper tributaries of the Rioni River (Kessler 1876). Much later, Bott (1950), Albrecht (1982), and Starobogatov (1995) compared species specific characteristics with other *Astacus* species/subspecies, presenting descriptive figures or a simple dichotomous key. The distribution of this species is known to be restricted to the upper Rioni basin in western Georgia (Kessler 1876; Albrecht 1982). Although Holthuis (1961) also reported *A. colchicus* from northern Turkey (in a creek close to Ünye town), these crayfish were determined to be narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823) later on (Machino & Holdich 2006). Another east Ponto-Caspian crayfish thick-clawed crayfish *Pontastacus pachypus* Rathke, 1837, is also known from southern slopes of the eastern Great Caucasus (Azerbaijan) and was suggested to occur in Georgia though it has never been recorded there (Derzhavin 1951) or having disappeared as in most of area of its occurrence in Ukraine (Policar *et al*. 2018).

The systematics of European freshwater crayfish underwent significant improvement due to advanced molecular tools, with most of the studies dedicated to *Austropotamobius* species (Zaccara *et al*. 2004; Trontelj *et al*. 2005; Klobučar *et al*. 2013; Jelić *et al*. 2016; Pârvulescu 2019; Pârvulescu *et al*. 2019) and some to the noble crayfish *Astacus astacus* (Linnaeus, 1758) (Bláha *et al*. 2016; Laggis *et al*. 2017; Schrimpf *et al*. 2017). On the other hand, Eastern European species are still somewhat inaccessible and suffer from a lack of data; their taxonomy relying mostly on morphological data and historical records and their systematics still not fully resolved (however see, Maguire *et al*. 2014; Akhan *et al*. 2014; Bláha *et al*. 2017). Recently, an updated classification of the freshwater crayfish was published (Crandall & De Grave 2017), keeping the rich taxonomic nomenclature of European crayfish as suggested by Karaman (1962), Albrecht (1982), or Starobogatov (1995) with respect to species and subspecies within the genera *Astacus* (3 species and 2 subspecies) and *Pontastacus* (9 species and 1 subspecies), respectively. This recent study should minimize the differences in nomenclature used by some authors (Kouba *et al*. 2014; Maguire *et al*. 2014; Śmietana *et al*. 2006) and uncertainty as to which taxon name should be correctly used. On the other hand, some of these species and subspecies are defined solely based on morphological traits and/or zoogeography, but not tested with modern molecular or morphometric tools. It would help to exclude that these differences in morphology are not resulting only from high intraspecific variability and phenotypic plasticity of the species. One of such cases is that of *A. colchicus*. Although the main morphological differences from *A. astacus* were already mentioned (Karaman 1962; Albrecht 1982; Starobogatov 1995), those differences could be a consequence of morphological plasticity and not really species specific. Recently, a new species *Austropotamobius bihariensis* Pârvulescu, 2019 has been described from the Apuseni Mountains in Romania (Pârvulescu 2019). Morphological differences from the closest relatives were in the shape of the rostrum or antennal scale, that is, differences which could be easily overlooked and originally considered within the phenotypical plasticity of the *Austropotamobius torrentium* (Schrank, 1803). However, the author of the species description found high genetic divergences from other *A. torrentium* populations and then applied detailed morphometry to find significant differences between the new species and its closest relatives.

No molecular genetic methods have so far been applied to *A. colchicus*, and no relevant data exist about its genetic diversity, phylogenetic position, and morphometry. Therefore, we present here the molecular and morphological analysis of the *A. colchicus* sampled in Georgia with 2 main aims: (i) to provide morphological and genetic data for this species, and (ii) to describe its phylogenetic position and reveal whether it represents a separate lineage to *A. astacus* or is clustered within *A. astacus* species and thus any morphological differences should be accounted as high intraspecific variability only.

MATERIALS AND METHODS

Specimen collection

In total, 106 crayfish individuals from 10 sampling sites (Table 1; Fig. S1, Supporting Information) in Georgia were collected by hand or trapping during 2016. Individuals of *A. colchicus* ($n = 51$) were identified in only 6 of them (Tables S1 and S2, Supporting Information), *P. leptodactylus* occurred in the rest of sampling sites as well as was found in sympatry with *A. colchicus* at Sepa river. Each of the 6 sampling sites were located in the catchments of Churia (Papantskuri Lake), Khobi, Rioni (Lashe river), Sepa, Choloki, and Kintrishi rivers. One pereiopod from each animal was dissected and individually preserved in pure 96% ethanol until DNA extraction. Most individuals were released back at the locality and allowed to regenerate.

Morphometric analysis

Morphological analysis of a total number of 51 individuals of *A. colchicus* was carried out with a total number of 21 morphological characteristics recorded for each crayfish, following Sint *et al*. (2005). Particular characteristics were measured with an electronic caliper to the nearest 0.1 mm. Any injured, damaged and regenerated claws were not used for measurements. All measurements were inverted for a 17 indices: CPL/CLL—length of the claw palm to the claw length; CLW/CLL—claw width to the claw length; HEL/TL—head length to the total length; CEW/TL—width of the carapace at the hind edges to the total length; CPW/TL—carapace width to the total length; ABW/TL—abdomen width to the total length; ABH/TL—abdomen height to the total length; TEW/TL—telson width to the to the total length; ROL/TL—rostrum length to the total length; CLH/CLW—claw height to the claw width; CFL/CPL—length of the claw finger to the length of the claw palm; TEL/TEW—telson length to the telson width; ROL/ROW—rostrum length to the rostrum width; ABL/TL—abdomen length to the total length; CPX/TL—carapace length (rostrum length, head length, areolar length are included) to the total length; CPX/CPW—carapace length (rostrum length, head length, areolar length are included) to the carapace width; HEL/HEW—head length to the head width. Further, individuals of *A. astacus* ($n = 100$), originating in Podolský brook, Vápenný Podol village, Czech Republic, were also measured and analyzed to contrast morphological differences between species. Multivariate redundancy analysis (RDA) was performed to describe differences between *A. colchicus* and *A. astacus* using the software Canoco version 5.0 (ter Braak & Šmilauer 2012). Monte-Carlo permutation test (4999 permutations, blocks defined by covariates) was applied for testing significance of the RDA model, that is, differences in morphometrics between both species, with sex as a covariate. Analysis of covariance (ANCOVA), run in Statistica 12 (StatSoft Inc.), was used to compare differences of individual morphometric indices between 2 species with sex as a covariate as well. Since some of the data did not have normal distribution (tested by Shapiro–Wilks test), Box-Cox transformation was applied. Supplementary pictures of body habitus, abdominal pleura, and carapace were done using a male individual from Khobi river and a female from Lashe river.

Morphological characteristics described by Füreder and Machino (2002) and keys to palaearctic fauna (Rogers & Thorp 2019) were used to genera determination, while study of Albrecht (1982) and Starobogatov (1995) to check specific differences of *A. astacus*.

Molecular data collection

Genomic DNA extraction and PCR amplification was done according to Bláha *et al*. (2016) using 36 *A. colchicus* individuals. Three mitochondrial genes, cytochrome c oxidase I (COI), 16S and 12S rRNA, and nuclear histone H3 (H3) were applied (details in Table 2). Product purification and sequencing was performed by Macrogen Inc., Korea.

Phylogenetic analysis

All newly obtained sequences were deposited in GenBank under accession numbers listed in Table 1. Sequences were aligned with MAFFT version 7 (Katoh *et al*. 2002) implemented in GENEIOUS version 8.0.5 (www.geneious.com; Kearse *et al*. 2012); COI and H3 alignments were translated into amino acids to check for indels and stop codons. Analysis of synonymous and non-synonymous substitutions were done in DnaSP version 5.10.01 (Librado & Rozas 2009) to omit usage of pseudogenes. Pairwise model-corrected genetic distances were calculated for each gene in PAUP∗ version 4.02b (Swofford 2001), for which we report the mean genetic distance in order to compare the relative amounts of divergence of each gene and among species. In addition to the *A. colchicus* samples from Georgia, available sequences of 5 Astacidae species [*A. astacus*, *P. leptodactylus*, *P. pachypus*, *Austropotamobius pallipes* (Lereboullet, 1858), and *A. torrentium*] as an ingroup, and *Pacifastacus leniusculus* (Dana, 1852) as an outgroup

Table 1 Information on sampling locality, type of habitat, geographic coordinates, number of analyzed individuals (*N*), and GenBank accession number of particular haplotypes (H) together with GenBank accession number of other species used in this study

			COI			16S			12S			H3		
Locality	Habitat type	Geographic coordinates	*N*	H	GenBank Acc. number	*N*	H	GenBank Acc. number	*N*	H	GenBank Acc. number	*N*	H	GenBank Acc. number
Astacus colchicus														
Lashe river, Tkemlovani	Small, slow flowing mountain river	42°11′12.43″N 43°18′28.65″E	11	3	MT483861, MT483865	7	4	MN809183-186	11	2	MN809190, MN809194	6	2	MT237558, MT237559
Papantskuri Lake, Sakirio	Lake	42°22′26.1″N 1°49′50.0″E	9	2	MT483860, MT483862	9	2	MN809187, MN809189	8	1	MN809191	5	2	MT237559
Khobi river, Khobi	Big river	42°19′46.4″N 41°54′10.4″E	1	1	MT483864	1	1	MN809188	1	1	MN809198	1	1	MT237560
Tributary of Choloki river, Kakuti	Small brook	41°51′53.3″N 41°56′34.9″E	6	1	MT483863	6	1	MN809187	6	2	MN809192, MN809196	4	2	MT237559, MT237561
Kintrishi river, near Nakaidzeebi	Medium size mountain river	41°48′14.4″N 41°48′12.2″E	1	1	MT483860	1	1	MN809187	1	1	MN809197	1	1	MT237559
Sepa river, near Ekaldidi	Medium size lowland river	41°59′06.4″N 41°49′03.0″E	9	1	MT483860	3	1	MN809187	6	2	MN809193, MN809195	4	3	MT237558-560
Astacus astacus					KX279348			KX279348			KX279348			KX444601
Pontastacus leptodactylus					KX279350			KX279350			KX279350			KX444603
Pontastacus pachypus					KX018609			KX018612			KX018615			MT597429
Austropotamobius torrentium					NC033504			NC033504			NC033504			KX444605
Austropotamobius pallipes					NC026560			NC026560			NC026560			KX444604
Pacifastacus leniusculus					NC033509			NC033509			NC033509			KX444621

Table 2 Primer sequence used for amplification with annealing temperatures

Primer	Sequence (5′ – 3′)	Annealing temperature (°C)	Source
LCO 1490	GGTCAACAAATCATAAAGATATTGG	50	Folmer *et al*. (1994)
COI 703r	CCRCCMGCAGGRTCAAAGAA		This study
16S ar	CGCCTGTTTAACAAAAACA	55	Simon *et al*. (1994)
16S br	CCGGTCTGAACTCAGATCACGT		
16S brAst	CCGGTRTGAACTCAGATCACGT		This study
12S F5357	ATYTTGTGCCAGCAGTCGCG	61	This study
12S R5937	CTTAAATGAAAGCGACGGGC		
H3 AF	ATGGCTCGTACCAAGCAGACVGC	50	Colgan *et al*. (1998)
H3 AR	ATATCCTTRGGCATRATRGTGAC		

corresponding to the COI, 16S and 12S rRNA mitochondrial genes, and nuclear H3 genes were downloaded from NCBI's GenBank (Table 1). Therefore, phylogenetic relationships were reconstructed using the concatenated dataset from 7 crayfish species. The final length of particular sequences used for alignment was 648 bp for COI, 489 bp for 16S, 471 bp for 12S, and 327 bp for H3 or 1935 bp in concatenated alignment. jModel Test 2.1.7 (Darriba *et al*. 2012) was used to find the optimal model of substitution for a particular gene based on Bayesian information criterion. The optimal models found for COI, 16S, 12S, and H3 alignment were HKY + G, TPM1uf + I, HKY + G, and K80, respectively. A maximum likelihood (ML) tree was constructed in RAxML version 7.2.871 implemented in GENEIOUS, with each partition having its own GTRGAMMA model, and nodal support of the tree was tested via 2000 bootstrap replicates. Bayesian analyses were conducted in MrBayes 3.2.4. (Ronquist *et al*. 2012) applying the specific nucleotide substitution model for a particular gene sequence set. The generated log files were analyzed with TRACER (Rambaut *et al*. 2013) to confirm that effective sample size values were >200 for all parameters, and that stationarity between particular runs was ensured after the burn-in period.

RESULTS

Morphology

All 51 analyzed individuals of *A. colchicus* demonstrated following characteristics clearly indicating affiliation with genus *Astacus*: male pleopod II without ventral process (talon) and abdominal somites II and III with pleura rounded or angular, lacking spines. Individuals of *A. colchicus* demonstrated also more rounded abdominal somites in comparison to *A. astacus*, which have abdominal somites wedge-shaped (Fig. S2, Supporting Information). *Astacus colchicus* had well-developed posterior postorbital ridges, approximately 2 times longer than anterior ones and posteriorly curved inward (Fig. S3, Supporting Information). Details about morphometry and sex of individuals are shown in the Tables S1 and S2, Supporting Information. Habitus of the crayfish can be seen in Fig. S4, Supporting Information.

RDA analysis (Fig. 1) explained 28.43% of variation. Differences found between 2 analyzed species were highly significant ($P = 0.001$, F-like statistic value = 53.0). Comparison of individual indices revealed almost all indices differed significantly except ABW/TL, TEW/TL, CLH/CLW, TEL/TEW, and ABL/TL (Table S3, Supporting Information).

Sequence data and phylogenetic analysis

From 36 analyzed specimens of *A. colchicus*, 36 sequences were recovered for COI, 27 sequences for 16S, 33 sequences for 12S, and 21 sequences for H3 (Table 3). The combined mitochondrial and nuclear dataset consisted of 16 haplotypes of the ingroup (11 of *A. colchicus*, 1 of *A. astacus*, *P. leptodactylus, P. pachypus*, *A. torrentium*, and *A. pallipes*, respectively), and 1 haplotype (*P. leniusculus*) of the outgroup.

The mean model-corrected sequence distances among *A. colchicus* and *A. astacus* were 10.9% for COI, 9.6% for 12S, 6.5% for 16S, and 1.1% for H3, while mostly similar distances were recorded for *P. leptodactylus* or *P. pachypus* (Tables S4 and S5, Supporting Information).

All the combined mtDNA and nDNA phylogenetic analyses recovered sequences of *A. colchicus* comprising a monophyletic clade with high statistical support. The other 6 monophyletic clades represented rest of species

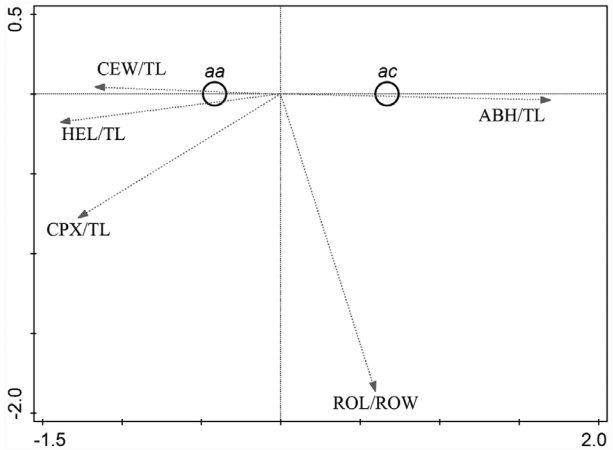

Figure 1 Morphometric characteristics-species biplot of RDA on standardized functional trait data. This diagram summarizes the variation in morphometric composition explained by species, after accounting for the effects of covariates (sex). The first 5 morphometric indices with highest fit are shown by given arrows and labeled by particular abbreviations (ABH/TL abdomen height to body total length, HEL/TL head length to body total length, CPX/TL carapace length to body total length, CEW/TL carapace width to body total length, and ROL/ROW rostrum length to rostrum width). The centroids of species are indicated by black empty circle (aa *Astacus astacus*, ac *A. colchicus*). The distance between the species centroids approximates the average dissimilarity of morphometric composition between these two species being compared as measured by their Euclidean distance

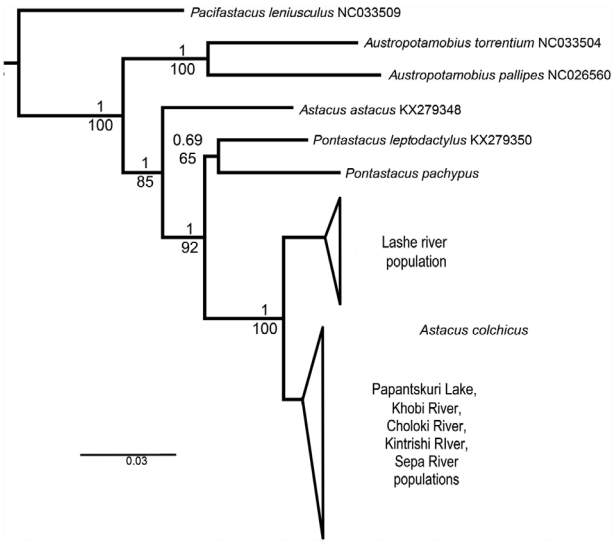

Figure 2 Bayesian tree reconstruction based on concatenated data set. Bayesian inference and Maximum likelihood bootstrap are displayed above and under each node, respectively

used in the analysis also with high statistical support in Bayesian analyses (Fig. 2). *Astacus colchicus* was shown to be a sister clade to *P. leptodactylus* and *P. pachypus*.

DISCUSSION

Our molecular and morphometric analysis revealed and indicated a unique phylogenetic and morphometric pattern of *A. colchicus* populations from Georgia, and corroborated correct taxonomic assignment to genus *Astacus* and species status as well. Despite a high morphological similarity with *A. astacus*, morphometric analysis revealed several characteristics useful for the differentiation from this species, namely ABH/TL, HEL/TL CPX/TL, CEW/TL, and ROL/ROW showing the most obvious differences (Fig. 1; Tables S1 and S2, Supporting Information). The different ratio of head length comparing to carapace or total length could be easily recognized from the drawings of Albrecht (1982) or pictures of these two crayfish species presented in this study (Figs S2–S4, Supporting Information). Besides characteristics dependent on measuring of individuals and calculating the particular indices, for most field researchers, there are also several distinguishing morphological characteristics without need of measuring or keeping the animals for necessary time. Especially, shape and length of posterior pair of postorbital ridges and shape of abdominal somites are well distinctive (Albrecht 1982; Starobogatov 1995).

Table 3 Nucleotide polymorphism of *Astacus colchicus* sequences based on mitochondrial (COI, 16S, 12S) and nuclear (H3) data

Gene	Length (bp)	VS	PI	N	H	Hd (SD)	π (SD)
COI	648	24	22	36	8	0.862 (0.022)	0.015 (0.002)
16S	484	7	5	27	7	0.598 (0.108)	0.004 (0.001)
12S	465	24	21	33	9	0.856 (0.030)	0.018 (0.001)
H3	327	2	2	21	4	0.628 (0.092)	0.002 (0.001)

VS, number of variable sites; PI, number of parsimony informative sites; N, number of sequences used; H, number of haplotypes determined; Hd, haplotype diversity; π, nucleotide diversity.

Although sympatry of these 2 species is improbable regarding mainly to endemism of *A. colchicus*, described morphological characteristics could be useful especially when analyzing old museum samples. The characteristics used to distinguish genera *Astacus* and *Pontastacus*, the shape of abdominal somites being with acute spines at their ventral ends and presence of abdominal process in second male pleopod in *Pontastacus*, are simply summarized in Rogers and Thorp (2019) or Füreder and Machino (2002). Furthermore, comparing to *P. leptodactylus*, *A. colchicus* has immovable finger of chela with incision in median part of inner margin, with *P. leptodactylus* having no such an incision. Sometimes, the incision of *Astacus* could be only weakly expressed; then there are clearly visible tubercles at the end of immovable finger. All these characteristics seem to be solid enough across a wide area of occurrence of both genera/species.

The results of molecular study clearly indicate deep molecular divergence with relatively high molecular distance for particular genes (Tables S4 and S5, Supporting Information). Although the high morphological similarity with *A. astacus* has led some scientists to assign the populations from Georgia as its subspecies (Albrecht 1982; Bott 1950), the others correctly appraise all indicia to assign it to valid species status (Karaman 1962; Starobogatov 1995; Crandall & De Grave 2017). At the same time, it automatically brings up a question about the phylogenetic position and species status of *A. balcanicus balcanicus* Karaman, 1929 populations, also presented by the authors of the recent updated classification of freshwater crayfishes as a valid species (Crandall & De Grave 2017). This species has a similar status, being morphologically very similar to *A. astacus* and by occurrence restricted to the area of the Vardar river system (Greece, Macedonia) and Ohrid Lake (Macedonia) (Albrecht 1982, 1983). Recently, Laggis *et al*. (2017) have analyzed *A. astacus* populations from Greece (thought to be *A. balcanicus*) at the southernmost area of the species distribution and identified 2 new phylogroups different from other known European ones. However, molecular distances recorded were much lower (up to 4.1% for COI and 1.9% for 16S) compared to those recorded between *A. colchicus* and *A. astacus* in our recent study, so not suggesting species status (Laggis *et al*. 2017). The *A. balcanicus* issue could hopefully be resolved by sampling and genetic analysis of *Astacus* species from the type locality (Ohrid Lake). This lake harbors many endemic organisms; however, the only crayfish species is referred to as *A. astacus* and its population density is quite low (Albrecht & Wilke 2008). Nevertheless, the past history of this area in Europe is very rich in geological processes (the Alpine–Carpathian–Dinaric orogeny) affecting the establishment of many aquatic species including crayfish (Copilaş-Ciocianu & Petrusek 2015; Mráz & Ronikier 2016; Pârvulescu 2019; Pârvulescu *et al*. 2019). Moreover, the Balkan region is considered one of the major glacial refugia for many species during the Pleistocene climatic oscillations (Hewitt 2004), its high genetic diversity of species later spreading to the rest of the unglaciated areas. A further revision of particular species/subspecies within *Astacus* is still needed to clarify the taxonomy of this dominant European crayfish taxon.

The high molecular divergence between *A. colchicus* and *A. astacus* is most likely caused by past paleogeographical events in the Ponto-Caspian region and thus a relatively long-time separation. Regarding the fauna of the Caucasus and their evolutionary relationships to other European relatives, there is a certain pattern driven by climatic and landscape changes shaping the establishment of a new species (Tarkhnishvili 2014). The earliest range fragmentation between the Caucasus, Western Europe, and Mediterranean area was linked to the early and middle Miocene (22–13 Mya) (Popov *et al*. 2004). This event caused a split between the *Mertensiela caucasica* and its closest relatives *Chioglossa lusitanica* Bocage, 1864 (Veith *et al*. 1998; Weisrock *et al*. 2001) as well as a split between *P. caucasicus* and its closest western European relative, *Pelodytes punctatus* (Daudin, 1802) (Garcıa-Parıs *et al*. 2003; Veith *et al*. 2006). Further Miocene–Pliocene range fragmentation linked to the Messinian salinity crisis (ca. 6 Mya) resulted in a global decline of humidity, environment instability and further landscape fragmentation, and finally, middle and late Pliocene range fragmentation after the Messinian salinity crisis and before the first glacial waves (Tarkhnishvili 2014). It caused a later separation between Caucasian populations of *Lissotriton vulgaris lantzi* (Krasavtzev, 1940) and its European populations (Babik *et al*. 2005) as well as between *Rana macronemis* Boulenger, 1885, and its closest western European relatives (*Rana* group, Veith *et al*. 2003). All of these events caused a decline in temperature followed by declines of evaporation and precipitation, resulting in landscape fragmentation (Zachos *et al*. 2001; Tarkhnishvili 2014), which might also have substantial effects for the origin of *A. colchicus*. Consequently, repeated isolation/connection between ancient Balkan and Anatolian (Pontides) lands throughout the Miocene could be thought as a basis for split between ancestors of *A. astacus* and eastern Ponto-Caspian crayfish clade, while a subsequent orogeny and range fragmentation in Anatolian/Caucasian areas might cause a divergence between *A. colchicus* and other *Pontastacus* lineages.

Nevertheless, it is still not easy task to suggest the most likely scenario about the origin of *A. colchicus* as well as of other European crayfish species without understanding the context of European crayfish species evolutionary relationships. Moreover, depending on the methods applied, recent studies using significant part of the European crayfish species have resulted in quite a wide frame of their origin, encompassing the period from the Cretaceous (Porter *et al*. 2005; Toon *et al*. 2010; Bracken-Grissom *et al*. 2014) to the Miocene (Klobučar *et al*. 2013; Jelić *et al*. 2016). Pârvulescu *et al*. (2019) pointed out discrepancies between age estimates based on molecular clocks, using common standard arthropod substitution rates for mtDNA genes (Knowlton & Weigt 1998; Schubart *et al*. 1998), and those originating from applying fossil calibrations or paleogeographic events (Porter *et al*. 2005; Breinholt *et al*. 2009; Bracken-Grissom *et al*. 2014; Pârvulescu *et al*. 2019). According to recent studies (Parham *et al*. 2012; Warnock *et al*. 2015), usage of proper fossil calibration or paleogeographic events is the most suitable way to obtain the most realistic age estimates of particular nodes in a time tree. However regarding European crayfish history, only a few available fossil records exist (Garassino 1997; Taylor *et al*. 1999; Rode & Babcock 2003). Moreover, most of them have a too unclear taxonomic status to be obvious what current species are their descendants (Rode & Babcock 2003; Karasawa *et al*. 2013) or do not fit into current theories about the origin of European crayfish (Buscalioni & Poyato-Ariza 2016). Therefore, disentangling the origin and history of European or, in the more general context, of Northern Hemisphere crayfish is a challenging task requiring advanced analysis of most of the European crayfish species and careful choice of appropriate calibrations for age estimates.

CONCLUSION

This study provides a novel insight into European crayfish phylogeography including the Caucasian endemic crayfish *A. colchicus*. Both morphological and molecular analyses corroborated valid species status of the populations from Georgia and mentioned helpful characteristics used for species identification. Moreover, molecular part, comprising most of the European crayfish species in the genera *Astacus* and *Pontastacus*, has also resulted in different topology compared to previous studies using assemblages of fewer European species. The phylogenetic position of *A. colchicus* together with its zoogeography matches current ideas about the origin of European crayfish species. Future studies should aim at revision of *A. balcanicus* morphology and phylogenetic position. Furthermore, specific information is still missing with respect to the status of rich taxa assigned to Eastern European crayfish *Pontastacus*, mostly characterized morphologically without molecular methods.

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Map of Georgia showing sampling sites during the crayfish survey. Red full circle indicates population of *Astacus colchicus*, orange triangle - sympatric population of *A. colchicus* and *Pontastacus leptodactylus* while green square - population of *P. leptodactylus*.

Figure S2 Pleura of abdominal somites of Astacus colchicus male (A), female (B) and *A. astacus* male (C).

Figure S3 Carapace of *Astacus colchicus* (A) and A. astacus (B) male.

Figure S4 Habitus of *Astacus colchicus* male (A), female (B) and *A. astacus* male (C).

Table S1 Comparison of the morphometric indices of *Astacus astacus* and *A. colchicus* individuals with mean values and standard deviation (SD)

Table S2 Comparison of the morphometric indices of *Astacus astacus* and *A. colchicus* individuals with mean values and standard deviation (SD)

Table S3 ANCOVA results for comparison of particular morphometric indices between *A. colchicus* and *A. astacus*

Table S4 Mean model corrected sequence distances among species

Table S5 Mean model corrected sequence distances among species

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Supplement 4

Bláha, M., Žurovcová, M., Kouba, A., Policar, T., Kozák, P., 2016. Founder event and its effect on the genetic variation in translocated populations of noble crayfish (*Astacus astacus*). Journal of Applied Genetics 57: 99-106.

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ANIMAL GENETICS • ORIGINAL PAPER

Founder event and its effect on genetic variation in translocated populations of noble crayfish (*Astacus astacus*)

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Abstract Establishing translocated populations is a common process to preserve and maintain genetic diversity of threatened species. In 2001, three translocated populations of noble crayfish (*Astacus astacus*) were established in the Czech Republic, founded by either adult or juvenile individuals from three particular source populations. We assessed genetic diversity at seven microsatellite loci after one decade (assumed three generations) from establishment. Although the translocated populations exhibited a slight but non-significant reduction in genetic diversity (A_R=2.2–5.0; H_O=0.11–0.31), the most striking result was generally very low genetic diversity in source populations (A_R=3.0–5.3; H_O=0.15–0.38). Similarly, a high degree of inbreeding (F_{IS}=0.36–0.60) demonstrates the nature of source populations, already affected by isolation and small size. In spite of that, based on the results of this study, the establishment of new translocated noble crayfish populations was successful, since there is no significant decline in genetic variability and all populations are still viable. Although source populations did not exhibit high genetic diversity, their distinctiveness makes them possible to use for conservation purposes. Continued monitoring is necessary to track the long-term progress of the translocation program, including other parameters describing the state of the population, such as the occurrence and frequency of diseases or morphological changes.

Keywords Bottleneck · Conservation · Homozygote excess · Microsatellites

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Introduction

It is widely assumed that the present distribution of native crayfish species in Europe has been mostly determined by the last ice age and species recolonization from glacial refugia afterwards (Hewitt 1996). Recently, their distribution has been heavily affected by human translocations, especially in the region of central and northern Europe (Albrecht 1983; Skurdal et al. 1999; Stefani et al. 2011). This is particularly the case for noble crayfish (*Astacus astacus*), which has been the target of extensive relocations, being a valuable trade article from the distant past to recent times (Skurdal and Taugbøl 2002; Sint and Füreder 2004). Moreover, central European populations of noble crayfish were substituted during the first recorded outbreaks of crayfish plague in the 19th century from the eastern part of the species distribution (Skurdal and Taugbøl 2002; Jussila et al. 2015).

The knowledge about the present European population structure and genetics of noble crayfish has recently been substantially improved (Schrimpf et al. 2011, 2014; Gross et al. 2013). Still, very little research has been conducted on the translocation and translocated populations of native crayfish, mostly without genetic background information (e.g., Keller 1999; Sint and Füreder 2004; Horton 2009). In spite of that, repatriation of native crayfish species have been suggested as

an essential part of management and conservation strategies in Europe (Schulz et al. 2002; Souty-Grosset and Reynolds 2009). However, without the known genetic structure of source populations and influence of a limited number of transferred specimens, these activities should be considered carefully with respect to the genetic diversity of crayfish in the area of interest. The use of genetically diverse populations for reintroduction to new localities has been proposed (Souty-Grosset and Reynolds 2009; Kozák et al. 2011). Translocations should respect larger geographic units as separate management units, as suggested by Weiss et al. (2002) and corroborated by Schrimpf et al. (2014) when analyzing noble crayfish, such that each river catchment should be treated as a distinct management unit. On the other hand, mixing different populations, which could lead to genetic homogenization, is considered a negative aspect (Schrimpf et al. 2014).

The other point is the current state of noble crayfish populations, which are, in most cases, isolated or reduced by diseases or inconvenient habitat conditions, and their long-term survival is mostly dependent on the extension and improvement of natural habitats (Meyer et al. 2007). Finding and creating so-called "ark sites" with suitable conditions for crayfish and isolated from non-indigenous species should be taken into consideration (Peay 2009), since there is a permanent strong threat from the rapid spreading of non-indigenous crayfish species (Gherardi 2006; Scalici et al. 2010; Kouba et al. 2014). These species can out-compete indigenous ones, but mainly are carriers of a pernicious pathogen, *Aphanomyces astaci*, which usually eliminates all survivors (Bohman et al. 2006; Kozubíková et al. 2009).

The aims of this study were to evaluate the successfulness of translocation of particular noble crayfish populations, and to measure the genetic diversity among individuals in translocated populations and make a comparison with the genetic diversity and structure in source populations of noble crayfish.

Materials and methods

Source area

The source area was located in two different sites. Crayfish specimens were taken from three small reservoirs: Světlohorská [SV; see Policar and Kozák (2005) for detailed information about the water chemistry and population status] and Kramata (KR), both in the Šumava National Park close to the town of Vimperk, and Zámecký pond (ZA) in the Janovice forest park close to the town of Chrudim (Fig. A in the supplementary material). All of the ponds are around 1 ha in size and are placed in the forest area.

Translocated populations

The first repatriation attempts took place during 2000. More than 40 different small brooks and reservoirs were monitored for water quality and convenient conditions for introduction in South Bohemia. After this assessment, crayfish specimens from donor populations were transferred into two ponds in the Natural Monument Písecké Mountains. The first pond, "U Včelníku" (VC), was stocked with adult females and males (30 and 40 individuals, respectively) from the source population Kramata, whereas the second pond, "U Sudu" (SU), was stocked with 0+ juveniles (800 individuals from 20 females) from the Zámecký source pond. Both ponds are used for extensive fish culture and any alterations in the water level or draining must be approved by local authorities from the municipal board of nature protection. Borová Lada (BL) is a population stocked in a small pond in NP Šumava close to the village of the same name. Stocked juveniles originated from both Kramata and Světlohorská reservoir stocks. Úbislav (UB) is a newly established population in a small pool, where the originally stocked individuals were only four berried females in 1988. This population underwent restriction to ca. 50 % of adults in 2002, resulting in 170 adult specimens presented in the pool. Further, the source population was extinct; thus, we could not include it in the analyses. This population was added to the analyses for comparison of the extremely low numbers of the initial stocking. All of the information is summarized in Table 1.

Sample collection and DNA isolation

During routine monitoring in 2009 and 2012, samples of pereiopod were taken from specimens of noble crayfish. This does not threaten the crayfish integrity, as appendages regenerate upon moulting. Sampled individuals were then released back to minimize the stress.

Total genomic DNA was extracted from 20 to 50 mg of muscle tissue dissected from samples of pereiopod stored in pure ethanol until DNA extraction using the NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), following the manufacturer's protocol.

Microsatellite analyses

A set of seven microsatellite loci, each with dinucleotide repeat (Aas2, Aas5, Aas6, Aas11, Aas766, Aas1198, and Aas3950), was applied following Kõiv's polymerase chain reaction (PCR) protocol (Kõiv et al. 2008, 2009). Fluorescent detection genotyping was performed with each primer separately for a given locus labeled with WellRED fluorescent dyes (Proligo, Boulder, CO) to enable the determination of allele sizes on a CEQ 2000XL (Beckman Coulter, Fullerton,

Table 1 List of localities, geographic location of noble crayfish (*Astacus astacus*) populations, and their characteristics

Population	Size (m^2)	Coordinates N/E		Year of establishing/ sampling	Repatriation	Sample size	Origin of translocated population
Source							
KR	10,136	49.061	13.717	NA/2009 and 2011		20	–
SV	3,200	49.006	13.729	NA/2009 and 2012		25	–
ZA	19,500	49.940	15.664	NA/2012		34	–
Translocated							
VC	1,771	49.222	14.267	2000/2012	40 ♂, 30 ♀	14	KR
BL	470	48.983	13.661	2001/2012	Three times tens of juveniles	19	KR + SV
SU	3,015	49.207	14.282	2000/2012	800 individuals 0+ from 20 ♀	21	ZA
UB	116	49.120	13.659	1988/2012	Four berried ♀	21	–

KR Kramata, *SV* Světlohorská, *ZA* Zámecký, *VC* U Včelníku, *BL* Borová Lada, *SU* U Sudu, *UB* Úbislav

CA), an automated DNA sequencer with a 400-bp internal size standard.

Population genetic diversity

Analyses were made using GenAlEx 6.5 (Peakall and Smouse 2006, 2012) and Genepop (Rousset 2008). Overall deviations from Hardy–Weinberg equilibrium (HWE) were tested using the exact probability test of Guo and Thompson (1992) and pairwise linkage disequilibrium between loci was tested using Fisher's exact test, also in GenePop (Rousset 2008). These tests use a Markov chain method (1,000 dememorization steps, 100 batches, and 1,000 iterations per batch). When applicable, statistically significant levels were confirmed applying a sequential Bonferroni correction (Rice 1989). Null alleles and scoring errors were verified using MICROCHECKER 2.3.3 (van Oosterhout et al. 2006) for each population at each locus, performing 1,000 randomizations. The parameter θ (Weir and Cockerham 1984), which is analogous to Wright's F_{ST} (Wright 1965), and its significance was calculated for individual loci using the FSTAT v1.2 program (Goudet 1995).

Utilizing the R package poppr (Kamvar et al. 2013), all samples were also analyzed as multilocus genotypes (MLGs), i.e., genotypes resulting from combining alleles at all microsatellite loci detected. This package allows the calculation of numbers of unique MLGs and their distribution in populations, as well as the evenness and the adjusted Shannon–Weaver diversity index (H) (Shannon and Weaver 1949). This latter index (H) was calculated as $H=-\Sigma p_i \ln p_i$, where p_i is the relative frequency of the ith MLG and expressed as e^H to obtain a parameter proportional to the actual genotypic richness in each population (Llewellyn et al. 2003).

The effective population size (N_e) was assessed using the molecular coancestry method (N_eCo) of Nomura (2008) and the bias-corrected version of the method based on linkage disequilibrium (N_eLD; Waples 1989; Waples and Do 2008), as implemented in NeEstimator V2 (Do et al. 2014). To reduce bias due to low-frequency variants, a threshold of 0.05 for the allelic frequency was chosen.

A possible bottleneck effect was investigated in all populations (the source populations were inspected for comparison) using the program BOTTLENECK (Cornuet and Luikart 1996; Piry et al. 1999). As recommended by the software authors, the most powerful two-phase mutation model (TPM) was utilized assuming 90 % stepwise mutation, and the final analysis was based on the Wilcoxon signed-rank test (Luikart et al. 1998).

Allelic richness, observed heterozygosity, F_{IS} estimates, and effective population size obtained in the previous analyses were compared by either the Kruskal–Wallis or the one-way analysis of variance (ANOVA) tests to assess the possible differences between the source and translocated populations.

Population structure analysis

Basic genetic differences over all loci among all population pairs were estimated using Nei's standard genetic distance (Nei 1973) and F_{ST} (Wright 1965). To visualize the patterns of genetic relationships contained in the distance matrix, the grouping of populations was performed with a principle coordinate analysis (PCoA) in GenAlEx 6.5 (Peakall and Smouse 2006, 2012). Since the Mantel test of correlation F_{ST} versus Nei's genetic distance was significant (r=0.5402, P<0.001), parameters can be used interchangeably and, in that case, we adopted Nei's distance for the mentioned analyses.

Results

Genetic diversity

Overall, a total of 154 individuals were analyzed with seven polymorphic microsatellite loci, yielding 60 different alleles.

Monomorphic loci were observed in the source population SV (*Aas6*) and translocated populations VC (*Aas776*), BL (*Aas11*), and UB (*Aas2*, *Aas11*). The characteristics of the loci are given in Table 2.

The highest number of private alleles had source population ZA, with translocated population SU being the second highest, while none were found in VC and UB (both translocated populations; Table 3).

In all populations, significant departures from HWE were detected, with no clear pattern across loci and populations. Approximately two-thirds of the tests showed a significant deficiency of heterozygotes (Table A in the supplementary material). Null alleles were detected at all loci, although their distribution in populations was not uniform (Table B in the supplementary material). Since all individuals were amplified for all loci tested and no genotypic failure or double-null homozygotes were observed, we did not discard any loci from further analysis. No significant linkage disequilibrium was detected between pairs of loci (only one test remained significant after Bonferroni correction).

The genetic diversity characteristics of particular populations considering all microsatellite loci are given in Table 3.

There were 145 MLG detected in the whole data set, with 142 unique MLGs and only three MLGs found repeatedly. Of these, the first MLG was shared by two individuals from BL and UB, the second was recorded in VC and UB, and the third MLG was found in BL, VC, and UB. The distribution of the MLGs is reflected in the evenness, which equals 1 when all genotypes occur at the same frequency, regardless of richness.

The effective population size (N_e) calculated using the coancestry method gave less contrasting results, with KR showing the lowest effective population size (1.4) and ZA the largest (11.4); both these populations are the source populations (Table C in the supplementary material). The LD method resulted in the lowest N_e for source population SV (6.3), again with ZA reaching the highest value (82.2). N_e in translocated populations appears reduced compared to the donor ZA and translocated SU; however, in other translocated and source populations, the pattern was opposite. Moreover, under the TPM, the Wilcoxon test did not reveal a significant pattern of heterozygosity excess or deficiency (P=0.05–1.00) (Table C in the supplementary material).

Comparisons of genetic diversity indices between the source and translocated populations were conducted in one-way ANOVA (N_eCo, N_eLD), and within source, within translocated, and among all populations with the Kruskal–Wallis test (Ar, H_O, F_{IS}). None of these tests yielded any significant differences at any level of comparisons (Table D in the supplementary material).

Table 2 Microsatellite loci scored in noble crayfish (*Astacus astacus*) with their number of alleles, observed heterozygosity (H_O), and θ_{ST} values for the total data set. *SE* standard error

Locus	Number of alleles	H_O	θ_{ST} (SE)
Aas2	7	0.276	0.209 (0.083)
Aas5	14	0.133	0.132 (0.068)
Aas6	9	0.316	0.326 (0.091)
Aas11	3	0.142	0.137 (0.042)
Aas776	7	0.021	0.223 (0.048)
Aas1198	8	0.136	0.071 (0.028)
Aas3950	12	0.330	0.077 (0.023)

Population differentiation

Pairwise F_{ST} estimates were all significant, indicating the moderate differentiation among populations (Table 4). The pairwise F_{ST} of source populations ranging from 0.158 to 0.230 is comparable to the range of translocated populations (0.078 to 0.259), with the smallest and largest difference found between source and translocated populations (0.035 for ZA vs. SU; 0.269 for SV vs. SU).

The genetic relationship of populations is depicted in the PCoA plot (Fig. B in the supplementary material). The first coordinate accounts for 50.54 % and the second for 22.47 % of the total variance, and distinguish several groups. The most distinct is the pair of populations ZA and SU, which is clearly separated from all the other populations. The pair KR and VC appears to be closer to the rest of the translocated populations, while SV stands somewhat separated (reflecting the translocation process as well as geographic position).

Discussion

Levels of genetic diversity

The genetic diversity of source populations revealed in this study was similar to those found in noble crayfish populations from central and northern Europe (Gross et al. 2013). In terms of the overall observed heterozygosity (H_O=0.19), the analyzed populations showed lower values than usual for central and western Europe, except for populations ZA and SU (H_O=0.502; Gross et al. 2013; H_O=0.306; Schrimpf et al. 2014). Furthermore, source populations (SV, KR) and one translocated population (SU) appeared to have higher values from previously published results (Schrimpf et al. 2014). Although decrease in the genetic diversity is a common phenomenon in translocated populations (e.g., Frankham 1995; Witzenberger and Hochkirch 2011), we found no significant decline in genetic diversity and differences between pairs of source and translocated populations (Table D in the supplementary material).

Table 3 Summary statistics of noble crayfish (*Astacus astacus*) populations. Sample size (*N*); number of multilocus genotypes (*MLGs*); Shannon–Weaver index of diversity (e^H); evenness (*E*); average allelic richness (*A*r); average number of different alleles (*A*a); total number of effective alleles over seven microsatellite loci (*A*e); number of private alleles per population (*A*pr); observed heterozygosity (H_O); expected heterozygosity (H_E); F-statistics (F_{IS}) considering all microsatellite loci

Population	*N*	MLGs	e^H	*E*	*A*r	*A*a	*A*e	*A*pr	H_O	H_E	Multilocus F_{IS}
KR	20	20	20.00	1.00	3.62	3.86	2.02	2	0.164	0.455	*0.601*
SV	25	22	21.18	0.95	3.07	3.29	2.14	1	0.154	0.412	*0.541*
ZA	34	34	33.99	1.00	5.28	6.29	3.78	11	0.382	0.666	*0.364*
VC	14	12	11.07	0.87	2.86	2.86	2.16	0	0.112	0.384	*0.637*
BL	19	19	18.99	1.00	3.38	3.57	2.38	1	0.105	0.511	*0.798*
SU	21	21	21.01	1.00	4.98	5.57	2.88	3	0.313	0.611	*0.486*
UB	21	16	15.09	0.94	2.21	2.29	1.61	0	0.122	0.273	*0.695*
Total/mean	154	142	134.42	0.94	5.13	3.96	2.42	2.57	0.193	0.473	0.579

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On the contrary, we recorded high rates of inbreeding (F_{IS}, 0.364–0.798) across all populations, which strongly contrasted with other noble crayfish European populations (−0.207–0.198; Gross et al. 2013); on the other hand, similar and even higher values (0.261–1.000) were found in white-clawed crayfish (*Austropotamobius italicus*; Matallanas et al. 2012). The authors explained this state as partly an effect of the low number of sampled specimens per population (6–15) and partly as a bottleneck across all populations caused mainly by droughts, diseases outbreaks, and competition with non-native crayfish species. Obviously, such high rates of inbreeding in the translocated populations analyzed can be partly explained by the limited number of stocklings and the lower number of sampled individuals. However, this phenomenon is rather surprising in source populations and is probably for other reasons—historical and ecological. Unfortunately, there are no relevant historical records about management not only in these localities, but in most of the sites with noble crayfish in the Czech Republic. We presume that especially the small size of localities and mating of close relatives cause an increase of inbreeding. Also, other factors can account for the detected homozygotes excess, namely null allele presence, Wahlund effect, or non-random sampling. However, none of these scenarios likely influenced the analyzed population to such an extent like the two main effects mentioned previously.

Bottleneck and effective population size

A recent bottleneck affects the number of alleles rather than the rate of heterozygosity (Cornuet and Luikart 1996; Luikart et al. 1998; Spencer et al. 2000). Although we presumed that translocated populations have been affected by a bottleneck, since a slightly decreased number of alleles was found especially in VC, examination using the Wilcoxon signed-rank test under the TPM mutation model revealed no significant possibility that particular populations have experienced recent bottlenecks. Current analysis likely lacks the power to detect a bottleneck even if one occurred. A bottleneck's signature might be disguised by genetic drift caused by the rapid growth of a population after its establishment (Bonhomme et al. 2008) or by the addition of several new specimens (immigrants) (Keller et al. 2001). A batch of noble crayfish juveniles was added three times into the BL population; however, no apparent effect was found on bottleneck detection; contrariwise, the

Table 4 Pairwise population matrix of F_{ST} (*below diagonal*) and Nei genetic distance (*above diagonal*) (GAE). All F_{ST} were highly significant ($P<0.001$) except for VC vs. UB ($P<0.002$; 999 permutations)

Population	KR	SV	ZA	VC	BL	SU	UB
KR	***	0.287	0.302	0.123	0.212	0.419	0.200
SV	*0.230*	***	0.425	0.229	0.131	0.507	0.158
ZA	*0.158*	*0.221*	***	0.264	0.257	0.103	0.300
VC	*0.111*	*0.213*	*0.158*	***	0.142	0.314	0.054
BL	*0.149*	*0.110*	*0.122*	*0.115*	***	0.255	0.132
SU	*0.216*	*0.269*	*0.035*	*0.194*	*0.130*	***	0.313
UB	*0.231*	*0.204*	*0.228*	*0.078*	*0.160*	*0.259*	***

KR Kramata, *SV* Světlohorská, *ZA* Zámecký, *VC* U Včelníku, *BL* Borová Lada, *SU* U Sudu, *UB* Úbislav

probability of heterozygote excess or deficiency was the second lowest. Moreover, the UB population, where a 50 % decrease in census size occurred in 2002, did not show any significant probability of heterozygote excess or deficiency. In addition, none of the analyzed populations (except KR) exhibited a departure from an L-shaped distribution of alleles, a phenomenon usually seen in bottlenecked populations (Luikart et al. 1998; England et al. 2003).

Reduced effective population size (N_e) could also be a major reason for such heterozygotes deficiency, as reported for *Austropotamobius italicus* (Matallanas et al. 2012). The number of N_e, depending on how it is calculated, is about ten to a hundred times lower than the real census size of source populations analyzed in this study. In the case that N_e has always been low relative to the census size, the population could experience a large reduction in the census size (Lawler 2008). There is no available information that source populations have undergone a drastic reduction of the census size in the past. Nevertheless, all of these populations are isolated and small, which makes them susceptible to any negative effects, including inbreeding and decreasing heterozygosity within the population. A high rate of inbreeding would also cause linkage disequilibrium, or at least affect most of the loci in a similar way. Conversely, no linkage disequilibrium was detected in our study, and results of HWE showed that the pattern of homozygote excess is not uniform across the loci and populations. Moreover, the recorded high number of MLGs speaks against pure inbreeding, since we would expect to see more individuals of the same genotypes similarly to the parthenogenetic mode in crayfish species (Yue et al. 2008; Buřič et al. 2011).

Population differentiation

Significant genetic structure was revealed by all the conducted methods. The analyses distinguished populations that originated from Central Bohemia (ZA, SU) from the remaining ones, those that originated from Southern Bohemia (Fig. B in the supplementary material), and matched the source and translocated populations too.

Reduced N_e, found especially in the Southern Bohemia populations, strengthens the effect of genetic drift to populations, as reported by Cardoso et al. (2009). Nevertheless, the number of generations since establishing the translocated populations is quite low (likely 2–3), so that we can still find low genetic differentiation between source and translocated populations (ZA vs. SU; Table 4). The genetic differentiation based on F_{ST} estimates (0.035–0.269) was in the range of values found in noble crayfish populations within particular countries, especially comparing close geographic areas such as the Czech Republic and Germany (0.100–0.202; Gross et al. 2013). The translocated population VC, founded by 30 females and 40 males, was moderately differentiated from the source population KR (F_{ST}=0.111); however, in the case of SU founded by 800 0+ individuals from 20 females originated in ZA, the differences were lower (F_{ST}=0.035). It is hard to say whether the different numbers or different life stages of stocklings accounted more to the level of differentiation. Nevertheless, population ZA with higher genetic diversity and translocated SU differentiate only slightly, whereas the others demonstrate deeper genetic differences, likely owing to the lower genetic diversity and, thus, stronger effect of genetic drift. Although tens of juveniles were added two times from the source population SV and/or KR after establishing the population BL, there is no clear effect in the translocated population, demonstrating moderate differentiation from both source populations (F_{ST}=0.110 and 0.149, respectively). PCoA analysis placed the population BL more closely to SV than KR (Fig. B in the supplementary material). Repeated releasing of new stocklings might have a rather negative effect on genetic diversity, as shown by Sigg (2006). The low genetic differentiations between populations VC and UB (F_{ST}= 0.078), together with other characteristics, could demonstrate naturally reduced genetic diversity in translocated populations.

Conclusion

Establishing translocated populations is a common procedure to preserve and maintain genetic diversity as a main goal in conservation genetics (Frankham et al. 2002; Souty-Grosset et al. 2003) and, thus, the source population should have high genetic diversity (Taugbøl and Peay 2004). Based on the results of this study, establishing new translocated noble crayfish populations was successful, even though the genetic variability and other characteristics of the source populations were generally lower compared to western and central European populations (Gross et al. 2013; Schrimpf et al. 2014). In spite of that, significant genetic structure was found among populations that originated from Central (ZA, SU) compared to Southern Bohemia populations (KR, SV, VC, BL, UB), and match the source and translocated populations too. In our opinion, the distinctiveness of a population is an important clue that a particular population is suitable for conservation management purposes and makes it reasonable to treat populations of noble crayfish as a single genetic unit. Therefore, genetic screening should be accomplished in advance when considering any population for conservation purposes in the area of interest. Further research is advisable and the screening should be repeated after an extended time period. Continued monitoring will be necessary to track the long-term progress of the translocation program, including other parameters describing the state of the population, such as the occurrence and frequency of diseases or morphological changes.

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Supplement 5

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Cherax (*Astaconephrops*) *gherardii*, a new crayfish (Decapoda: Parastacidae) from West Papua, Indonesia

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Abstract

Cherax (*Astaconephrops*) *gherardii* **n. sp.** is a moderate burrowing crayfish endemic to the Ajamaru Lakes of West Papua, Indonesia. This species is one of the crayfish species from this region that are exploited for ornamental purposes. Its commonly used commercial name in the pet trade is "Rainbow Crayfish" or "Blue Moon Crayfish", and its native name is "udang kuku biru". The new species is genetically and morphologically similar to *Cherax boesemani*, however, both species may be easily distinguished morphologically or by using sequence divergence, which is substantial for considering *C. gherardii* **n. sp.** to be a valid species.

Key words: *Cherax gherardii* **n. sp.**, new species, taxonomy, morphology, phylogeny, pet trade

Introduction

Crayfish from the genus *Cherax* belong to a group of freshwater decapod crustaceans that are exploited for ornamental purposes (Chucholl 2013; Papavlasopoulou *et al.* 2014; Patoka *et al.* 2014). *Cherax* crayfish from West Papua are captured in the field and subsequently exported by Indonesian wholesalers to European, USA and Japanese pet markets (Lukhaup & Herbert 2008; Patoka *et al.* 2015). Inasmuch as certain traded *Cherax* crayfish from West Papua are scientifically undescribed and their captured quantities are not registered by relevant authorities, the related potential decline of abundance of these species can be easily overlooked. Scientifically undescribed species are advertised only under trade names as noted by Patoka *et al.* (2014). The new species of *Cherax* crayfish presented in our paper is known under the commercial name "Rainbow Crayfish" (Mendoza Alfaro *et al.* 2011) and "Blue Moon Crayfish" (Schäfer 2014). However these names are also used for certain other scientifically undescribed *Cherax* crayfish. Three crayfish species native in regions of West Papua and adjoining Papua (formerly known as Irian Jaya), *Cherax boesemani* Lukhaup and Pekny, 2008, *C. holthuisi* Lukhaup and Pekny, 2006, and *C. peknyi* Lukhaup and Herbert, 2008, were described following their ornamental exploitation in recent years (Lukhaup & Pekny 2006; Lukhaup & Herbert 2008; Lukhaup & Pekny 2008). The new species complements this collection and its description is crucial for proper management of this crayfish in its native range.

The new species, *Cherax* (*Astaconephrops*) *gherardii* **n. sp.**, is genetically and morphologically most similar to *Cherax boesemani*, which is endemic to the Ajamaru Lakes and the Ajamaru River in West Papua, Indonesia (Lukhaup & Pekny 2008). Both species may be easily distinguished using sequence divergence or by coloration; chelae shape; position and color of the uncalcified patch on the outer margin of chelae of adult males; rostral reaching; and large teeth on propodal cutting edges.

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Material and methods

All specimen morphometric measurements were taken with digital calipers with an accuracy 0.1 mm (e.g. Cooper & Boyko 2006; Thoma *et al.* 2014). Weight was taken using a digital pocket scale with an accuracy of 0.01 g. The following abbreviations are used below: TL, total body length; TCL, total carapace length; PCL, postorbital carapace length.

Specimen and tissue collection. Obtained crayfish were captured in the field for ornamental purposes in West Papua, Indonesia and consequently imported with other *Cherax* species into the Czech Republic between October 2013 and February 2014. We collected altogether three individuals (two adult males and one adult female) from one of the leading Czech wholesalers of ornamental aquatic animals, including crayfish. All specimens were photographed and kept alive separately in indoor tanks until samples of haemolymph were obtained for DNA analysis. After this procedure, the specimens were preserved in 80% ethanol. One male was designated as holotype, the female as allotype, and the second male as paratype.

DNA extraction, amplification and sequencing. DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG. Düren, Germany) following the manufacturer's protocol. Two molecular markers were amplified, namely cytochrome oxidase subunit I (COI) and 16S rRNA. Primers LCO and HCO (Folmer *et al.* 1994) and 1471 and 1472 (Crandall & Fitzpatrick 1996) were used for COI and 16S rRNA amplification, respectively. All PCR reactions were carried out in a Biometra T3000 thermocycler (Göttingen, Germany) with the following cycling conditions: 5 min at 95 °C; 40 cycles of 30 s at 94 °C, 30 s at 50 °C, 45 s at 72 °C; 10 min at 72 °C. PCR reactions were run in 10 µl of 5 µL of PPP Master mix [50 mM Tris–HCl, pH 8.8, 40 mM $(NH_4)2SO_4$, 0.02% Tween 20.5 mM $MgCl_2$, 400 lM dATP, 400 lM dCTP, 400 lM dGTP, 400 lM dTTP, and 100 U⁄mL Taq-Purple DNA polymerase], 0.3 µL of each primer (10 pmol ⁄ µL), 1 µL genomic DNA. For sequencing, the PCR products were run on an electrophoresis agarose gel, the relevant bands excised and purified using the Nucleospin® (Macherey-Nagel) kit. Purified products were subsequently sequenced on an ABI automatic capillary sequencer (series 373; Macrogene, Inc., Korea).

Genetic data analysis. Nucleotide sequences were aligned using MAFFT v7.017 (Katoh *et al.* 2002) implemented in GENEIOUS 8.0.5 (www.geneious.com, Kearse *et al.* 2012), further the alignment of COI sequences was checked by translating into aminoacids. For the concatenated dataset, partial gene fragments were downloaded from the National Center for Biotechnology Information (NCBI) available sequences (*C. holthuisi* KJ950520, KJ950521—COI, KJ920804; KJ920805—16S, *C. boesemani* KJ950507—COI, KJ920783—16S; and *C. peknyi* KJ950533—COI, KJ920835—16S). Further particular gene fragments were extracted from available *Cherax* mitogenom sequences available on NCBI to get fragments corresponding to ours (*C. monticola* KF649851; *C. quadricarinatus* KF649850; *C. bicarinatus* KM501041; *C. robustus* NC023478; and *Euastacus spinifer* NC026214). The sequence divergences were estimated in MEGA6 (Tamura *et al.* 2013) using the Kimura 2-parameter model. The HKY+G model of evolution was chosen by AIC and BIC (Akaike and Bayesian information criterion, respectively) estimated in jModelTest 2.1.7 (Darriba *et al.* 2012) for combined dataset as well as for both gene fragments datasets. A maximum likelihood (ML) tree was constructed in PHYML (Guindon & Gascuel 2003) implemented in GENEIOUS 8.0.5 (Kearse *et al.* 2012), while Bayesian analyses was conducted in MrBayes 3.2.4. (Ronquist *et al.* 2012).

Systematics

Cherax (*Astaconephrops*) *gherardii* Patoka, Bláha and Kouba, new species

Figs. 1–2

Diagnosis. Carapace surface smooth with exception of one to five small spiniform tubercles posterior cervical groove on lateral carapace. Eyes large, pigmented, cornea slightly broader than eyestalk. Rostrum lanceolate in shape with excavated margins. Rostral margins with three prominent teeth. Rostral carinae prominent. Postorbital ridges prominent with one acute tubercle at anterior terminus. Scaphocerite regularly narrows into apex with a single distinct spine at terminus. Antennular peduncle reaching slightly behind acumen, antennal peduncle reaching slightly behind apex of scaphocerite. Uncalcified patch on lateral margin of chelae of adult male pale, translucent, extending from about middle of palm to about one fifth of opposable propodus (fixed finger). Propodal

cutting edge with row of small granules and one large tubercle. Chelipeds blue with orange joints. Palm of chelae blue in basal part, pale in distal part. Fingers orange, in distal third black with hooked orange tips. Row of blunt spines on inner lateral margin of palm light blue. Other walking legs deep blue in color. Gonopores of both sexes normal in shape and position.

Description of holotypic male. (Figs. 1, 2B–G, 3A). Body and eyes pigmented. Eyes not reduced. Body subovate, slightly compressed laterally. Cephalothorax 1.2 times broader than pleon.

FIGURE 1. *Cherax gherardii* **n. sp.**, holotype.

Rostrum (Fig. 2D) relatively slender, lanceolate in shape, 3.6 times as long as wide, reaching slightly beyond end of second segment of antennular peduncle. Terminus of acumen straight, not deflected or upturned. Median carina absents. Rostral margins elevated, anteriorly convergent throughout length to acumen, posteriorly forming rostral carinae. Each lateral margin bearing three slightly upturned prominent teeth on distal half. Upper surface smooth and without setae, sparsely short setose hairs present on outer rostral margins and on ventral side of rostrum. Rostral carinae prominent, extending as slight elevation posteriorly on to carapax, gradually fading and indistinct behind middle of PCL (a well-developed rostral carinae is characteristic to subgenus *Astaconephrops*). Postorbital ridges (Fig. 2D) prominent, strongly elevated posteriorly, gradually fading, remaining 1/3 of PCL indistinct. Anterior terminus of postorbital ridges with slightly upturned spiniform tubercle. Eyes (Fig. 2D) relatively large; cornea globular, darkly pigmented, about as long as eyestalk and slightly broader.

Antennulae and antennae normal in shape; the antennae similarly long as TL. Antennular peduncle reaching slightly behind acumen, antennal peduncle reaching slightly behind apex of scaphocerite. Coxicerite of antennal peduncle with spiniform tubercle anteriorly; basicerite with one lateral and one ventral spiniform and hooked tubercles (Fig. 2B). Scaphocerite (Fig. 2G) horizontal, with lamina 2.7 times as long as broad, broadest at midlength; convex in distal part becoming narrower at base, but otherwise is straight; reaching slightly behind the antennular peduncle; regularly narrows into the apex; thickened outer lateral margin with prominent spiniform tubercle at apex reaching distinctly beyond the lamina; rounded inner margin strongly covered by setae.

Epistome (Fig. 2F) with subcordiform cephalis lobe bearing weak cephalomedian projection and constricted at base; lateral margins of lobe not thickened; each lateral margin covered with two groups of small tubercles

separated by smooth area; central part smooth with fovea, not pitted; inner side of cephalomedian projection strongly setose, ventral surface smooth with sparse short hairs, not pitted; epistomal zygoma prominent and thick, moderately arched with oblique arms.

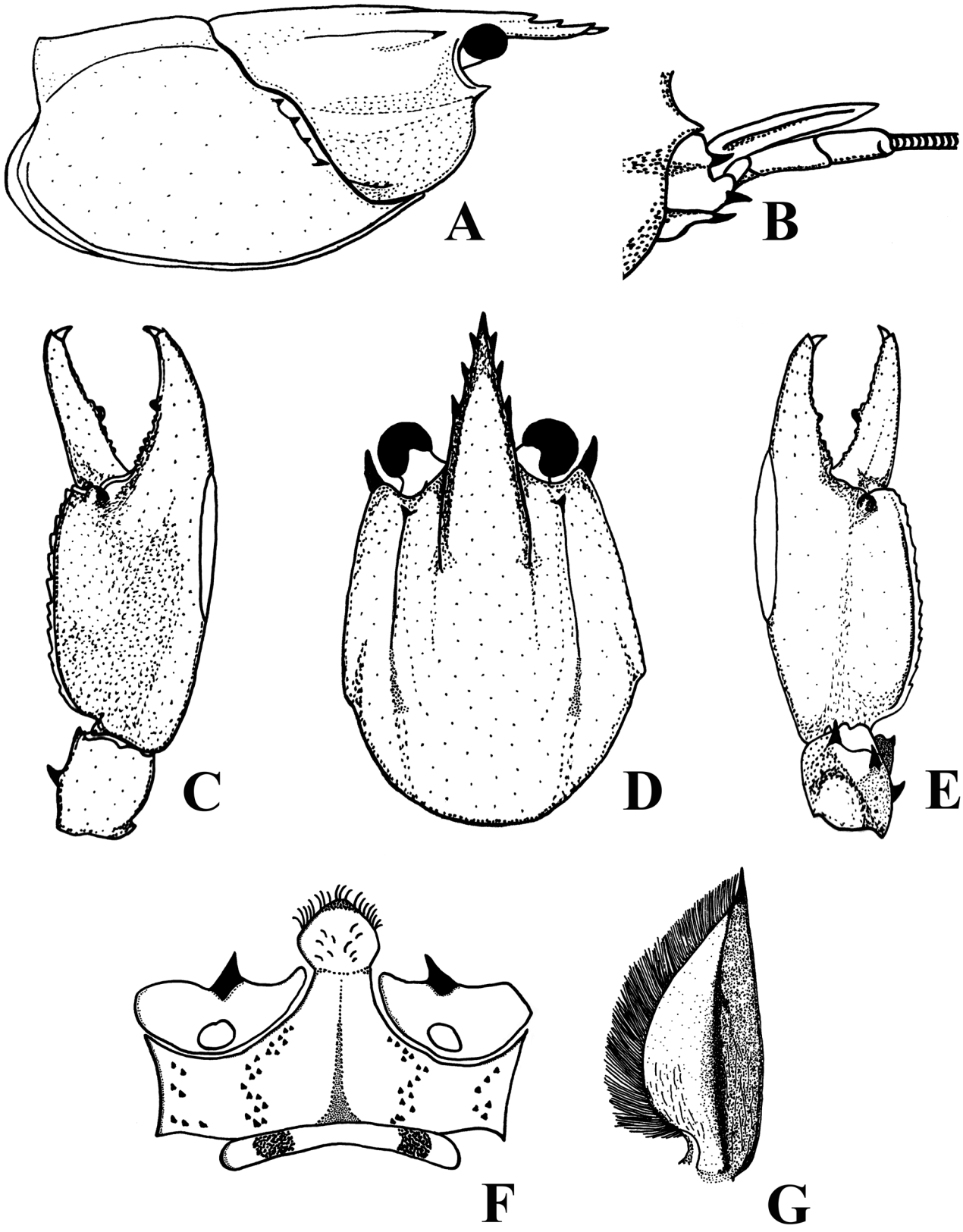

FIGURE 2. *Cherax gherardii* **n. sp.**: A. lateral view of carapace; B. lateral view of antennal peduncle; C. dorsal view of right chela; D. dorsal view of carapace; E. ventral view of right chela; F. epistome and coxicerite of antennal peduncle; G. dorsal view of right scaphocerite; A from allotype, B–G from holotype.

Areola 1.8 times as long as broad at narrowest part. Length of areola 28% of TCL; surface smooth and pitted. Cervical groove distinct, non-setose. Carapace surface smooth, pitted, with set of 4 anteriorly directed small spiniform tubercles laterally just posteriorly to cervical groove at level of antennae and below, only the lowest one prominent.

Male chelipeds and chelae (Fig. 2C, E, 3A) equal in form and size. Chelae 2.6 times as long as broad and 7.1 times as long as deep, strongly compressed; chela surface smooth, pitted; palm 1.6 times longer than fingers; carapace 1.2 times longer than chela; fingers slightly gaping; dactyl broad at base, tapering slightly towards tip; opposable propodus triangular, merging gradually into palm of chela; opposable propodus 1.8 times broader than dactyl at base. Outer lateral margin of chelae with swollen soft and uncalcified patch which extends from about middle of palm to about one fifth of opposable propodus, surface of the uncalcified patch slightly pitted (Fig 3); entire inner lateral margin of palm covered with slender row of more than ten bluntly topped teeth. Dactyl cutting edge with small granular teeth mainly near base, and with one large prominent tooth near middle of cutting edge; setose in posterior part of ventral surface. Dactyl tip with acute, hooked spine pointing outwards at an angle of approx. 45°. Propodal cutting edge with numerous denticles which are more distinct near base; one large prominent tooth at middle of cutting edge; setose in posterior part of ventral surface. Propodal tip with acute, moderate hooked spine. Propodal and dactyl tips slightly crossing when fingers clasp. Carpus smooth, pitted; with one well-developed acute and hooked spiniform tubercle in the middle of dorsolateral inner margin (mentioned tubercle is characteristic for genus *Cherax*); terminated with one spiniform tubercle oriented straight. Ventral carpal surface covered with tiny hairs and with fovea; fovea not pitted; margins slightly elevated; inner margin with set of 3 or 4 small granules and one acute spiniform tubercle oriented almost straight; outer margin with one spiniform tubercle oriented straight. Merus laterally depressed in basal part; surface smooth and pitted; single directly oriented spiniform tubercle present on dorsal surface; row of three directly oriented spiniform tubercles present on ventral surface; row of small granules on entire inner ventrolateral margin; chela 2.0 times longer than merus. Merus laterally strongly depressed; surface smooth and pitted; single spiniform tubercle present on ventral margin.

Second pereiopod reaching slightly behind apex of scaphocerite. Palm as long as fingers; fingers and palm sparsely setose; tips of fingers hooked. Carpus 2.0 times longer than palm. Merus 1.6 times longer than carpus and 2.7 times longer than ischium.

Third pereiopod 1.4 times longer than second pereiopod. Palm 1.2 times longer than fingers. Fingers sparsely setose; tips of fingers hooked. Carpus 1.5 times longer than palm. Merus 1.6 times longer than carpus and 2.6 times longer than ischium.

Fourth pereiopod reaching in to middle of the scaphocerite. Propodus and dactyl setose. Dactyl slightly hooked. Propodus 1.7 times longer than carpus. Merus 2.1 times longer than carpus and 2.1 times longer than ischium.

Fifth pereiopod reaching proximal end of scaphocerite. Propodus and dactyl setose. Dactyl slightly hooked. Propodus 2 times longer than carpus. Merus 2.4 times longer than carpus and 2 times longer than ischium.

Dorsal surface of pleon smooth in median region; pleura smooth, densely pitted. Each pleomere strongly setose with short hairs on posterior margin. Telson with two posteriorly directed spiniform tubercles in caudolateral corners. Protopod of uropod with single posteriorly directed spiniform tubercle on distal margin. Endopod of uropod with two posteriorly directed spiniform tubercles in middle and outer margin of mesial lobe. Exopod of uropods with transverse row of posteriorly directed diminutive spiniform tubercles ending in two bigger posteriorly directed spiniform tubercles on outer margin of mesial lobe.

Description of allotypic female. (Fig. 2A, 3B). Differing from the holotype in the following respects: soft uncalcified patch on palm absent; the chelae 3.0 times as long as broad, 8.7 times as long as deep; palm of chela 1.2 times longer than fingers; pleon equally broad as cephalothorax; tubercles on propodal cutting edges smaller and less prominent than in holotype; cervical groove with set of four (right side) and three (left side) anteriorly directed prominent tubercles.

Description of paratypic male. Differing from the holotype in the following respects: left chela 3.4 times as long as broad and 7.5 times as long as deep; one large tooth at about middle propodal cutting edge of left chela not so prominent; single straight spiniform tubercle on dorsal surface of ischium of left cheliped poorly developed. Cervical groove with set of four (left side) and five (right side) anteriorly directed small tubercles. Endopod of uropods without spiniform tubercles.

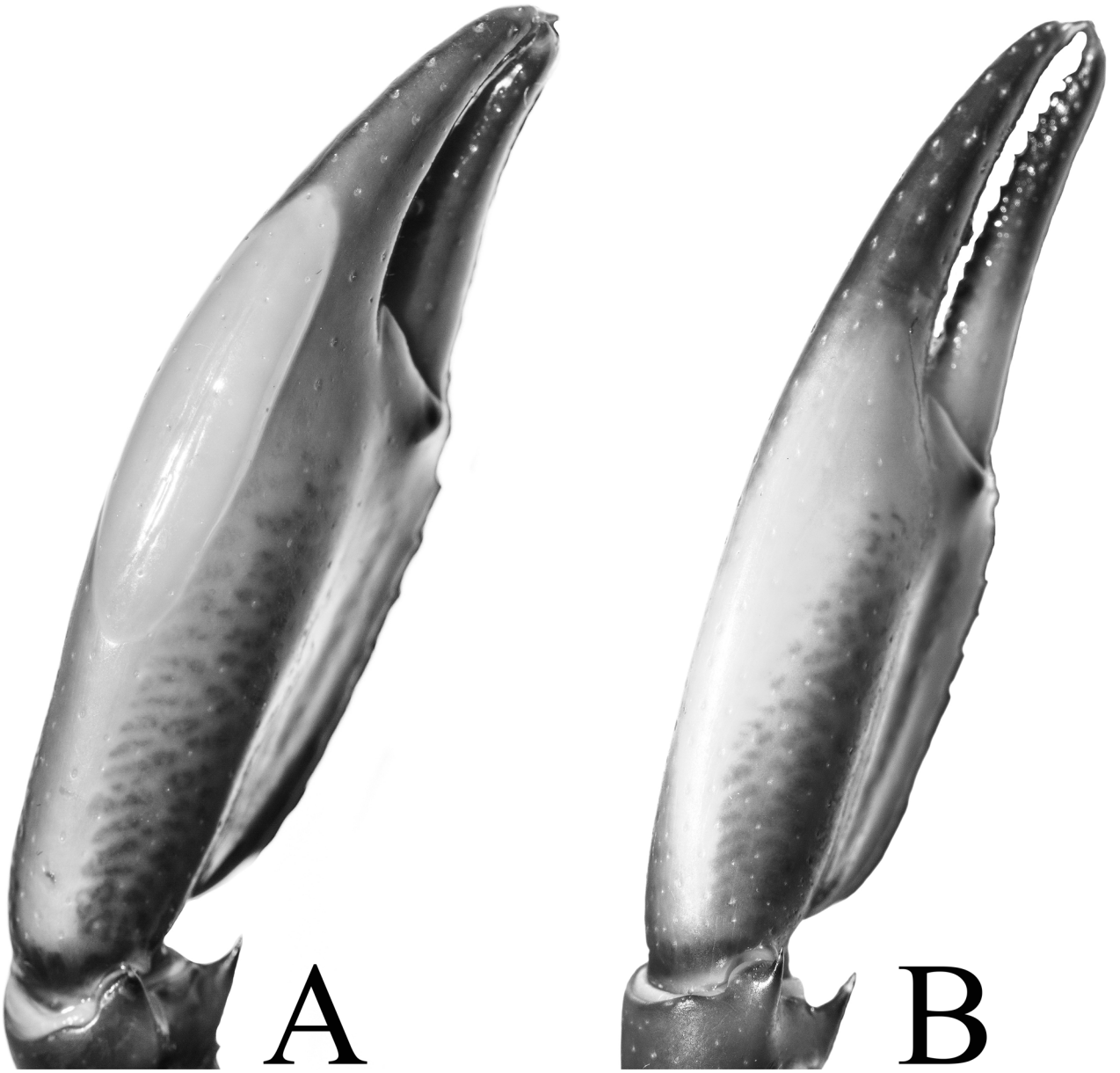

FIGURE 3. Outer lateral margin of chela: A. holotype (adult male); B. allotype (adult female).

Remarks. The single well-developed acute and hooked spiniform tubercle in the middle of dorsolateral inner margin of carpus is characteristic for the genus *Cherax*. The well-developed rostal carinae and triangular shape of scaphocerite is characteristic for adult males from the subgenus *Astaconephrops*. Both holotype and allotype chelae were without visible damage. The paratype has a regenerated right chela, left chela 1.5 times longer than right chela; this specimen has prominent erosion with soft tissue on inner lateral side of ischium of left cheliped, right chela with indistinct tubercles on propodal and dactyl cutting edges; uncalcified patch on outer lateral margin of palm of right chela absent; the anterior part of the carapace, before cervical groove on left lateral side with large swollen ulcer.

Size. Holotype TL = 94 mm, TCL = 43 mm, PCL = 31 mm, and weight = 20.61 g; allotype female TL = 97 mm, TCL = 45 mm, PCL = 32 mm, and weight = 27.07 g; paratype TL = 78 mm, TCL = 35 mm, PCL = 26 mm, and weight = 18.09 g.

Coloration of live specimens. Background color of live individuals dark brown, marbled on sides of carapace with pale brown spots. Cervical groove and distal end of carapace orange. Pleon with prominent orange spot on both lateral sides on each pleomere. Soft distal part of caudal fan orange. Chelipeds blue with orange joints, palm of propodus blue in basal part, pale in distal part. Fingers orange, distal third black with orange tips. Row of blunt

spines on inner lateral margin of palm light blue. Ventral surface of chela pale orange with bluish basal margin, fingers black in distal third with orange tips. Remaining pereiopods deep blue. Both antennal and antennular peduncle blue, flagella reddish-brown. Swollen uncalcified patch on outer lateral margin of palm pale and translucent, the rest of the margin whitish. Maxillipeds deep blue, ventral surface of cephalothorax and pleon pale.

Deposition of types. Holotype, allotype, and paratype are deposited at the Czech University of Life Sciences Prague. Holotype, No. JP2014/10-20: ♂, Indonesia, West Papua; collected by anonymous supplier of John's Aquatic wholesaler, TL 94 mm. Allotype, No. JP2014/10-21: ♀, Indonesia, West Papua; collected by anonymous supplier of John's Aquatic wholesaler, TL 97 mm. Paratype, No. JP2014/10-24: ♂, Indonesia, West Papua; collected by anonymous supplier of John's Aquatic wholesaler, TL 78 mm.

Systematic position. *Cherax gherardii* belongs to the subgenus *Astaconephrops* due to well-developed rostral carinae and triangular shape of scaphocerite (Holthuis 1949, 1950, 1982; Munasinghe *et al.* 2004). This subgenus includes eight Papuan species, namely: *Cherax* (*Astaconephrops*) *albertisii* (Nobili, 1899), *C.* (*A.*) *boesemani* Lukhaup and Pekny, 2008, *C.* (*A.*) *lorentzi* Roux, 1911, *C.* (*A.*) *minor* Holthuis, 1996, *C.* (*A.*) *misolicus* Holthuis, 1949, *C.* (*A.*) *monticola* Holthuis, 1950, *C.* (*A.*) *quadricarinatus* (von Martens, 1868), and *C.* (*A.*) *rhynchotus* Riek, 1951. The new species, *Cherax* (*A.*) *gherardii* **n. sp.**, differs from all others in the *Astaconephrops* subgenus in its coloration.

Cherax (*A.*) *gherardii* is morphologically most similar to *C.* (*A.*) *boesemani* and differs from this species in the following characters: chelae in *C.* (*A.*) *boesemani* are 2.3 to 2.4 times as long as broad and 5.4 times as long as deep while 2.6 to 3.4 times as long as broad and 7.1 to 8.7 times as long as deep in *C.* (*A.*) *gherardii*; uncalcified patch on outer lateral margin of chelae of adult males extends from middle or distal third of opposable propodus to about middle of palm and is yellowish or pale to white in *C.* (*A.*) *boesemani* while it is pale, translucent and extends from about middle of palm to about one fifth of opposable propodus in *C.* (*A.*) *gherardii*; in *C.* (*A.*) *boesemani* rostrum reaches close to the end of the ultimate antennular peduncle while reaching slightly beyond end of second segment of antennular peduncle in *C.* (*A.*) *gherardii*; propodal cutting edge without large teeth in *C.* (*A.*) *boesemani* while there is one prominent large tooth in *C.* (*A.*) *gherardii*; no setose hairy parts present on chelae except for ventral cutting edge of opposable propodus in *C.* (*A.*) *boesemani* while setose hairs developed in posterior ventral surface of dactyl in *C.* (*A.*) *gherardii*.

Cherax (*A.*) *gherardii* differs from *C.* (*A.*) *albertisii* in shape of chelae, and color of uncalcified patch on outer lateral margin of chelae of adult males. Chelae 5.0 to 5.8 times as long as broad in *C.* (*A.*) *albertisii* while 2.6 to 3.4 times in *C.* (*A.*) *gherardii*. Uncalcified patch red in *C.* (*A.*) *albertisii* while pale and translucent in *C.* (*A.*) *gherardii*.

Cherax (*A.*) *gherardii* differs from *C.* (*A.*) *lorentzi* in shape of chelae, number of rostral teeth, and color of uncalcified patch on outer lateral margin of chelae of adult males. Chelae in *C.* (*A.*) *lorentzi* 2.1 to 3.3 times as long as broad while 2.6 to 3.4 in *C.* (*A.*) *gherardii*. Each lateral margin of the rostrum with 2 teeth in *C.* (*A.*) *lorentzi* while with 3 teeth in *C.* (*A.*) *gherardii*. Uncalcified patch red in *C.* (*A.*) *lorentzi* while pale and translucent in *C.* (*A.*) *gherardii*.

Cherax (*A.*) *gherardii* differs from *C.* (*A.*) *minor* in shape of chelae, size of eyes, number of rostral teeth, and position of uncalcified patch on outer lateral margin of chelae of adult males. In *C.* (*A.*) *minor* chelae less than 2.0 times as long as broad while 2.6 to 3.4 in *C.* (*A.*) *gherardii*. Eyes are small and cornea is narrower than eyestalk in *C.* (*A*) *minor* while eyes large and cornea slightly broader than eyestalk in *C.* (*A.*) *gherardii*. Each rostral lateral margin bears no teeth except for 2 or 3 small subapical denticles in *C.* (*A*) *minor* while 3 large teeth present in distal third of rostrum in *C.* (*A.*) *gherardii*. Uncalcified patch extends from middle or distal third of opposable propodus to about middle of palm in *C.* (*A.*) *minor* while from about middle of palm to about one fifth of opposable propodus in *C.* (*A.*) *gherardii*.

Cherax (*A.*) *gherardii* differs from *C.* (*A.*) *misolicus* in shape of chelae, number of rostral teeth, and in spination on lateral carapax. Chelae of *C.* (*A.*) *misolicus* 2.0 to 2.4 times as long as broad while 2.6 to 3.4 in *C.* (*A.*) *gherardii*. Each rostral lateral margin with 2 to 3 teeth in *C.* (*A.*) *misolicus* while with 3 in *C.* (*A.*) *gherardii*. Both lateral sides of carapax with 7 to 8 tubercles in *C.* (*A.*) *misolicus* while 3 to 5 spiniform tubercles in *C.* (*A.*) *gherardii*.

Cherax (*A.*) *gherardii* differs from *C.* (*A.*) *monticola* in shape of chelae, number of rostral teeth, and in number, position and color of uncalcified patch of chelae in adult males. Chelae 2.3 to 2.7 times as long as broad in *C.* (*A.*) *monticola* while 2.6 to 3.4 times in *C.* (*A.*) *gherardii*. Each rostral margin with 0 to 3 small but distinct lateral teeth in *C.* (*A*) *minor* while with 3 large teeth in *C.* (*A.*) *gherardii*. In *C.* (*A.*) *monticola* one large whitish uncalcified patch extending from extreme anterior part of palm proper to short distance before top of opposable propodus.

Furthermore, one minor uncalcified area present in proximal half of the lower margin of palm. In *C.* (*A.*) *gherardii* only one pale and translucent uncalcified patch extending from about middle of palm to about one fifth of opposable propodus.

Cherax (*A.*) *gherardii* differs from *C.* (*A.*) *quadricarinatus* in shape of chelae, length and elevation of rostral carinae, and in color and position of uncalcified patch on outer lateral margin of chelae of adult males. Chelae slender and long in *C.* (*A.*) *quadricarinatus* while 2.6 to 3.4 times as long as broad in *C.* (*A.*) *gherardii*. Rostral carinae with strongly elevated margins reach behind end of postorbital ridges in *C.* (*A.*) *quadricarinatus* while rostral carinae gradually fade before postorbital ridges, margins are not so elevated in *C.* (*A.*) *gherardii*. Uncalcified patch consists of a red to whitish-orange membrane, extending close to tip of propodus in *C.* (*A.*) *quadricarinatus* while it is pale and translucent, extending from about middle of palm to about one fifth of opposable propodus in *C.* (*A.*) *gherardii*.

Cherax (*A.*) *gherardii* differs from *C.* (*A.*) *rhynchotus* in width of areola, size of eyes, number of rostral teeth, and color of uncalcified patch on outer lateral margin of chelae of adult males. Areola narrow, 4.0 to 5.0 times as long as broad in *C.* (*A.*) *rhynchotus* while 1.8 times as long as broad in *C.* (*A.*) *gherardii*. Eyes small in *C.* (*A.*) *rhynchotus* while large in *C.* (*A.*) *gherardii*. In *C.* (*A.*) *rhynchotus*, each rostral margin with two teeth while three in *C.* (*A.*) *gherardii*. Color of uncalcified patch white in *C.* (*A.*) *rhynchotus* while pale and translucent in *C.* (*A.*) *gherardii*.

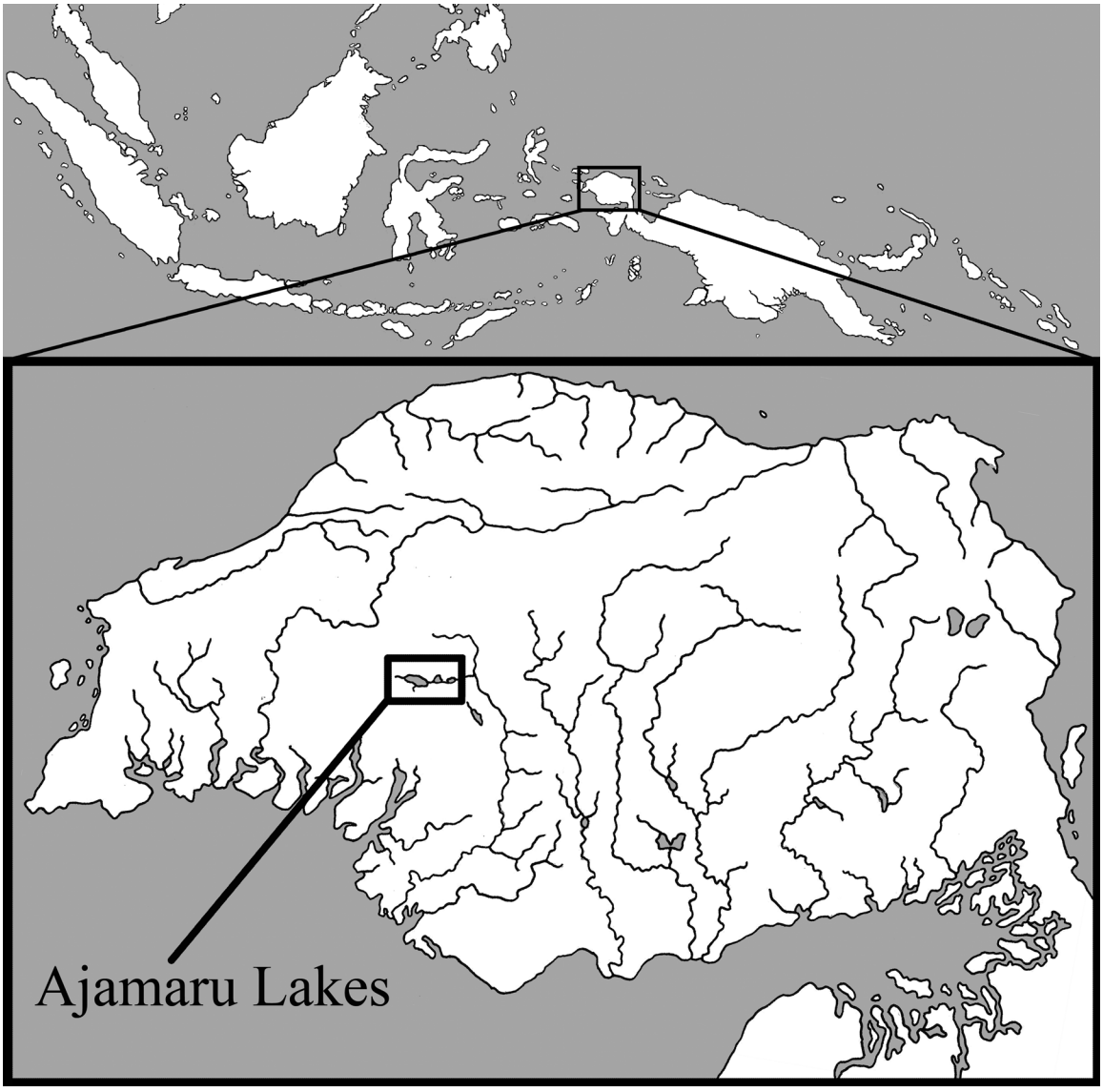

FIGURE 4. The Bird's Head Peninsula, West Papua, Indonesia, and the indicated locality of the Ajamaru Lakes.

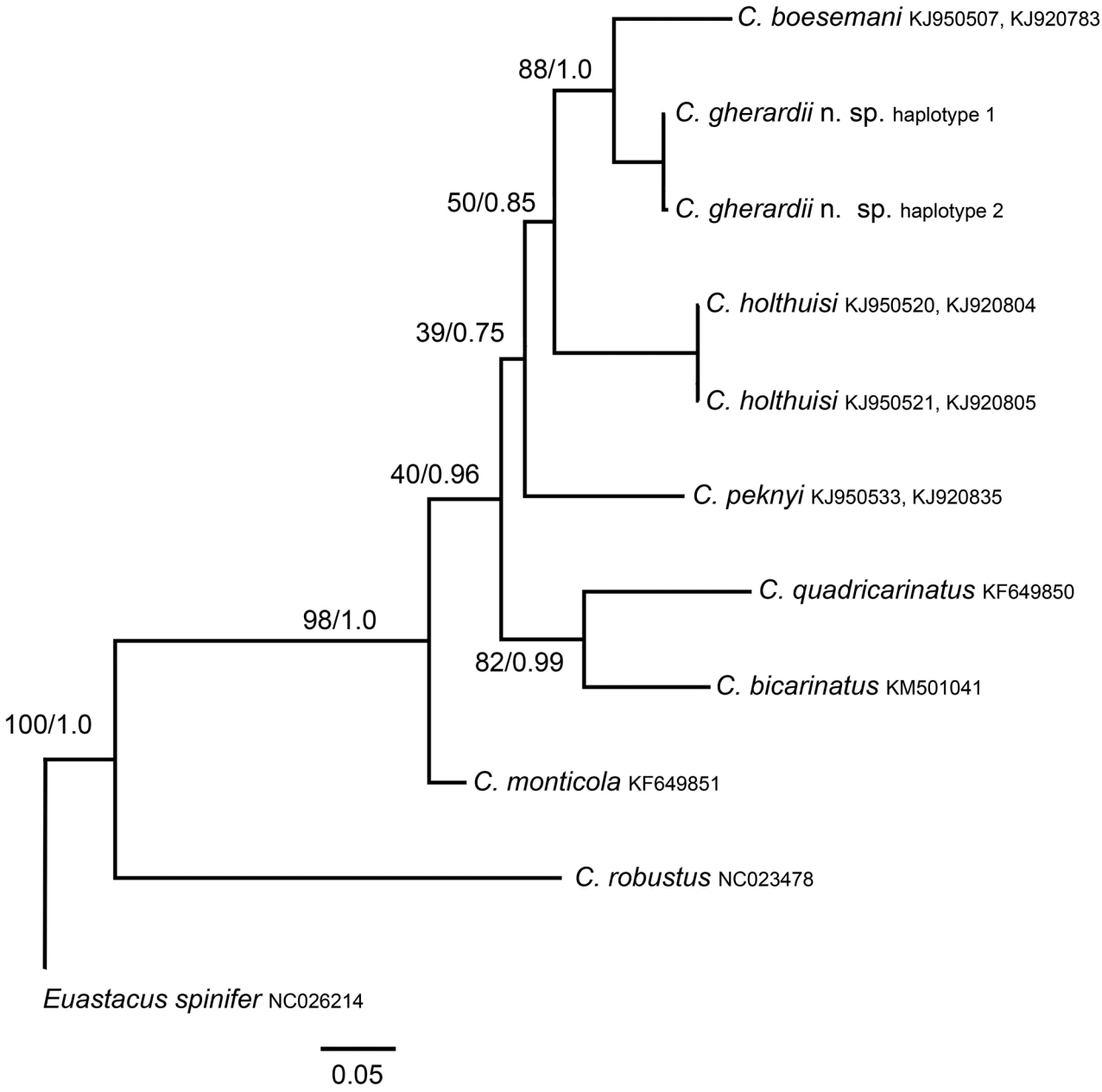

FIGURE 5. Bayesian analysis consensus phylogram of selected *Cherax* species based on combined COI and 16S dataset. ML bootstrap values and posterior probabilities are displayed next to each node.

Etymology. The specific name corresponds to the Latin form, singular genitive of Gherardi, in honor of Francesca Gherardi (Florence, Italy, 1955–2013), Associate Professor at the University of Florence, a brilliant astacologist and ethologist, interested in the behavior and ecology of freshwater decapod crustaceans including crayfish.

Common name. Both trade names of the new species, “Rainbow Crayfish” and “Blue Moon Crayfish,” are used for other scientifically undescribed *Cherax* species. The local name used by native inhabitants is "udang kuku biru" (crayfish with blue legs). Therefore we proposed a new name, Blue-Legged Crayfish, as a common name for the new species, *Cherax* (*A.*) *gherardii* **n. sp.**

Distribution. Based on information from the supplier, *C. gherardii* occurs in surrounding tributary streams to Ajamaru (also Ayamaru or Aiamaru) Lakes, West Papua, Indonesia (GPS S1°16'23.18" E132°12'21") (Fig. 4), where also *Cherax boesemani* occurs (Lukhaup and Pekny, 2008). The three connected Ajamaru Lakes are located in the west-central part of the Bird's Head Peninsula at the western extremity of West Papua on the Ajamaru limestone plateau about 250 m a.s.l. The shallow well-vegetated lakes are situated at the headwaters of the Ajamaru

River which is a tributary of the Kais River. The lakes are surrounded by low and rounded hills covered with low rainforest and the gardens of the Mejprat people who live close by and in a relatively dense population (Allen & Boeseman 1982; Bartstra 1998). A collecting trip along with a detailed survey is recommended to improve the knowledge of *C.* (*A.*) *gherardii* distribution.

Phylogenetics. The phylogenetic relationship inferred from two mitochondrial gene fragments (COI and 16S) results in a phylogram with a clearly defined species, *C. gherardii* **n. sp.** (Fig. 5). The new species forms a strongly supported (88–100%) monophyletic clade with *C. boesemani* differing at 9.2% (COI+16S dataset) from each other. *Cherax gherardii* and *C. boesemani* form a sister clade to *C. holthuisi* and, together with *C. peknyi*, *C. quadricarinatus*, *C. bicarinatus*, and *C. monticola*, belong to the northern group of *Cherax* species occurring in Papua and North Australia. *Cherax robustus* and *Euastacus spinifer* (NC026214.1) here represent an outgroup. The detailed phylogenetic relationships within the northern *Cherax* species group are described in Bláha *et al.* (In Prep). From three analyzed specimens, two haplotypes were identified at COI sequence; however all three specimens share the same haplotype for 16S rRNA. In addition, patristic distance based on the COI data set among *C. gherardii* and the others ranges from 0.280 (*C. boesemani*) to 0.781 (*C. robustus*). These values are beyond the crustacean species level threshold of a 0.16 substitutions per site (Lefébure *et al.* 2006). Both the high level of sequence divergence, along with the morphological differences described above, suggests that *C. gherardii* **n. sp.** is distinct from the closely related *C. boesemani* and supports the view that it can be described as a separate species.

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Supplement 6

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BRILL

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Journal of Crustacean Biology

CHERAX (*CHERAX*) *SUBTERIGNEUS*, A NEW CRAYFISH (DECAPODA: PARASTACIDAE) FROM WEST PAPUA, INDONESIA

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ABSTRACT

Cherax (*Cherax*) *subterigneus* n. sp., is a crayfish endemic to the Aitinjo Lake of West Papua, Indonesia. This species is one of the field-captured species from this region that are exploited for ornamental purposes. Its commonly used commercial name in the pet trade is "Black Orange Tip Crayfish," "Orange Tip Crayfish," or "Red Tip Crayfish." The new species is genetically and morphologically similar to *Cherax holthuisi*, however, both species can be easily distinguished by certain morphological characteristics or by using sequence divergence, which is substantial, for considering *C. subterigneus* n. sp. as a valid species. We have also added a note about the probable incorrect subgeneric assignment of the *Cherax peknyi* and mandatory change of incorrect original spelling of recently described *C. gherardii* as *C. gherardiae*.

KEY WORDS: *Cherax*, morphology, New Guinea, Parastacidae, pet trade, phylogeny, taxonomy

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INTRODUCTION

Many species of freshwater decapod crustaceans, including crayfish of the genus *Cherax*, have been exploited for ornamental purposes in recent years (Chucholl, 2013; Papavlasopoulou et al., 2014; Patoka et al., 2014). *Cherax* from West Papua are captured in field and pet-traded in Europe, the USA, and Japan (Lukhaup and Herbert, 2008; Patoka et al., 2015a). Part of these species are advertised under commercial names only, as noted by Patoka et al. (2014), and thus potential conservation management and regulation of capture of these species cannot be realized as long as they remain scientifically undescribed. This is also the case in the current paper, where the new species of *Cherax* described herein is known under various commercial names "Red Tip," "Orange Tip Crayfish," or "Black Orange Tip" (Patoka et al., 2014). Five crayfish species are known from this region of West Papua (formerly known as Irian Jaya), *Cherax* (*Astaconephrops*) *boesemani* Lukhaup and Pekny, 2008, *C.* (*A.*) *gherardiae* Patoka et al., 2015 (mandatory change of incorrect original spelling as *Cherax* (*Astaconephrops*) *gherardii* Patoka et al., 2015), *C.* (*Cherax*) *holthuisi* Lukhaup and Pekny, 2006, *C.* (*C.*) *peknyi* Lukhaup and Herbert, 2008, and *C.* (*A.*) *pulcher* Lukhaup, 2015 were described due to imports for ornamental purposes (Lukhaup and Pekny, 2006, 2008; Lukhaup and Herbert, 2008; Lukhaup, 2015; Patoka et al., 2015b).

The new species, *Cherax* (*Cherax*) *subterigneus* is genetically and morphologically similar to *Cherax* (*C.*) *holthuisi*, the latter is endemic to the Aitinjo Lake in West Papua, Indonesia (Lukhaup and Pekny, 2006). Both species can be distinguished using sequence divergence, and morphologically by the body and chelae color (in live individuals), narrow gap between the fingers when closed, and rows of setose hairs present on dactyl and fixed finger of the chela.

MATERIALS AND METHODS

Specimen and Tissue Collection

Obtained crayfish were field-captured for ornamental purposes in West Papua, Indonesia and consequently imported with other *Cherax* into the Czech Republic between October 2013 and February 2014. We collected nine individuals altogether (five adult males, four adult females) from one of the leading Czech wholesalers for ornamental aquatic animals, including crayfish. All individuals were weighed, photographed, and kept alive separately in indoor tanks until samples of haemolymph were obtained for DNA analysis. After this procedure, the specimens were preserved in 80% ethanol. One male was examined as a holotype, the female as allotype, and the other individuals as paratypes.

Morphometric Analysis

The morphometric measurements of all nine individuals were taken using a DigiMicro Profi portable USB-microscope and recorded to an accuracy of 0.1 mm. Weight was taken using a digital pocket scale with an accuracy of 0.1 g. The following abbreviations are used below: TL, total body length; TCL, total carapace length; PCL, postorbital carapace length.

DNA Extraction, Amplification and Sequencing

DNA was extracted from hemolymph using the NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG., Düren, Germany) following the manufacturer's protocol. Two molecular markers were amplified, namely cytochrome *c* oxidase subunit I (COI) and 16S rRNA at eight specimens. Primers LCO1490 and HCO2198 (Folmer et al., 1994) and 1471 and 1472 (Crandall and Fitzpatrick, 1996) were used for COI and 16S rRNA

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amplification, respectively. PCR reactions were run in 10 μl or 5 μl of PPP Master mix (50 mM Tris-HCl, pH 8.8, 40 mM $(NH_4)_2SO_4$, 0.02% Tween, 20.5 mM $MgCl_2$, 400 μM dATP, 400 μM dCTP, 400 μM dGTP, 400 μM dTTP, and 100 U/ml *Taq*-Purple DNA polymerase), 0.3 μl of each primer (10 pmol/μl), 1 μl genomic DNA. For sequencing, the PCR products were run on an electrophoresis agarose gel, the relevant bands excised and purified using the Nucleospin® kit. Purified products were subsequently sequenced on an ABI automatic capillary sequencer (series 373; Macrogen, Seoul, South Korea). Nucleotide sequences were aligned using MAFFT v7.017 (Katoh et al., 2002) implemented in GENEIOUS 8.0.5 (www.geneious.com, Kearse et al., 2012). Furthermore, the alignment of COI sequences was checked for presence of pseudogenes translating into amino acids. For the concatenated dataset, partial gene fragments were downloaded from GenBank (*C. holthuisi* KJ950520, KJ950521 (COI), KJ920804-KJ920805 (16S); *C. boesemani* KJ950507 (COI), KJ920783 (16S); *C. peknyi* KJ950533 (COI), KJ920835 (16S); *C.* sp. KJ950549-KJ950550, KJ950552 (COI), KJ920853-KJ920854, KJ920857 (16S)). In addition, particular gene fragments were extracted from available *Cherax* and *Euastacus* mitogenom sequences available on GenBank to get fragments corresponding to ours (*C. monticola* KF649851; *C. quadricarinatus* KF649850; *C. bicarinatus* KM501041; *C. robustus* NC023478; and *Euastacus spinifer* NC026214). The sequence divergences were estimated in MEGA6 (Tamura et al., 2013) using the Kimura 2-parameter model. The HKY+G model of evolution was chosen by Bayesian information criterion (BIC) estimated in jModelTest 2.1.7 (Darriba et al., 2012) for the combined dataset, as well as for both gene fragment datasets. A maximum likelihood (ML) tree was constructed in PHYML (Guindon et al., 2010) implemented in GENEIOUS 8.0.5 (Kearse et al., 2012), while Bayesian analyses were conducted in Mr.Bayes 3.2.4 (Ronquist et al., 2012). Additionally, the bPTP model with non-ultrametric gene trees was performed using its webserver (http://species.h-its.org/) for species delimitation using the COI dataset (Zhang et al., 2013).

SYSTEMATICS

Cherax* (*Cherax*) *subterigneus n. sp.
(Figs. 1-4)

Diagnosis.—Carapace surface smooth, no tubercles posteriorly behind cervical groove on lateral sides of carapace. Eyes small and pigmented, eyestalk at its base slightly broader than cornea. Rostrum broad in shape, 1.8-2.1 ($\overline{x}$ = 1.9, SD = 0.61) times as long as wide, with setose hairs covering distal parts of margins. Rostral margins slightly excavated. Each rostral margin with two small indentations, rostral teeth absent. Rostral carinae slightly developed. Postorbital ridges prominent with one acute tubercle at anterior terminus. Semicircular scaphocerite gradually narrows into the apex with a single distinct spine at terminus. Antennular peduncle reaching behind acumen, antennal peduncle reaching behind apex of scaphocerite. Areola 2.4-3.5 ($\overline{x}$ = 2.9, SD = 0.37) times as long as wide at the narrowest part. Cervical spines always absent. Carapace 1.2-1.5 ($\overline{x}$ = 1.4, SD = 0.46) times longer than chela. Chela 2.4-3.5 ($\overline{x}$ = 2.4, SD = 0.76) times as long as broad, and 4.7-7.3 ($\overline{x}$ = 5.9, SD = 2.10) times as its depth. Uncalcified patch on lateral margin of chelae of adult males absent. Both propodal and dactylar cutting edges with row of small granules and one large tubercle. Fingers slightly gaping in distal half; anthracite in color, in distal part orange with hooked tips. Two proximal thirds of fixed finger with a row of setose hairs, covering densely ventrally and less so on the dorsal surface of cutting edge of both fingers, sometimes less obvious. Terminal half of caudal fan soft and membranous.

Description of Holotypic Male (Fig. 1).—Body and eyes pigmented. Body subovate, slightly compressed laterally. Cephalothorax 1.2 times broader than pleon.

Rostrum (Fig. 2E) broad in shape, 1.9 times as long as wide, reaching slightly beyond the end of second segment of antennular peduncle. Upper rostral surface smooth; distal part with short setose hairs. Acumen with anteriorly oriented spine at terminus. Median carina absents. Rostral margins slightly elevated, anteriorly convergent throughout length to acumen, posteriorly continuing in very short rostral carinae ending before level of postorbital spines. Each lateral margin bearing two indentations on each side in distal part, no rostral teeth, distal part covered with dense

Fig. 1. *Cherax subterigneus* n. sp., holotype.

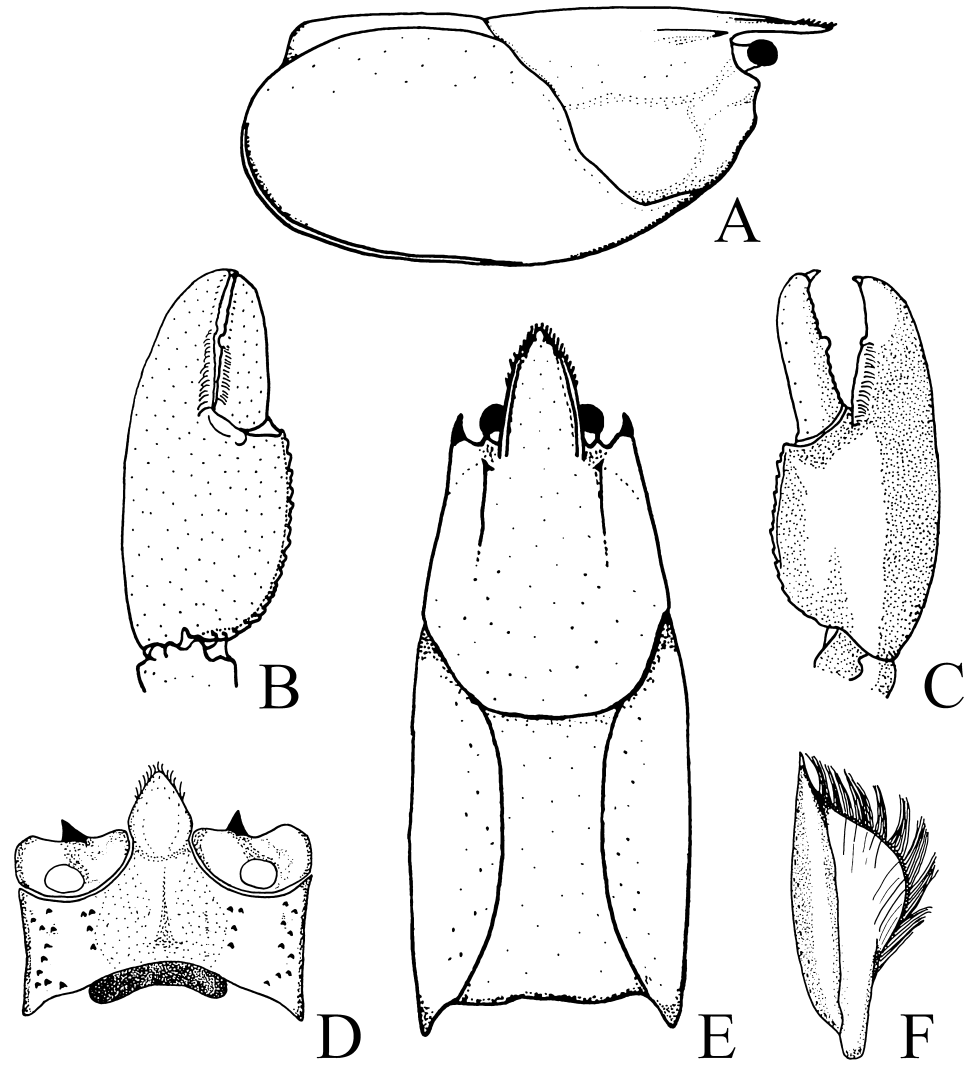

Fig. 2. *Cherax subterigneus* n. sp., from holotype. A, lateral view of carapace; B, dorsal view of left chela; C, ventral view of left chela; D, epistome and coxa of antennal peduncle; E, dorsal view of carapace; F, dorsal view of left scaphocerite.

setose hairs. Postorbital ridges (Fig. 2A, E) prominent, strongly elevated, posteriorly fading and beyond middle of PCL indistinct. Anterior terminus of postorbital ridges with slightly upturned spiniform tubercle. The eyes (Fig. 2A, D) small; cornea globular, darkly pigmented, about as long as eyestalk; eyestalk slightly narrower than cornea, but at its base 1.3 times broader than cornea in diameter.

Antennulae and antennae normal in shape; the antennae similarly long as TL. Antennular peduncle reaching behind acumen, antennal peduncle reaching behind apex of scaphocerite. Coxa of antennal peduncle with spiniform tubercle anteriorly; basis with one lateral and one ventral spiniform and hooked tubercles. Scaphocerite (Fig. 2F) flat and horizontal, semicircular in shape, narrower in basal part, with lamina 2.7 times as long as broad, broadest at midlength; reaching behind antennular peduncle; gradually narrows into the apex; thickened outer lateral margin with prominent acute spiniform tubercle at apex which reaches distinctly beyond the lamina; rounded inner margin strongly covered by setae.

The oral parts typical; epistome (Fig. 2D) with subcordiform cephalis lobe anteriorly bearing lanceolate cephalomedian projection constricted at base; lateral margins of lobe not thickened; each lateral margin covered with two groups of small tubercles separated by a smooth place; central part smooth with fovea, not pitted; posterior margin of cephalomedian projection with short setose hairs, not pitted; epistomal zygoma darkly pigmented, prominent and thick, moderately arched with oblique arms.

Dorsal surface of carapace smooth and pitted, with no tubercles laterally behind cervical groove at level, ventrolaterally with scattered small indistinct tubercles. Areola 3 times as long as wide at the narrowest part. Length of areola 41% of TCL; surface smooth and slightly pitted. Cervical groove distinct, non-setose.

Male chelipeds and chelae (Figs. 2B, C, 3A) equal in form and size, but the holotype has right chela regenerated and 1.5 times shorter than left chela. Left chelae 2.5 times as long as broad (high) and 5.3 times as long as deep; chela surface smooth, pitted; palm 1.4 times longer than

fingers; carapace 1.2 times longer than chela; fingers slightly gaping in distal part; dactyl about same height throughout its length, slightly narrower in its distal third; cutting edge with rather small granular teeth over the full length, one large blunt tooth at mid-length. Dactyl setose near its base. Fixed finger triangular, merging gradually into palm of chela; fixed finger 2.4 times broader than dactyl at base; cutting edge with rather small granular teeth over the full length and one tooth larger and more prominent at mid-length. Fixed finger with row of short setose hairs covered two thirds of ventral surface of cutting edge, dorsal surface sparsely setose with very short hairs. Tips of fingers with acute, hooked spines slightly crossing when fingers clasp. Entire inner lateral margin of palm covered with slender row of numerous bluntly topped teeth. Carpus smooth and pitted; with one well-developed acute and hooked spiniform tubercle in the middle of dorsolateral inner margin (mentioned tubercle is characteristic for genus *Cherax*); terminated with one spiniform tubercle oriented anteriorly. Ventral carpal surface non-setose and with fovea; fovea not pitted; margins slightly elevated; inner margin with set of 4 small granules and one acute spiniform tubercle oriented almost anteriorly; outer margin smooth with one spiniform tubercle oriented anteriorly. Merus laterally depressed in basal part; surface smooth and pitted; single anteriorly oriented spiniform tubercle and row of small tubercles (serrate carina) present on dorsal surface; two anteriorly oriented spiniform tubercles present on ventral surface at mid-length and other two spiniform tubercles present on lateral margins of fovea in broad distal part of the merus; row of small granules on entire inner ventrolateral margin, group of seven small granules on outer ventrolateral margin between two aforementioned spiniform tubercles at mid-length.

Second pereiopod reaching slightly behind apex of scaphocerite. Palm equally long as fingers; fingers and palm sparsely setose; tips of fingers hooked, surface smooth and pitted. Carpus 1.6 times longer than palm. Merus 1.6 times longer than carpus and 2.4 times longer than ischium.

Third pereiopod 1.3 times longer than second pereiopod. Palm 1.7 times longer than fingers; fingers and palm sparsely setose; tips of fingers hooked, surface smooth and pitted. Carpus 1.2 times longer than palm. Merus 1.5 times longer than carpus and 3.3 times longer than ischium.

Fourth pereiopod reaching middle of the scaphocerite. Propodus and dactyl setose. Dactyl slightly hooked. Propodus 1.5 times longer than carpus. Merus 1.7 times longer than carpus and 2.8 times longer than ischium.

Fifth pereiopod reaching base of scaphocerite. Propodus and dactyl setose. Dactyl slightly hooked. Propodus 1.7 times longer than carpus. Merus 1.8 times longer than carpus and 3 times longer than ischium.

Dorsal surface of pleon smooth in median region; pleura smooth, pitted. Each pleomere strongly setose with short hairs on ventral posterior margin. Telson with two posteriorly directed spiniform tubercles in caudolateral corners. Protopod of uropod with single posteriorly directed spiniform tubercle on distal margin. Endopod of uropod with two posteriorly directed spiniform tubercles in the middle and on outer margin of mesial lobe. Exopod of uropods with transverse row of posteriorly directed diminutive spiniform tubercles ending in two more prominent posteriorly directed spiniform tubercles on outer margin of mesial lobe. Terminal half of caudal fan soft and membranous.

Description of Allotypic Female.—Differing from the holotype in the following respects: the chelae (Fig. 3B) 2.1 times as long as broad and 6.6 times as long as deep; the palm of chela 1.1 times longer than fingers; carapace 1.4 times longer than chela. Large tubercles on propodal and dactylar cutting edges smaller, acute, and less prominent than in holotype. Three anteriorly oriented spiniform tubercles present on ventral surface at mid-length of the merus of the chelipeds. Pleon equally broad as cephalothorax; areola 3.4 times long as wide at the narrowest part.

Remarks.—The single, well-developed, acute and hooked spiniform tubercle in the middle of the dorsolateral inner margin of carpus is characteristic of *Cherax*. Both the semicircular shape of the scaphocerite and a poorly-developed rostral carinae are characteristics of the subgenus *Cherax* (Holthuis, 1949, 1950). The holotype was missing both the second and fifth right pereiopods, being cut through the merus near its base. The right chela is regenerated and small in size in comparison with left; the entire flagellum of left antenna is missing. The allotype was without any visible damages.

Size and Weight.—Holotype, ♂, TL = 84.3 mm, TCL = 39.0 mm, PCL = 31.2 mm, weight = 13.6 g. Allotype, ♀, TL = 99.0 mm, TCL = 46.7 mm, PCL = 39.6 mm, weight = 22.8 g. Paratypes: ♂ (n = 4), TL = 63.7-93.0 mm ($\overline{x}$ = 73.7 mm, SD = 13.8), TCL = 33.9-42.0 mm ($\overline{x}$ = 38.5 mm, SD = 3.4), PCL = 27.5-35.1 mm ($\overline{x}$ = 32.2 mm, SD = 3.3), weight = 9.8-18.3 g ($\overline{x}$ = 15.1 g, SD = 3.7); ♀ (n = 3), TL = 68.1-85.0 mm ($\overline{x}$ = 77.9 mm, SD = 8.8), TCL = 29.5-38.3 mm ($\overline{x}$ = 34.1 mm, SD = 4.4), PCL = 23.6-31.9 mm ($\overline{x}$ = 29.0 mm, SD = 4.7), weight = 7.9-13.8 g ($\overline{x}$ = 10.8 g, SD = 3.0).

Color of Live Individuals.—Background color is dark anthracite, occasionally not fully saturated. Cervical groove, ventral side of pereiopods including chelipeds, outer margin of caudal fan as well as both ventrolateral margins of each pleomere and posterior margin of sixth pleomere are fiery orange. Soft distal part of caudal fan orange. Lateral side of cephalothorax anthracite or dark yellowish. Chelipeds anthracite or yellowish with orange joints. Dorsal side of chelae anthracite with orange distal third of fingers, outer margin pale. Finger tips orange (Fig. 4), the row of blunt spines on inner lateral margin of palm anthracite. Ventral surface of chela pale orange. Dorsal side of remaining pereiopods anthracite with orange joints. Antennae with orange flagella. Ventral surface of the cephalothorax, pleon and pleopods pale. The variance in coloration is expected and is supported by one pale colored paratype (Fig. 5).

Deposition of Types.—The holotype, allotype, and paratypes are deposited at the Czech University of Life Sciences Prague. Holotype, No. JP2014/10-34 (♂), West Papua, Indonesia; collected by anonymous supplier of John's Aquatic wholesaler. Allotype, No. JP2014/10-30 (♀), West Papua, Indonesia; collected by anonymous supplier of John's Aquatic

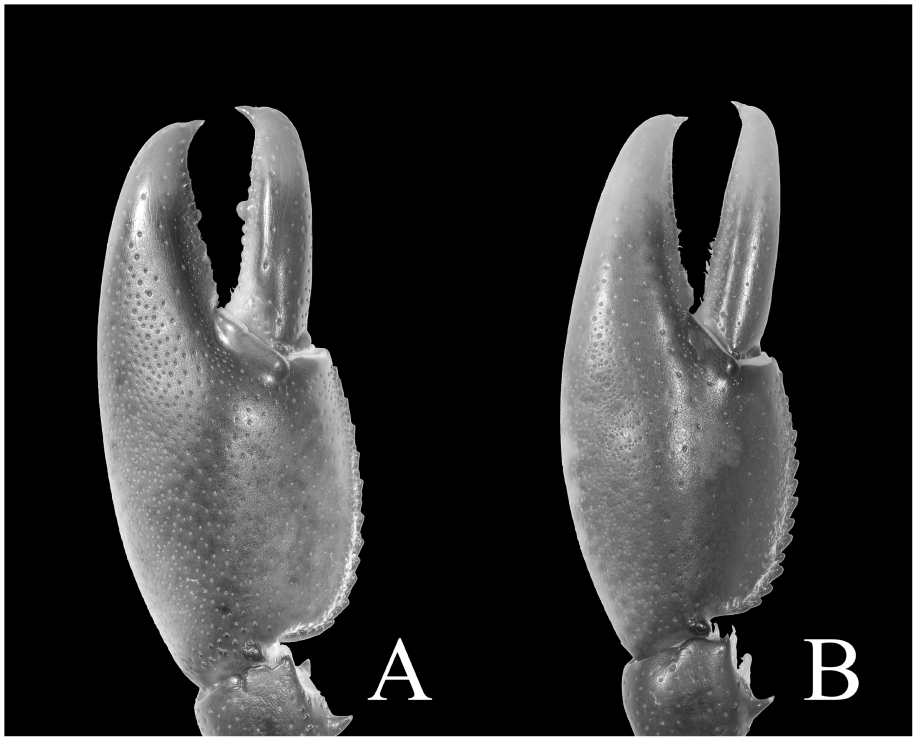

Fig. 3. Outer lateral margin of chela: A, holotype (adult male); B, allotype (adult female).

wholesaler. Paratypes, No. JP2014/10-01 (♀), JP2014/10-02 (♀), JP2014/10-31 (♂), JP2014/10-32 (♂), JP2014/10-33 (intersexual individual), JP2014/10-35 (♂), JP2014/10-36 (♀), JP2014/10-37 (♂), West Papua, Indonesia; collected by anonymous supplier of John's Aquatic wholesaler.

Etymology.—The specific name corresponds to the Latin form for fiery orange color and bottom side, in reference to the color of ventrolateral parts of the body, chelae and other pereiopods.

Common Name.—The common trade names of the new species, "Black Orange Tip Crayfish," "Orange Tip Crayfish," and "Red Tip Crayfish," are related to the color of the tips of the fingers. Therefore, we proposed the name Orange Tip Crayfish as a common name for the new species, *Cherax subterigneus*.

Distribution.—Indonesia: Based on information from the supplier, the new species occurs in the Kais River Drainage, at the shoreline of Aitinjo (also Aitinyo) Lake, situated about 20-25 km southeast of Ajamaru Lakes, West Papua

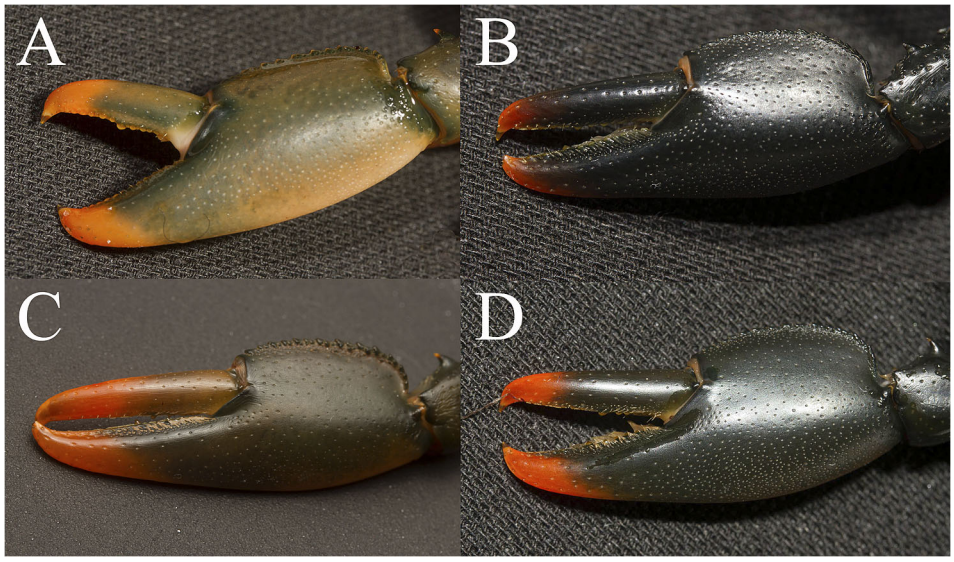

Fig. 4. Variability in shape and chela color of *Cherax subterigneus* n. sp. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1937240x.

Fig. 5. *Cherax subterigneus* n. sp.: pale color of adult male. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1937240x.

(GPS 1°25′55.895″S, 132°22′55.996″E) (Fig. 6). The lake is located in the west-central part of the Bird's Head Peninsula at the western extremity of West Papua. Boeseman (1963) described the locality in detail as follows: altitude about 90 m a.s.l.; a subterranean connection with the Kais River is uncertain but expected; pH of water 6.5; depth 15-20 m; large shallow parts; bottom rocky, sandy and muddy; aquatic vegetation dense. A collecting trip, along with a detailed survey, is recommended in order to improve our understanding of the geographic distribution of this new species (Fig. 6).

DISCUSSION

Systematic Position.—The new species belongs to the subgenus *Cherax* based on the poorly developed rostral carinae and semicircular shape of scaphocerite (Holthuis, 1949, 1950, 1996). This subgenus includes eleven Papuan species, namely: *Cherax boschmai* Holthuis, 1949, *C. buitendijkae* Holthuis, 1949, *C. communis* Holthuis, 1949, *C. holthuisi* Lukhaup and Pekny, 2006, *C. longipes* Holthuis, 1949, *C. murido* Holthuis, 1949, *C. pallidus* Holthuis, 1949, *C. paniaicus* Holthuis, 1949, *C. papuanus* Holthuis, 1949, *C. peknyi* Lukhaup and Herbert, 2008, and *C. solus* Holthuis, 1949. The new species, *C.* (*C.*) *subterigneus* n. sp., differs from all others in this subgenus by coloration of live individuals.

In comparison to all species of the subgenus *Cherax*, the new species is morphologically most similar to *C.* (*C.*) *holthuisi* and differs from this species in the following characters: body and chelae coloration of live individuals, rows of setose hairs on both fingers of the chela and narrow gaping of fingers in distal part. The rostrum in *C.* (*C.*) *holthuisi* is 1.5 times as long as basal width while 1.8-2.1 times in the new species. The chelae in *C.* (*C.*) *holthuisi* are 2.2-2.5 times as long as broad and 4.2-4.5 times as long as deep while 2.4-3.5 times as long as broad and 4.7-7.3 times as long as deep in the new species.

The new species differs from *C.* (*C.*) *boschmai* in the numbers of rostral teeth, the size of eyes, and in the shape of chelae. The new species has no rostral teeth while *C.* (*C.*) *boschmai* has 5-7 teeth on each rostral margin. The eyes are smaller in the new species than in *C. boschmai*. The chelae are 4.5-6.0 times as long as broad in *C.* (*C.*) *boschmai* while 2.4-3.5 times in the new species.

The new species differs from *C.* (*C.*) *buitendijkae* in the number of rostral teeth, the size of eyes, and in the shape of chelae. In *C.* (*C.*) *buitendijkae* each rostral margin bears 4-6 (seldom 3) teeth near apex while the new species has no prominent rostral teeth. The eyes are smaller in the new species than in *C.* (*C.*) *buitendijkae*. The chelae are 3.5-5.0 times as long as broad in *C.* (*C.*) *buitendijkae* while 2.4-3.5 times in the new species.

The new species differs from *C.* (*C.*) *communis* in the number of rostral teeth, the carapace surface, and in the shape of chelae. In *C.* (*C.*) *communis* each rostral margin bears 4-7 (seldom 3) teeth in proximal part while the new species has no prominent rostral teeth. The numerous small tubercles are present behind the cervical groove in *C.* (*C.*) *communis* while the new species has smooth carapace surface behind the cervical groove. Chelae are 2.0-3.0 times as long as broad in *C.* (*C.*) *communis* while 2.4-3.5 times in the new species.

The new species differs from *C.* (*C.*) *longipes* in the number of rostral teeth, the carapace surface, and in the shape of chelae. In *C.* (*C.*) *longipes*, each rostral margin bears 3-5 teeth in proximal part while the new species has no prominent rostral teeth. The numerous small tubercles are present behind the cervical groove in *C.* (*C.*) *longipes* while the new species has smooth carapace surface behind the cervical groove. Chelae are 4.0-4.5 times as long as broad in *C.* (*C.*) *longipes* while 2.4-3.5 times in the new species.

The new species differs from *C.* (*C.*) *murido* in the number of rostral teeth, the size of eyes, the carapace surface,

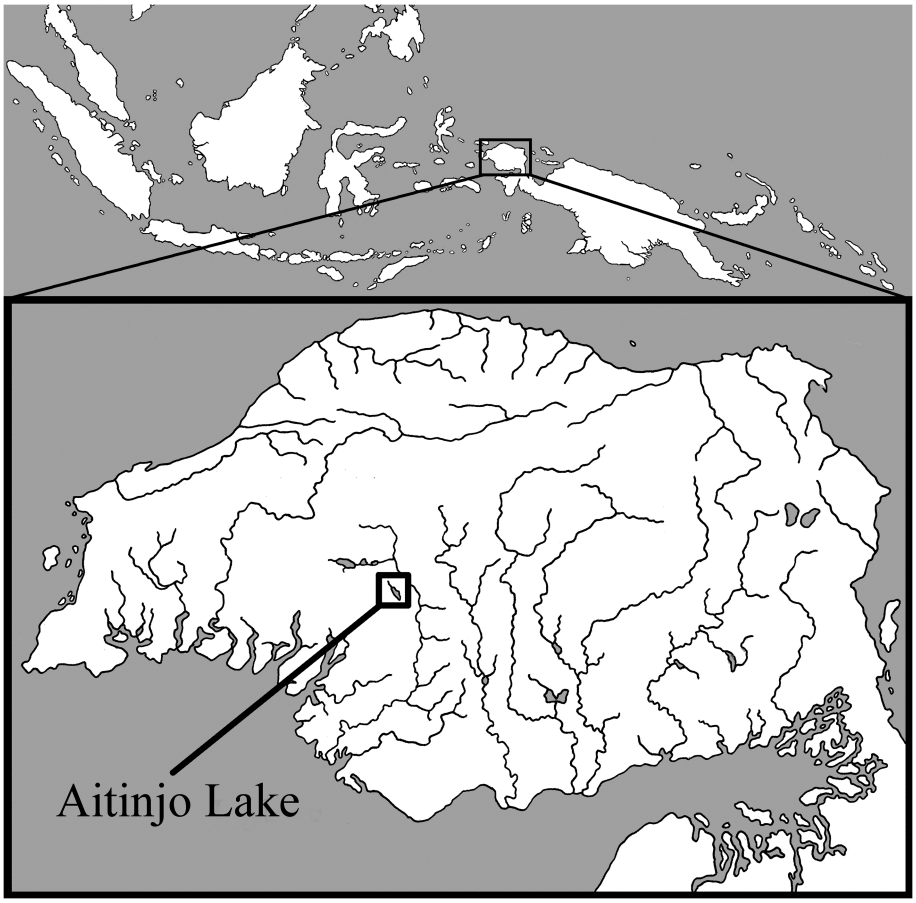

Fig. 6. The Bird's Head Peninsula, West Papua, Indonesia, and the indicated type locality of Aitinjo Lake.

the pleon surface, and in the shape of chelae. In *C.* (*C.*) *murido* each rostral margin bears 4-6 (seldom 3 or 7) teeth in proximal part while the new species has no prominent rostral teeth. The eyes are smaller in the new species than in *C.* (*C.*) *murido*. The carapace is covered with numerous widely spaced tubercles in *C.* (*C.*) *murido* while carapace surface is smooth in the new species. The pleon is distinctly tuberculated on the pleurae in *C.* (*C.*) *murido* while smooth in the new species. Chelae are 4.0 times as long as broad in *C.* (*C.*) *murido* while 2.4-3.5 times in the new species.

The new species differs from *C.* (*C.*) *pallidus* in the number of rostral teeth, the size of eyes, the carapace surface, and in the shape of chelae. In *C.* (*C.*) *pallidus* each rostral margin bears 2-3 (seldom 4) teeth in proximal part while the new species has no prominent rostral teeth. The eyes are smaller in the new species than in *C.* (*C.*) *pallidus*. Numerous and densely tubercles placed on almost the entire carapace in *C.* (*C.*) *pallidus* while carapace surface is smooth in the new species. Chelae are long (6.5-8.0 times as long as broad) in *C.* (*C.*) *pallidus* while only 2.4-3.5 times in the new species.

The new species differs from *C.* (*C.*) *paniaicus* in the number of rostral teeth, carapace shape, and in the shape of chelae. In *C.* (*C.*) *paniaicus* each rostral margin bears 5-8 teeth while the new species has no prominent rostral teeth. Numerous small tubercles placed close together on the carapace in *C.* (*C.*) *paniaicus* while carapace surface is smooth in the new species. Chelae are 3.5-4.0 times as long as broad in *C.* (*C.*) *paniaicus* while 2.4-3.5 times in the new species.

The new species differs from *C.* (*C.*) *papuanus* in the number of rostral teeth and slightly in the shape of chelae. In *C.* (*C.*) *papuanus* each rostral margin bears 2 large teeth in the distal half while the new species has no prominent rostral teeth. Chelae are 2.5 times as long as broad with fingers equally wide in *C.* (*C.*) *papuanus* while 2.4-3.5 times as long as broad with fixed finger 2.4 times broader than dactyl in the new species.

The new species differs from *C.* (*C.*) *peknyi* in the shape of rostrum, the number of rostral teeth, the length of rostral carinae, the shape of scaphocerite, the number of cervical spines, the setose level at base of fingers, the size of eyes, and the shape of chelae. Rostrum of *C.* (*C.*) *peknyi* is slender with well-developed rostral carinae. These characteristics together with triangular shape of scaphocerite place this species into the subgenus *Astaconephrops* rather than in the subgenus *Cherax*; the incorrect classification of this species by Lukhaup and Herbert (2008) was probably caused by the absence of a soft uncalcified patch on outer margin of chelae in adult males, but this character is not crucial for placement in the subgenus *Astaconephrops* (Holthuis, 1949). Moreover Bláha et al. (unpublished data) noted that species from the subgenus *Astaconephrops* are placed in very different clades, but the position of both subgenera is unclear and a detailed revision using molecular data is recommended.

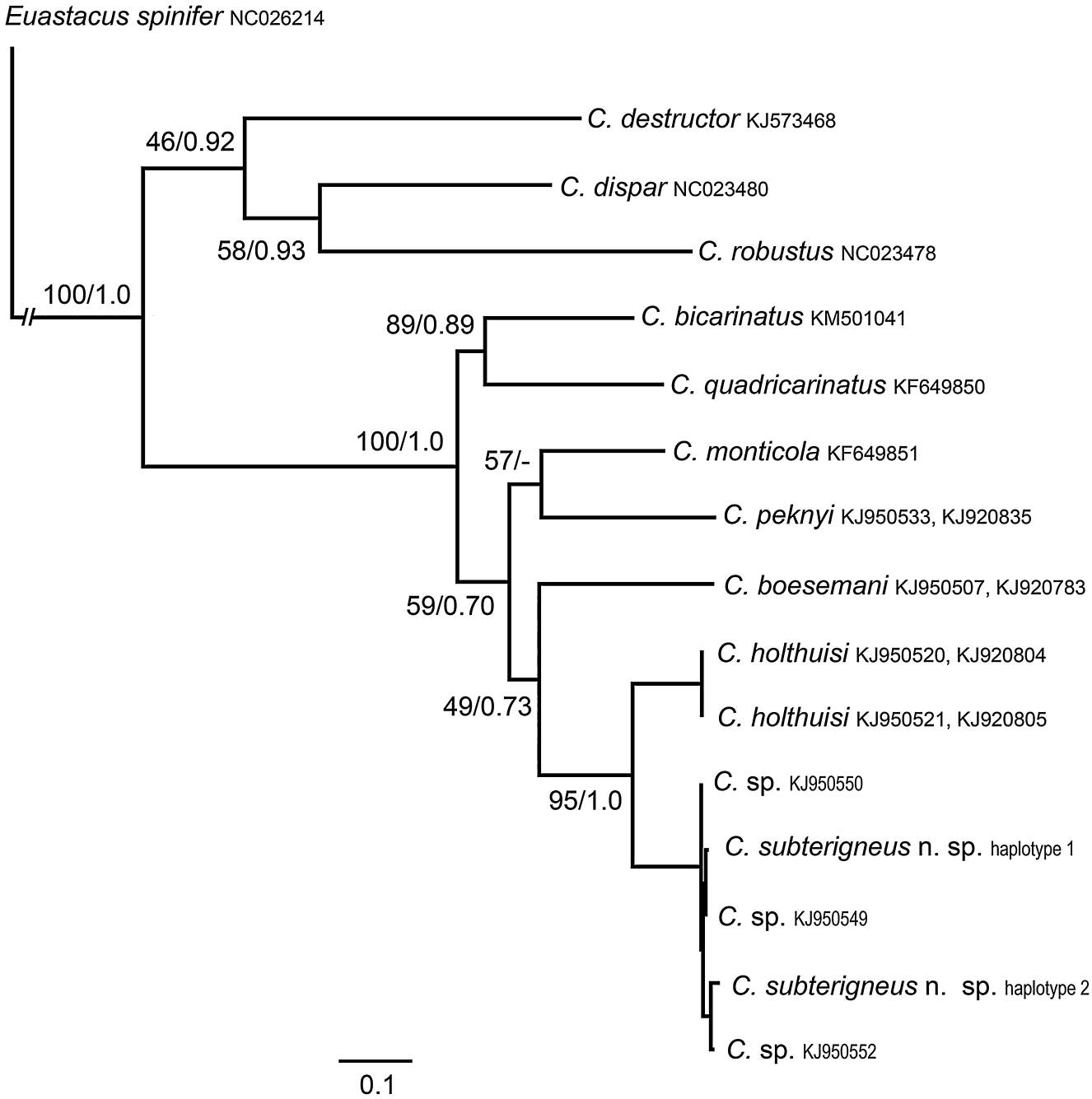

Fig. 7. Maximum likelihood phylogram of selected species of *Cherax* based on combined COI and 16S dataset. ML bootstrap values and posterior probabilities are displayed at each node.

In comparison, with the aforementioned characteristics, the new species has a broad rostrum with a poorly developed rostral carinae and a scaphocerite that is semicircular in shape. In *C.* (*C.*) *peknyi*, each rostral margin bears 2 teeth while the new species has no prominent rostral teeth. In *C.* (*C.*) *peknyi* 3-4 anteriorly directed spines are found clustered together laterally just behind the cervical groove, while no cervical spines are present in the new species. Prominent and dense setose piles at base of fingers are present in *C.* (*C.*) *peknyi* and differentiate this species from the new species, which has far less prominent setose hairs on the surface of the fingers. The eyes are smaller in the new species than in *C.* (*C.*) *peknyi*. Chelae are 4.5 times as long as broad in *C.* (*C.*) *peknyi*, while only 2.4-3.5 times in the new species.

The new species differs from *C.* (*C.*) *solus* in the number of rostral teeth, the size of eyes, the carapace surface, and in the shape of chelae. In *C.* (*C.*) *solus* each rostral margin bears 3-4 teeth in the distal half while the new species has no prominent rostral teeth. The eyes are smaller in the new species than *C.* (*C.*) *solus*. Behind the cervical groove the carapace is densely tuberculated in *C.* (*C.*) *solus* while smooth in the new species. Chelae are 4.0 times as long as broad in *C.* (*C.*) *solus* with the fixed finger only slightly

broader than dactyl while 2.4-3.5 times as long as broad with the fixed finger being 2.4 times broader than the dactyl in the new species.

Phylogenetics.—The phylogenetic relationship inferred from two mitochondrial gene fragments (COI and 16S) results in a phylogram with a clearly defined species *C. subterigneus* n. sp. (Fig. 7). The new species forms a strongly supported (95-100%) monophyletic clade with *C. holthuisi* differing at 10.9, 4.1 and 7.5% (COI, 16S and combined dataset, respectively) from each other (K2P distance). Moreover, our haplotypes were clustered with three GenBank sequences assigned as *C.* sp. (KJ950549-KJ950550, KJ950452). The new species together with *C. holthuisi*, *C. boesemani*, *C. peknyi*, *C. quadricarinatus*, *C. bicarinatus*, and *C. monticola*, belongs to the northern group of *Cherax* species occurring in New Guinea and North Australia. The *C. dispar*, *C. destructor*, and *C. robustus* here represent members of western species group, while *Euastacus spinifer* (NC026214.1) here represents an outgroup. From eight analysed specimens, two haplotypes were identified at each mitochondrial locus (COI and 16S) (GenBank accession numbers KT387669-KT387670 for COI; and KT387671-KT387672 for 16S rRNA). The bPTP analysis identified all species shown in the phylogram, including the new species, *C. subterigneus* n. sp. Both the high level of sequence divergence, along with the morphological differences described above, suggests that *C. subterigneus* n. sp. is distinct from closely related *C. holthuisi*, and supports the view it should be described as a separate species.

Note Added in Proof

In the interim since this article was accepted for publication (received 26 March 2015, accepted 5 August 2015, available online 8 September 2015), the following paper was published: Lukhaup, C., J. Panteleit, and A. Shrimpf. 2015. *Cherax snowden*, a new species of crayfish (Crustacea, Decapoda, Parastacidae) from the Kepala Burung (Vogelkop) Peninsula in Irian Jaya (West Papua), Indonesia. ZooKeys 518: 1-14 (received 17 July 2015, accepted and published 24 August 2015). *Cherax subterigneus* Patoka, Bláha, and Kouba, 2015 appears to be indistinguishable from *C. snowden* Lukhaup, Panteleit, and Shrimpf, 2015, and is probably a junior subjective synonym of the latter.

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ORIGINAL ARTICLE

Unrecognized diversity in New Guinean crayfish species (Decapoda, Parastacidae): The evidence from molecular data

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Abstract

The phylogenetic relationships among imported ornamental crayfish belonging to the genus *Cherax* were inferred from a combined dataset of 3 mitochondrial genes (COI, 16S and 12S) and by comparison with available GenBank sequences of 14 *Cherax* species. Furthermore, the concordance of previously described species obtained from a wholesaler (*Cherax boesemani*, *C. holthuisi* and *C. peknyi*) with available GenBank sequences was verified based on COI with special respect to comparison with sequences assigned as *Cherax* species. Recently described species *C. gherardiae*, *C. pulcher* and *C. subterigneus* belong to the northern group of *Cherax* species. Comparison and analysis with other GenBank COI sequences show previously unreported diversity of New Guinean species, suggesting 5 putative new species. Surprisingly, species assigned to the subgenus *Astaconephrops* do not form a monophyletic clade; this subgenus should be reappraised relative to the purported typical morphological characteristic of the uncalcified patch on male chelae. Increasing importation of crayfish underscores the importance of accurate species identification. Use of basic molecular methods is a necessary requisite for documenting occurrence, abundance and population trends of target species. Consequently, it helps to support eventual conservation decision-making by stakeholders.

Key words: *Cherax*, cytochrome c oxidase, DNA barcoding, mitochondrial DNA, ornamental crayfish

INTRODUCTION

The genus *Cherax* (Decapoda: Parastacidae) comprises a group of moderately burrowing crayfish species that is most widespread across Australia and southern New Guinea and is divided into 2 subgenera, *Astaconephrops* and *Cherax* (Austin 1996). All New Guinean crayfish belong to this genus (Holthuis 1982). Fetzner (2005) recorded 53 species in the genus *Cherax*, but *C. minor* Holthuis, 1996 is missing from this checklist. We can state that 22 species have their native range in New

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Guinea and 2 of them also occur in Northern Australia (Fig. 1, Table 1).

In contrast to the Australian *Cherax* species, New Guinean species have been at the edge of scientific interest for a long time. The systematics of Australian *Cherax* crayfish has been described in detail (e.g. Austin 1996; Austin & Knott 1996; Munasinghe *et al.* 2004), but in the case of the New Guinean group, we have only publications based on results of zoological expeditions and trips over the period 1858–1959 (Holthuis 1949, 1982, 1996). Despite numerous expeditions, knowledge about these crayfish relative to taxonomy, ecology, distribution, conservation status and value to local communities has remained fragmentary (Holthuis 1982).

The situation began to change when the pet trade with crayfish started in the 1990s (Chucholl 2013). Certain *Cherax* crayfish native to New Guinea, have been exploited for ornamental purposes in recent years (Chucholl 2013; Papavlasopoulou *et al.* 2014; Patoka *et al.* 2014). The vast majority of these crayfish were field

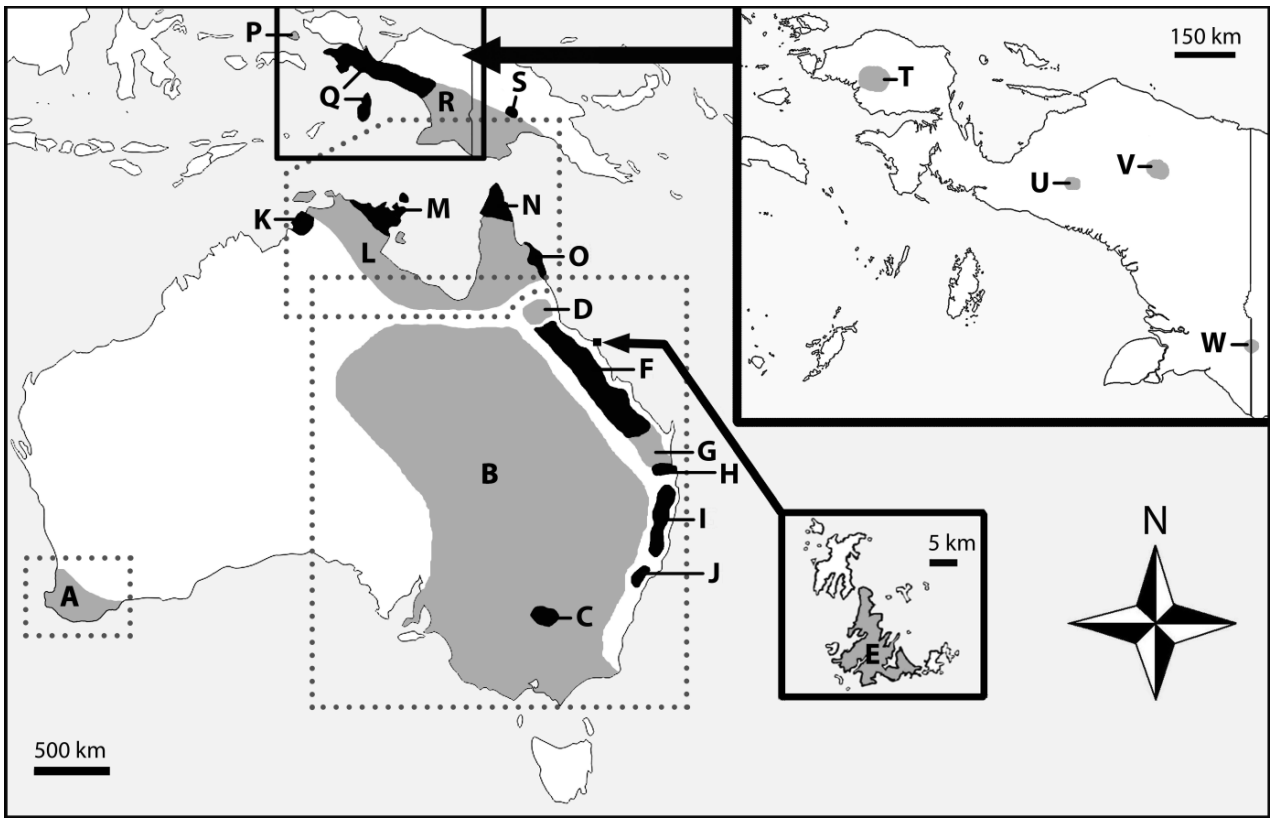

Figure 1 Known distribution of *Cherax* species in Australia and New Guinea (according to Riek 1967; Holthuis 1982, 1996; Munasinghe *et al.* 2004; Coughran 2005; Coughran *et al.* 2008, 2012). Shaded areas and letters indicate group or single species distribution. Dotted lines contain species belonging to northern, south-western and eastern species group. Note that this map still contains many gaps and should be understood as an indication of general distribution patterns rather than an accurate distribution map, especially in New Guinea, where distribution information is particularly scrappy. (**A**) *C. quinquecarinatus*, *C. cainii*, *C. tenuimanus*, *C. glaber*, *C. glabrimanus*, *C. crassimanus*, *C. neocarinatus*, *C. plebejus*, *C. preissii*; (**B**) *C. destructor*; (**C**) *C. rotundus*; (**D**) *C. parvus*; (**E**) *C. austini*, *C. cid*; (**F**) *C. cairnsensis*, *C. gladstonensis*; (**G**) *C. punctatus*, *C. dispar*, *C. depressus*, *C. robustus*; (**H**) *C. leckii*, *C. gladstonensis*; (**I**) *C. cuspidatus*; (**J**) *C. setosus*; (**K**) *C. nucifraga*; (**L**) *C. quadricarinatus*; (**M**) *C. barretti*, *C. bicarinatus*; (**N**) *C. wasselli*, *C. rhynchotus*; (**O**) *C. cartacoolah*; (**P**) *C. misolicus*; (**Q**) *C. lorentzi*; (**R**) *C. albertisii*, *C. quadricarinatus*, *C. rhynchotus* (2 latter distributed in New Guinea only in sector of dotted line); (**S**) *C. papuanus*; (**T**) *C. boesemani*, *C. gherardiae*, *C. holthuisi*, *C. pulcher*, *C. subterigneus*; (**U**) *C. boschmai*, *C. buitendijkae*, *C. communis*, *C. longipes*, *C. murido*, *C. pallidus*, *C. paniaicus*, *C. solus*; (**V**) *C. monticola*, *C. minor*; (**W**) *C. peknyi*.

Table 1 Taxa examined for this study with number of analyzed individuals (*N*) and haplotypes (H) found in particular mitochondrial genes (COI, 16S, 12S), GenBank accession numbers and voucher numbers for all and originally analyzed individuals in this study, respectively

	Voucher number	Authority	Geographic distribution	COI		GenBank accession number	16S		GenBank accession number	12S		GenBank accession number
				N	H		*N*	H		*N*	H	
Samples originally analyzed in our study												
C. boesemani	JP2014/10-D23 JP2014/10-D22	Lukhaup and Pekny 2008	NG	7	3	KU821414-16	6	3	KU821429-31	6	2	KU821441-42
C. gherardiae	JP2014/10-20 JP2014/10-21 JP2014/10-24	Patoka, Bláha and Kouba 2015	NG	3	2	KU821417-18	3	1	KU821432	3	2	KU821443-44
C. holthuisi	—	Lukhaup and Pekny, 2006	NG	5	3	KU821419-21	5	2	KU821433-34	4	1	KU821445
C. peknyi	—	Lukhaup and Herbert, 2008	NG	2	2	KU821422-23	2	2	KU821435-36	2	2	KU821446-47
C. pulcher	JP2014/02-D25 – D29	Lukhaup, 2015	NG	5	3	KU821424-26	4	2	KU821437-38	4	2	KU821448-49
C. quadricarinatus	—	(von Martens, 1868)	NG, N-AU	1	1	KU821427						
C. subterigneus	JP2014/10-31 – 37	Patoka, Bláha and Kouba 2015	NG	8	2	KT387671-2	5	2	KT387669-70	8	3	KU821450-51
C. sp. Black Scorpion	JP2014/02-BS01 – BS04	-	NG	4	4	KU821410-13	3	1	KU821428	3	21	KU821439-40
Used sequences available at GenBank												
C. boesemani		Lukhaup and Pekny 2008	NG	1		KJ950507 KM501042 NC026224			NC026224			NC026224
C. communis		Holthuis, 1949	NG	10		KJ950508-12 & 14-18						
C. holthuisi		Lukhaup and Pekny, 2006	NG	2		KJ950520-21 NC026227 KM501039			NC026227			NC026227
C. leckii				1		KM039114						
C. monticola		Holthuis, 1950	NG	2		NC022938, KF649851	1		NC022938	1		NC022938
C. murido		Holthuis, 1949	NG	2		KJ950526-27						
C. paniaicus		Holthuis, 1949	NG	5		KJ950528-32						
C. peknyi		Lukhaup and Herbert, 2008	NG	1		KJ950533						

Table 1 Continued

	Voucher number	Authority	Geographic distribution	COI		GenBank accession number	16S	GenBank accession number	12S	GenBank accession number
C. sp.		-	NG	31	22	KJ950505-6 & 22-25 & 38-52				
C. cairnsensis		Riek, 1969	E-AU	3		KM039106-7 NC023480	1	NC023480	1	NC023480
C. cuspidatus		Riek, 1969	E-AU	2		KM039109-10				
C. destructor destructor		Clark, 1936	E-AU	3		NC023478, KJ950555, KJ573468		NC023478, KJ573468		NC023478, KJ573468
C. destructor albidus		Clarke, 1936	E-AU	1		FJ965956				
C. dispar		Riek, 1951	E-AU	3		NC023480, KJ950554 KM039113	1	NC023480	1	NC023480
C. parvus		Short and Davie, 1993	E-AU	1		DQ006293				
C. preissii		(Erichson, 1846)	E-AU	2		AF493622-3				
C. robustus		Riek, 1951	E-AU	2		NC023478, KM039115	1	NC023478	1	NC023478
C. rotundus		Clark, 1941	E-AU	1		KM039116				
C. setosus		(Riek, 1951)	E-AU	1		KM039117				
C. bicarinatus		(Gray, 1845)	N-AU	4		KM501041, KJ950502-4	1	KM501041	1	KM501041
C. cainii		Austin and Ryan, 2002	SW-AU	1		KF649849	1	KF649849	1	KF649849
C. crassimanus		Riek, 1967	SW-AU	2		AF493624-25				
C. glaber		Riek, 1967	SW-AU	2		KF649852, FJ965958	1	KF649852	1	KF649852
C. quadricarinatus		(von Martens, 1868)	NG, N-AU	2		KF649850, DQ006294	1	KF649850	1	KF649850
C. quinquecarinatus		Gray, 1845	SW-AU	3		NC023479, AF493618,20	1	NC023479	1	NC023479
C. tenuimanus		(Smith, 1912)	SW-AU	7		NC026559, AF493626-29, KJ950558	1	NC026559	1	NC026559
Euastacus yarraensis		(McCoy, 1888)		1		NC023811	1	NC023811	1	NC023811
E. spinifer		(Heller, 1865)		1		NC026214	1	NC026214	1	NC026214
Astacopsis gouldi		Clark, 1936		1		KD006289				
A. tricornis		Clark, 1936		1		DQ006290				

captured and exported by Indonesian wholesalers into the European, US and Japanese pet markets (Lukhaup & Herbert 2008; Patoka *et al.* 2015). Unfortunately, some of these species are scientifically undescribed and they are advertised under misnomers or trade names only (Chucholl 2013; Patoka *et al.* 2014). The population statuses and trends of New Guinean crayfish species are not known, so a potential decline of abundance because of intensive capture can be easily overlooked. Therefore, accurate species identification is very important for monitoring details on occurrence, abundance and trends of populations, and/or consequently for support of eventual conservation decision-making by stakeholders. Moreover, risk assessment for potential invasive crayfishes in trade would also benefit from accurate and timely species identification (Tricarico *et al.* 2010; Zeng *et al.* 2015).

In regards to the import of ornamental crayfish, 6 new *Cherax* species from New Guinea have been scientifically described in the past decade; 3 of them belong to subgenus *Cherax*: *C. holthuisi* (Lukhaup & Pekny 2006), *C. peknyi* (Lukhaup & Herbert 2008) and *C. subterigneus* (Patoka *et al.* 2015b); 3 belong to subgenus *Astaconephrops*: *C. boesemani* (Lukhaup & Pekny 2008), *C. pulcher* (Lukhaup 2015) and *C. gherardiae* (Patoka *et al.* 2015a). Most of these descriptions, except for *C. subterigneus* and *C. gherardiae*, were based only on morphometrics, although it is well known that crayfish show substantial variability in many morphological characters which can be associated with habitat variation (Austin & Knott 1996). A genetic analysis would help in species identification and clarification of phylogenetic relationships (Mathews *et al.* 2008; Breinholt *et al.* 2012; Thoma & Loughman 2014). In this study, we focused on phylogenetic analysis of recently described *Cherax* species from New Guinea and available *Cherax* sequences from the GenBank database.

MATERIAL AND METHODS

Collecting of individuals

Cherax crayfish were field-captured in New Guinea and subsequently imported from Indonesia into the Czech Republic between October 2013 and February 2014. We collected 35 individuals altogether from a leading Czech wholesaler of ornamental freshwater animals. These 35 animals included the recently described species *C. boesemani*, *C. gherardiae*, *C. holthuisi*, *C. peknyi*, *C. pulcher* and *C. subterigneus*, but also individuals distributed under the pet trade name *C.* sp. Black Scorpion. Not all analyzed specimens are vouchered because they were originally used for another study and partly damaged; however, those specimens with voucher numbers are deposited at the Czech University of Life Sciences Prague (codes are listed in Table 1).

Molecular analysis

Total genomic DNA was extracted from 200–500 μL of hemolymph taken from the ventral part of the abdomen with an insulin needle and syringe, then stored in pure ethanol until DNA extraction using a NucleoSpin® Tissue kit (Macherey-Nagel GmbH KG Düren, Germany) following the manufacturer's protocol. Three molecular markers were amplified using the polymerase chain reaction (PCR); namely, cytochrome c oxidase subunit I (COI), 16S and 12S rRNA. Primers LCO1490 and HCO2198 (Folmer *et al.* 1994), 1471 and 1472 (Crandall & Fitzpatrick 1996), and L13337 and H13845 (Machida *et al.* 2004) were used for COI, 16S and 12S rRNA amplification, respectively. All PCR reactions were carried out in Biometra T3000 thermocycler (Göttingen, Germany) with the following cycling conditions for all 3 molecular markers: 5 min at 95 °C; 40 cycles of 30 s at 94 °C, 30 s at 50 °C, 45 s at 72 °C; 10 min at 72 °C. PCR reactions were run in 30 μL of 15 μL of PPP Master mix [150 mM Tris–HCl, pH 8.8, 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 μM dATP, 400 μM dCTP, 400 μM dGTP, 400 μM dTTP and 100 U⁄mL Taq-Purple DNA polymerase], 1 μL of each primer (10 pmol/μL) and 1.5 μL genomic DNA. For sequencing, the PCR products were electrophoresed on agarose gel, then dissected and purified by the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. Düren, Germany). Purified products were subsequently sequenced on an ABI automatic capillary sequencer (series 373) (Macrogene, Korea) using LCO, 1472 and L13337 primers. Together with amplified gene fragments of analyzed individuals, available sequences of other *Cherax* species were used (details in Table 1). All newly obtained sequences were deposited in GenBank under the accession numbers listed in Table 1.

Data analysis

Nucleotide sequences were aligned using MAFFT v7.017 (Katoh *et al.* 2002) implemented in GENEIOUS 8.0.5 (Kearse *et al*. 2012), and sequences were checked visually for ambiguous peaks. Furthermore, to prevent usage of nuclear mitochondrial pseudogenes (numts) in analyses (Song *et al.* 2008), the sequences of protein coding gene (COI) were translated into aminoacids to check for indels and stop codons, and

compared to closely related species. In addition, the topological congruence between the trees obtained with 3 mitochondrial gene sequence sets was calculated according to the maximum agreement subtree (MAST) method using the online calculator of congruency index *I*cong (de Vienne *et al.* 2007). For the concatenated dataset, partial gene fragments were extracted from available *Cherax* mitogenom GenBank sequences (Table 1). Similarly, to reveal relationships among the New Guinean species mentioned in the introductory part of this study, COI gene fragments from the GenBank database were used (Table 1), including *Cherax* species assigned as *Cherax* sp. As an outgroup, *Euastacus yarraensis* (NC023811) and *Euastacus spinifer* (NC026214) were used because they resolved relationships of ingroup species well. Pairwise model-corrected genetic distances were calculated for each gene for all of our original samples of *Cherax* taxa in PAUP* v.4.02b (Swofford 2002), for which we report the mean genetic distance in order to compare the relative amounts of divergence of each gene and among particular species. The GTR+I+G model of evolution was chosen by Bayesian information criterion (BIC) estimated using jModelTest 2.1.7 (Darriba *et al.* 2012) for the combined dataset, while HKY+I+G, and HKY+G models were selected for particular COI and for 2 ribosomal subunits (16S and 12S rRNA) datasets, respectively. Furthermore, the GTR+I+G model fits the best for the COI dataset of the available sequence of *Cherax* species based on BIC. A maximum likelihood (ML) tree was constructed in PHYML (Guindon & Gascuel 2003) implemented in GENEIOUS 8.0.5 (Kearse *et al.* 2012) with 1000 bootstrap replications, while Bayesian analyses were conducted in MrBayes 3.2.4. (Ronquist *et al.* 2012) with at least one million generations and trees sampled every 200th generation. The first 25% from the cold chain was discarded. The trees were summarized into a consensus tree with nodal support estimated with posterior probabilities.

Bayesian implementation of the Poisson tree processes (PTP) model with non-ultrametric gene trees was performed using the bPTP web server (http://species.h-its.org/) for species delimitation (Zhang *et al.* 2013). The tree was assigned as rooted and the outgroup was excluded from the analysis. For species delimitation, the standalone PTP model generally outperforms the general mixed Yule coalescent (GMYC) method (Fujisawa and Barraclough, 2013). We ran the analysis independently on particular gene trees (COI, 16S, 12S). Because the results were consistent with the analysis of the combined dataset we presented the results from this combined dataset. The analysis was also performed on a COI dataset of most of the available sequences from New Guinean crayfish species.

RESULTS

Sequence variation and alignments

Sequences from the 35 analyzed individuals were amplified for 3 mitochondrial genes; however, amplification of 16S and 12S rRNA was less efficient in some individuals (Table 1). The mean model-corrected sequence distance for genes within the genus *Cherax* indicates that COI (39%) is the most variable followed by 12S (23.8%) and then 16S (16.7%). The overall phylogenetic topology based on the COI, 16S or 12S rRNA sequences was consistent (*I*cong = 2.736 – 2.740, $P = 2.07e^{-12} – 2.74e^{-13}$). Thus, 3 datasets were combined for subsequent analyses. The combined dataset (excluding the outgroup) consists of 1588 bp, containing 620 variable sites of which 528 are parsimony informative, while the COI dataset (excluding the outgroup) consists of 560 bp, containing 246 variable sites of which 224 are parsimony informative.

Combined dataset phylogenetic analysis

The recently described *C. boesemani*, *C. holthuisi*, *C. gherardiae*, *C. peknyi*, *C. pulcher* and *C. subterigneus* and individuals with the trade name *C*. sp. Black Scorpion) form monophyletic clades with strong bootstrap support (85–100%) obtained with both ML and BI methods; these are placed in the group of northern *Cherax* species previously defined by Munasinghe *et al.* (2004). Divergences among these clades range from 4% (*C. holthuisi vs C*. sp. Black Scorpion) to 13.8% (*C. peknyi vs C. boesemani*). The clade of *C. boesemani* contains 2 well-supported lineages represented by our samples and the GenBank sequences of *C. holthuisi* (NC026227) and *C. boesemani* (KM501042) differing at 4%. Similarly, the GenBank sequence of *C. boesemani* (NC026224) is embedded among our *C. holthuisi* sequences. Furthermore, all of the previously mentioned species belong to a strongly supported clade (94–100%), with *C. quadricarinatus*, *C. bicarinatus* (Gray, 1845) and *C. monticola* forming the northern group of *Cherax* species (Fig. 2).

Cytochrome c oxidase phylogenetic analysis

Because there is only a limited number of complete mitochondrial sequences available for *Cherax*, the

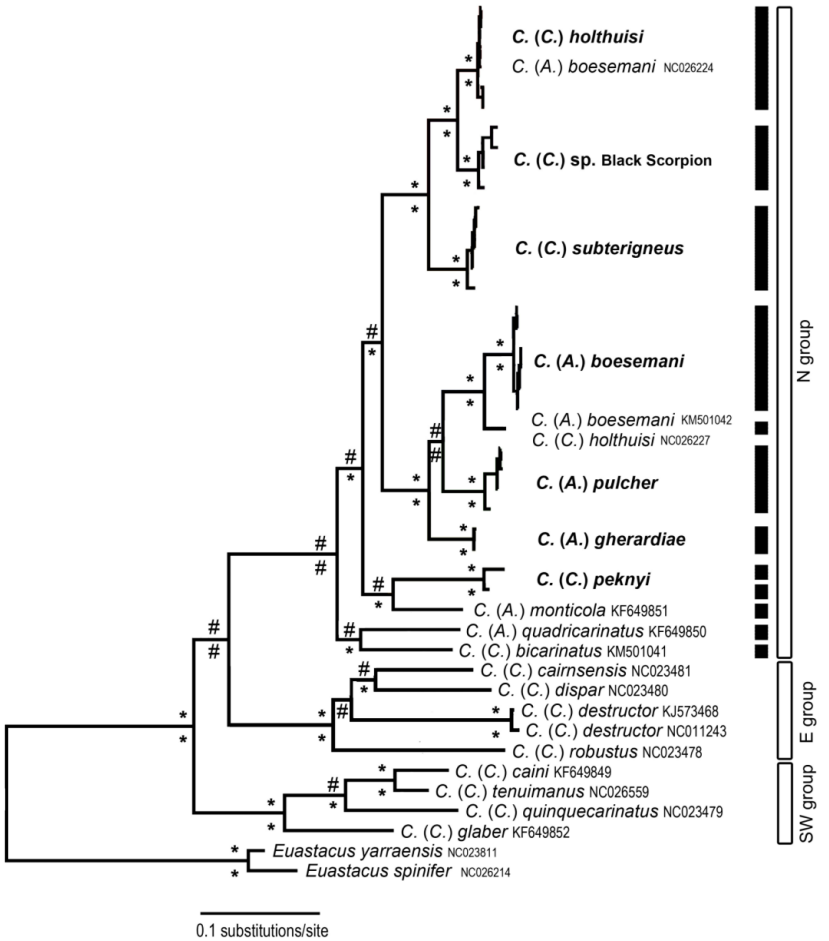

Figure 2 Maximum likelihood (ML) tree depicting relationship of *Cherax* species from mtDNA combined dataset (COI, 16S and 12S). Individuals originally analyzed in this study are written in bold. ML bootstrap and posterior probabilities are displayed above and under each node, respectively. The asterisk and hash represent values ≥95% and in the range from 75% to 94%, respectively. Each *Cherax* specimen from GenBank is labeled with its code. Specific subgenera (*C.* – *Cherax* or *A.* – *Astaconephrops*) are mentioned in known species. Full bars next to the particular individuals mean species status assigned by PTP methods, here displayed only for the north group of *Cherax* species, while empty bars mean division into 3 main lineages. E group, eastern species group; N group, north species group; SW group, south-western species group.

phylogeny based only on COI enables comparison with the rest of New Guinean species with known and available COI sequences, including specimens assigned as *Cherax* sp. The basic phylogram topology is consistent; that is, there are 3 main clades corresponding to the northern, eastern and southwestern species groups. The northern species group consists of 2 well defined and supported clades (100%). The first consists of *C. quadricarinatus* and *C. bicarinatus* individuals, and the other consists of the rest of the New Guinean species. All of the individuals assigned as *Cherax* sp. were embedded within the northern group being clustered either with already known species (specimens WAM1-4 with *C. monticola*, specimens SOR4, 5 and 7 with *C. subterigneus* and specimen RAC 1 with *C. quadricarinatus*, Fig. 3). The rest of the *Cherax* sp. specimens comprise 4 monophyletic clades with moderate to strong bootstrap support (≥55%) either closely related to the clade of *C. subterigneus*, *C. holthuisi* and *C.* sp. Black Scorpion, or to the clade of *C. boesemani*, *C. gherardiae* and *C. pulcher*, respectively (Fig. 3). The clade of *C. boesemani* comprises 2 lineages, the first composed of individuals analyzed in this study and the other by the GenBank sequence. This clade is consistently clustered with *C. pulcher* and *C. gherardiae*. Similarly, *C. holthuisi*, *C. subterigneus* and *C.* sp. Black Scorpion comprise a monophyletic clade with a consistent position according to BI and ML analysis. The same is true also for *C. peknyi*, which clustered consistently as a sister group to *C. monticola* (combined dataset) or to the clade of *C. monticola* with *Cherax* species from lakes Paniai and Tiki (*C. boschmai*, *C. communis*, *C. longipes*, *C. pallidus*, *C. paniaicus* and *C. murido*; Figs 1 and 3), respectively. The latter species comprise a cluster of very similar sequences differing at 5.1%.

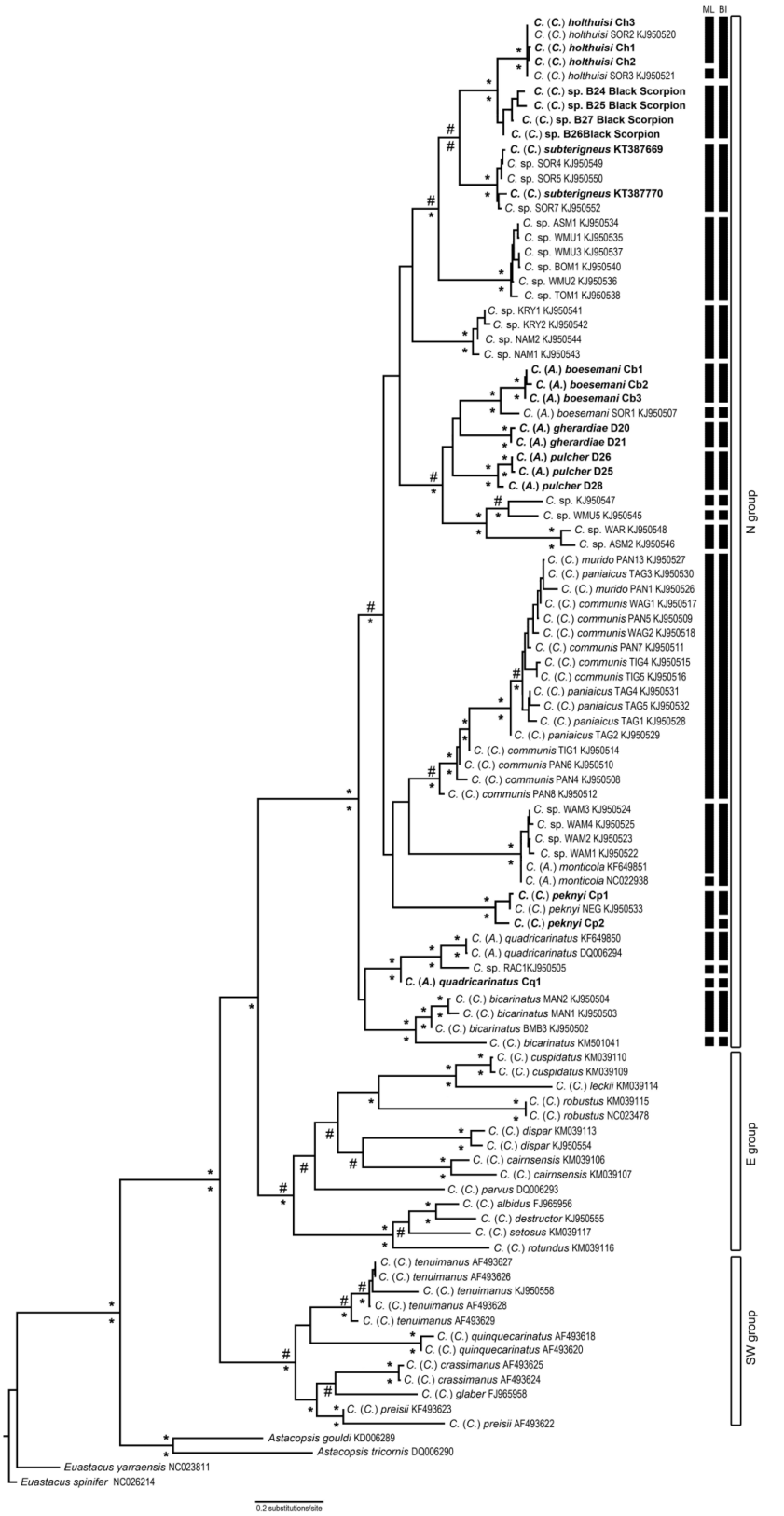

Figure 3 Maximum likelihood (ML) tree depicting relationship of *Cherax* species from COI sequences. Individuals originally analyzed in this study are written in bold. ML bootstrap and posterior probabilities are displayed above and under each node, respectively. The asterisk and hash represent values ≥95% and in the range from 75% to 94%, respectively. The specimens from GenBank are labeled with theirs codes. Specific subgenera (*C.* – *Cherax* or *A.* – *Astaconephrops*) are mentioned in known species. Full bars next to the particular individuals mean species status assigned by Poisson tree processes methods either by ML or Bayesian heuristic search (BI), here displayed only for north group of *Cherax* species.

Species delimitation

We ran 2 analyses, one based on an ML phylogram from the combined dataset, and the second based on COI sequences of New Guinean *Cherax* species available at GenBank. The PTP analysis assigned individuals from this study to species according to affiliation with particular monophyletic clades; that is, the previously described *C. boesemani*, *C. gherardiae*, *C. holthuisi*, *C. peknyi*, *C. pulcher* and *C. subterigneus*, although 2 haplotypes of *C. peknyi* were each assigned as particular species with strong support (0.81). Analysis of the combined dataset reveals the same number of identified species for both methods, either a simple heuristic search or ML (Fig. 2). In contrast, the number of estimated species differs using a simple heuristic search compared to the ML method for the COI dataset. Among others, compared to the ML method, the simple heuristic search identified more species within the clade of *C. peknyi*, while on the other hand, fewer in the clade of *C. holthuisi* and *C. monticola*. Furthermore, our individuals of *C. boesemani* are assigned as a different species to the GenBank sequences of *C. boesemani*. The specimens of *C. communis*, *C. boschmai*, *C. paniaicus* and *C. pallidus* are assigned as a single species. Undescribed species *Cherax* sp. which form 4 monophyletic clades, referred to in the previous subsection, are also assigned as 5 particular species with stong support (0.61–0.99; Fig. 3).

DISCUSSION

Phylogenetic relationships

The results of the phylogenetic analysis of 3 mitochondrial genes indicated that the 3 phylogenetic groups of *Cherax* species proposed by Munasinghe *et al.* (2004) is largely supported. Moreover, the present study provides a basic framework for New Guinean *Cherax* species phylogenetic relationships. The outcomes of the analyses also corroborate that eastern *Cherax* species are a sister group to the northern species group, however with only moderate support depending on the method used (52–82%). Moreover, the northern species group is split into 2 highly supported lineages represented by: (i) *C. quadricarinatus*, *C. bicarinatus*, (and also *C. albertisii*, *C. lorentzi* and *C. rhynchotus* based on GenBank 16S data analysis, not shown here), all mentioned species, excluding *C. bicarinatus*, occurring in Northern Australia and New Guinea; and (ii) the rest of the species occurring only in New Guinea. The assignment of *C. holthuisi* SOR3 strain (NC026227) and *C. boesemani* SOR1 strain (NC026224) to the opposite species clades in the combined dataset analysis was probably due to mislabeling by the authors of the GenBank submission (Fig. 2), because other available sequences of these 2 species correspond well with ours (*C. boesemani* [SOR1 KJ950507, SOR3 KM501042] and *C. holthuisi* [SOR1 KM501039, SOR2 KJ950520 and SOR3 KJ950521]). We contacted the GenBank submission office to communicate these findings to the authors of the GenBank submission.

Species delimitation

The clustering species to evolutionary significant units is robust enough comparing to the widely used GMYC method; moreover, the key advantage of PTP method is in using non-ultrametric trees and modeling speciation events relative to number of substitutions rather than time (Tang *et al.* 2014). This approach makes the assumption that the number of substitutions between species is significantly higher than the number of substitutions within species. The present study has corroborated 3 recently described *Cherax* species based on the PTP method used for the combined dataset, namely *C. gherardiae*, *C. pulcher* and *C. subterigneus*. The PTP method supports species status (0.63) of *Cherax* sp. Black Scorpion in most of the datasets (except 12S rRNA, where the individuals are joined with *C. holthuisi*), and also strongly supports (0.92) the lineage of *C. boesemani* GenBank sequences from our *C. boesemani* individuals and 2 haplotypes of *C. peknyi* (0.70), the latter only in the COI dataset. The other clue is whether there are any morphological differences among these individuals and closely-related species. Unfortunately, we have only had the opportunity to examine our individuals and compare them to the available morphological data on *Cherax* species (Holthuis 1982; Lukhaup & Pekny 2006) and among each other. Because we found no unambiguous characteristic to differentiate *Cherax* sp. Black Scorpion from *C. holthuisi*, except for the coloration, we will refer to these individuals as only having a different color morph of *C. holthuisi*. This finding is important for conservationists and also for importers and keepers of ornamental crayfish, because both of these crayfish are internationally traded as pets. Concerning *C. boesemani* and *C. peknyi*, we have only our analyzed individuals to evaluate, whose designations were verified according to Lukhaup and Herbert (2008) and Lukhaup and Pekny (2008). Certainly, future molecular analysis of more extensive specimen collections using suitable nuclear markers for crayfish (28S, Koizumi *et al.* 2012 or GAPDH, Mathews *et al.* 2008) will be necessary to clarify the status of the individuals referred to as *Cherax* sp. Black Scorpion and individuals within the clade of *C. boesemani*. Moreover, geometric mor-

phometry may help to differentiate species, as shown in the genus *Cambarus* (Helms *et al.* 2015) where genetic differences manifested in shape variation.

Concerning the other undescribed individuals forming monophyletic clades (Fig. 3) not clustered with already known species, all of these were assigned by PTP analysis as putative new species. This is all that we can assume about GenBank individuals assigned as *Cherax* sp. based on sequence analysis, because there are no available data on the sampling localities nor the voucher number or the morphology of particular individuals. Similarly, the cluster of several species, namely of *C. communis*, *C. murido* and *C. paniaicus*, demonstrates low genetic distance to each other, however, all of these species are morphologically well defined (Holthuis 1949). All of them occur in Paniai Lake, except for *C. longipes* and *C. solus*, which occur in Tigi Lake, all in the area known as Wissel Lakes (Holthuis 1949). The rest of the species from this area (*C. boschmai*, *C. buitendijkae*, *C. longipes*, *C. pallidus* and *C. solus*) are not shown in the COI phylogram; they demonstrate the same pattern using available 16S rRNA sequences from GenBank (not shown here). Although there is some substructure of this clade in both analyzed genes (COI and 16S, the latter not shown here), it does not correspond to a geographical pattern of species distribution.

Subgenus *Astaconephrops*: morphological versus molecular data

A surprising output of COI analysis is the position of species that have been considered in the subgenus *Astaconephrops* Nobili, 1899. However, our assumptions based only on one mitochondrial marker should be taken with caution. This subgenus includes 10 New Guinean species: *Cherax* (*Astaconephrops*) *albertisii*, *C.* (*A.*) *boesemani*, *C.* (*A.*) *gherardiae*, *C.* (*A.*) *lorentzi*, *C.* (*A.*) *minor*, *C.* (*A.*) *misolicus*, *C.* (*A.*) *monticola*, *C.* (*A.*) *pulcher*, *C.* (*A.*) *quadricarinatus* and *C.* (*A.*) *rhynchotus*. Although we have analyzed sequences from only *C. boesemani*, *C. gherardiae*, *C. monticola*, *C. pulcher* and *C. quadricarinatus*, these species are embedded in very different clades (Fig. 2). However, recently described species (Lukhaup *et al.* 2015; Patoka *et al.* 2015) of which we are sure about their morphology (*C. gherardiae*, *C. pulcher*) sharing the same clade with *C. boesemani*, have the same soft decalcified patch on the outer distal margin of adult male claws. Although this characteristic could be assigned as a distinctive morphological apomorphy (Lukhaup & Herbert 2008), there are also other characteristics originally mentioned by Holthuis (1949) that distinguish this subgenus. He proposed that the genus *Cherax* might be divided into 2 groups according to well-developed rostral and sometimes also the median carinae. He examined specimens of *C. albertisii*, originally described by Nobili (1899) and assigned them as *Astaconephrops albertisii*. Holthuis (1950) later described and assigned *C. divergens* into the *Astaconephrops* section of the genus *Cherax*; he referred to the uncalcified patch on the male chelae as well as previously mentioned characteristics. Subsequent authors accepted this as a primary distinguishing characteristic between these 2 groups (Lukhaup & Pekny 2006, 2008; Lukhaup & Herbert 2008). In contrast to this clearly visible characteristic, the evaluation of the rostral or median carinae in some species assigned to the subgenus *Astaconephrops* is very subjective (i.e. in the recently described *C. minor* Holthuis, 1996). The same is true for the shape of the scaphocerite, which is triangular, or rather circular for *Cherax* or the *Astaconephrops* group, respectively. A similar situation with the validity of *Cambarus* subgenera classifications based on morphological characteristics is currently being intensely debated among the North American community of crayfish researchers (e.g. Breinholt *et al.* 2012). We are afraid that only advanced morphometric methods, combined with molecular evidence using already mentioned nuclear markers, will help to elucidate this incongruence in *Cherax* species relative to 2 different subgenera of *Cherax* and justify or omit their usage in future taxonomic works.

CONCLUSION

This study provides a basic framework of New Guinean *Cherax* species phylogenetic relationships based on individuals imported into the Czech Republic and the available GenBank sequences; however, we are limited to using only mitochondrial markers. The existence of 2 highly supported lineages within the north group of *Cherax* as well as uncertainty in the assignment of particular species to the subgenus *Astaconephrops* has raised further questions that need to be answered through detailed molecular studies including nuclear markers of more extensive collections. It is worth remarking that several putative new species were identified herein based on analysis of the combined dataset and COI sequences from GenBank. With growing crayfish importation (Patoka *et al.* 2015), accurate species identification using basic molecular methods is a necessary requisite for a focus on occurrence, abundance and trends of populations, and, consequently, for support of eventual conservation decisions by stakeholders in the area of original distribution. Moreover, there is strong potential for crayfishes in trade to be released and become invasive and, thus, the risk assessment for crayfishes in trade benefits from better and more information on the taxonomy and ecology of these organisms.

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Supplement 8

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RESEARCH PAPER OPEN ACCESS

Hungary: a European hotspot of non-native crayfish biodiversity

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Abstract – There is a long history of crayfish introductions in Europe and numbers keep increasing. In Hungary, spiny-cheek crayfish *Faxonius limosus*, signal crayfish *Pacifastacus leniusculus*, red swamp crayfish *Procambarus clarkii*, marbled crayfish *P. virginalis* and Mexican dwarf crayfish *Cambarellus patzcuarensis* have become established. Here we report on monitoring at two localities with novel crayfish assemblages closely linked to releases associated with the pet trade. Florida crayfish *Procambarus alleni* were recorded from the Gombás brook near Vác living in syntopy with the established spiny-cheek crayfish. Dozens of Florida crayfish individuals including egg-carrying females have been detected. The short lifespan of this species and its documented presence including two overwintering in at least two years suggests possible establishment. However, the lack of juvenile records calls for further monitoring as long-term propagule pressure cannot be ruled out. We also identified a single marbled crayfish in the Danube floodplain at the end of the monitoring campaign. The second locality (Városliget thermal pond in Budapest) harbours an even more diverse crayfish assemblage. Here, we identified numerous red swamp and marbled crayfish in syntopy with dozens of monitored redclaws *Cherax quadricarinatus* and seven individuals of New Guinean *Cherax* species – *C. holthuisi*, *C. snowden,* as well as two scientifically undescribed species. These findings clearly indicate the attractiveness of urban and, especially, thermal waters for the release of even expensive aquatic pets and highlight the hitherto poorly known biodiversity of New Guinean crayfish species.

Keywords: pet trade / biological invasion / animal release / invasive species / thermal water

Résumé – Hongrie : un point chaud européen de la biodiversité des écrevisses non indigènes. Les introductions d'écrevisse en Europe est une longue histoire et leur nombre ne cesse d'augmenter. En Hongrie, l'écrevisse américaine *Faxonius limosus*, l'écrevisse signal *Pacifastacus leniusculus*, l'écrevisse rouge de Louisiane *Procambarus clarkii*, l'écrevisse marbrée *P. virginalis* et l'écrevisse naine mexicaine *Cambarellus patzcuarensis* se sont établies. Nous présentons ici un rapport sur la surveillance à long terme dans deux localités où des assemblages d'écrevisses nouvelles sont étroitement liés aux lâchers associés au commerce des animaux de compagnie. L'écrevisse bleue *Procambarus alleni* a été observée dans le ruisseau de Gombás, près de Vác, vivant en syntopie avec l'écrevisse américaine établie. Des dizaines d'individus d'écrevisses de Louisiane, y compris des femelles porteuses d'œufs, ont été détectées. La courte durée de vie de cette espèce et sa présence documentée, y compris deux hibernations en au moins deux ans, suggèrent une possible implantation. Toutefois, l'absence de données sur les juvéniles exige une surveillance plus poussée, car on ne peut exclure la possibilité d'une pression de propagation à long terme. Nous avons également identifié une seule écrevisse marbrée dans la plaine d'inondation du Danube à la fin de la mission de

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surveillance. La deuxième localité (l'étang thermal de Városliget à Budapest) abrite un assemblage d'écrevisses encore plus diversifié. Ici, nous avons identifié de nombreuses écrevisses rouges de Louisiane et marbrées en syntopie avec des dizaines de *Cherax quadricarinatus* et sept individus d'espèces de Cherax de Nouvelle-Guinée – *C. holthuisi, C. snowden*, ainsi que deux espèces non décrites scientifiquement. Ces résultats indiquent clairement l'attrait des eaux urbaines et, surtout, des eaux thermales pour le lâcher d'animaux aquatiques, même coûteux, et mettent en évidence la biodiversité jusqu'ici mal connue des espèces d'écrevisses de Nouvelle-Guinée.

Mots-clés : commerce d'animaux de compagnie / invasion biologique / lâcher d'animaux / espèces envahissantes / eaux thermales

1 Introduction

Biological invasions have a negative impact on native biodiversity and ecosystem functioning (Simberloff *et al.*, 2013). Rates of biological invasions are continuously accelerating worldwide among both taxa and regions (Seebens *et al.*, 2017). Crayfish, ecologically important animals and freshwater ecosystem engineers (Kouba *et al.*, 2016; Momot, 1995), are an integral part of these processes (Holdich *et al.*, 2009; Twardochleb *et al.*, 2013). Several crayfish species are known to be highly invasive (Gherardi and Acquistapace, 2007; Lodge *et al.*, 2000) and many native crayfish species may be directly or indirectly threatened by the appearance of their non-native counterparts (Crandall and Buhay, 2008; Richman *et al.*, 2015; Taylor *et al.*, 2019).

The total number of crayfish species native to Europe is relatively low (Holdich *et al.*, 2009; Kouba *et al.*, 2014). Nevertheless, species diversity in the genus *Austropotamobius* is just beginning to be fully appreciated (Jelić *et al.*, 2016; Klobučar *et al.*, 2013; Lovrenčić *et al.*, 2020; Pârvulescu *et al.*, 2019), which has among others resulted in the recently discovered idle crayfish *A. bihariensis* (Pârvulescu, 2019). The greatest degree of species diversity is expected to occur in Eastern Europe, essentially related to the genus *Pontastacus*. However, this question has never been investigated thoroughly and clear evidence for this hypothetical species diversity (Crandall and De Grave, 2017) remains lacking (Bláha *et al.*, 2020; Bláha *et al.*, 2017; Maguire *et al.*, 2014).

In Europe, the first outbreaks of the so-called crayfish plague appeared in 1860 and soon affected the then numerous stocks of native crayfish species. The resulting declines stimulated interest in stocking with alternative crayfish species that were resistant to the causative agent of the disease, an oomycete *Aphanomyces astaci* (Svoboda *et al.*, 2017). Initially, three species originating from North America (spiny-cheek crayfish *Faxonius limosus* in 1890, signal crayfish *Pacifastacus leniusculus* in 1959, and red swamp crayfish *Procambarus clarkii* in 1973) were introduced for use in fisheries and/or aquaculture (Holdich *et al.*, 2009). However, it became clear that the crayfish species native to North America are chronic carriers of this pathogen. As their ranges have expanded, these non-native species have spread this parasite, to which the crayfish species from other regions are usually highly susceptible (Svoboda *et al.*, 2017; Ungureanu *et al.*, 2020). The above-mentioned non-native crayfish species have become widespread in Europe (Kouba *et al.*, 2014) and are now classified as invasive species of European Union concern (EU, 2014, 2016) as they pose a serious threat not only to native crayfish but also to entire freshwater ecosystems.

Unfortunately, the number of non-native crayfish species has continued to increase, frequently due to both intentional and non-intentional releases of originally pet-traded individuals or their offspring that are kept for ornamental purposes (Patoka *et al.*, 2016; Weiperth *et al.*, 2019). In recent years, various monitoring programs including astacological surveys have yielded valuable distributional data on both native and non-native crayfish species in Hungary (Gál *et al.*, 2018; Ludányi *et al.*, 2016; Seprős *et al.*, 2018), including information regarding multiple releases of redclaw *Cherax quadricarinatus* in the wild (Weiperth *et al.*, 2019) and the confirmed establishment of marbled crayfish *Procambarus virginalis* and red swamp crayfish (Gál *et al.*, 2018; Kovács *et al.*, 2015; Lőkkös *et al.*, 2016; Weiperth *et al.*, 2015; Weiperth *et al.*, 2020). Additionally, the Mexican dwarf crayfish *Cambarellus patzcuarensis* was discovered in Budapest in 2017 (Weiperth *et al.*, 2017). Unfortunately, this increase in non-native species shows no signs of abating and further species are still appearing. As biomonitoring work continues in Hungary, interesting new discoveries are occurring. Here we report the results of long-term monitoring at two localities in Hungary that are home to three and seven co-occurring crayfish species.

2 Materials and methods

2.1 Gombás brook

Under the auspices of project NVKP_16-1-2016-0003 (National Research, Development and Innovation Office), the Gombás brook near Vác, Hungary, was monitored monthly in 2017–2018 using the EU Water Framework Directive (European Commission, 2009) and Hungarian Biodiversity Monitoring System (www.termeszetvedelem.hu) methodologies to investigate fish populations. During this survey, a well-established population of the spiny-cheek crayfish was regularly observed. On the last sampling day on 31 August 2018 two individuals of a new crayfish species, later identified as the Florida crayfish *Procambarus alleni,* were caught by electrofishing (DEKA 3000 Lord, Hans Grassl IG 600). This encouraged the setting up of a regular, usually monthly-based, monitoring program focused on crayfish presence (Tab. 1). Five monitoring points were established and during each sampling event a baited crayfish trap made from PET bottles was placed at each point and left for 24 hours. This monitoring was accompanied by manual searching using hand-held nets

Table 1. Gombás brook (near Vác, Hungary). The number of sampling point and the name of the locality with a description of the site and GPS coordinates are given.

No.	Locality	GPS
1	Gombás brook (inflow from a rainwater water pipe and surrounding waters)	47°46′8.57″N, 19°8′17.31″E
2	Gombás brook (pedestrian footbridge)	47°46′8.09″N, 19°8′15.95″E
3	Gombás brook (corner of sports field)	47°46′5.34″N, 19°8′8.42″E
4	Gombás brook (Euvovelo six-bicycle bridge)	47°46′1.69″N, 19°8′3.40″E
5	River Danube floodplain	47°45′55.32″N, 19°8′4.97″E

Table 2. Gombás brook (near Vác, Hungary). Detailed characteristics of the locality and sampling points with the range of each parameter during the survey period (August 2018–July 2020).

Environmental parameters	Sampling point				
	1	2	3	4	5
Water temperature (°C)	7.2– 24.2	4.9–26.5	4.2–24.6	4.0–24.8	3.2–26.8
Water depth (cm)	5–100	10–60	10–50	10–50	5–70
Distance from the bank (m)	0.1–1.5	0.5–2.0	0.5–2.0	0.5–2.0	0.5–5.0
Water velocity (m/s)	0–0.2	0–0.2	0–0.2	0–0.2	0–0.2
Submerged vegetation (%)	0–10	0	0–10	0	0–10
Emergent vegetation (%)	0–25	0–15	0–10	0–10	0–35
Woody debris (%)	0–15	0–20	0–15	0–10	0–30
Shading tree cover (%)	0–10	10–30	10–30	5–20	0–40
Depth of sediment (m)	0.3–0.8	0.05–0.5	0.1–0.6	0.2–0.6	0.2–0.5
Type of bottom	Concrete, stone, organic sludge	Concrete, mud	Mud, stone	Clay, mud	Clay, mud

by two field workers, one on each shore. Sampling took place usually once a month from August 2018 to July 2020. Sampled crayfish were collected for species identification (see below) and sexing, and their carapace (CL) and total body (TL) were measured using calipers to the nearest 0.1 mm. Females were checked for glair glands and attached eggs. Detailed characteristics of the localities and sampling points are given in Table 2.

2.2 Városliget pond

Városliget is one of the biggest thermal ponds in Budapest, Hungary, and is divided into three sections (Fig. 1). In general, the whole area has been heavily modified by human activity, although the lower section is still semi-natural. Inflow from the thermal spring located under the Széchényi thermal bath enters the upper section of the pond (47°31′4.14″N, 19° 4′43.34″E; sampling point 1, Fig. 1). A weir separates the middle and lower sections (47°30′57.00″N, 19°4′54.5"E). After draining, cleaning, and refilling, the middle section is used as a public ice rink in winter (from the end of November to mid-March), during which time the thermal water from the upper section bypasses and flows directly into the River Danube. The outflow also flows directly into the River Danube (47°31′0.115″N, 19°4′43.044″E, close to sampling point 3). This locality was previously known to harbor both marbled and red swamp crayfish, with the marbled crayfish first appearing earlier (Weiperth *et al.*, 2015). Since 2019, there have been occasional records of redclaws (Weiperth *et al.*, 2020). An individual of another New Guinean *Cherax* species was first detected on 10 January 2019 in a crayfish trap at sampling point 4, close to a local restaurant (see Fig. 1). This initiated a regular monthly-based monitoring program focused on the presence of *Cherax* crayfish species. Five baited crayfish traps made from PET bottles were left for 24 hours at specific sites along the shores of the upper section every month in January 2019–July 2020. The greater water depth, the structure of the embankment, and the high conductivity of the water prevented electrofishing at this locality. Additionally, manual searching using hand-held nets was conducted in the middle and lower section at night for one hour by two people. The sampling of the middle section was discontinued during the ice-skating season (see above). The only exception was November 2019, when the middle section was drained and crayfish could be collected by hand in the shallow water. This explains why more crayfish were detected that month (see Results). The numerous red swamp and marbled crayfish were only categorized (females/males/juveniles smaller ca. 25 mm of TL), while *Cherax* individuals (the prime target of this monitoring effort) were measured

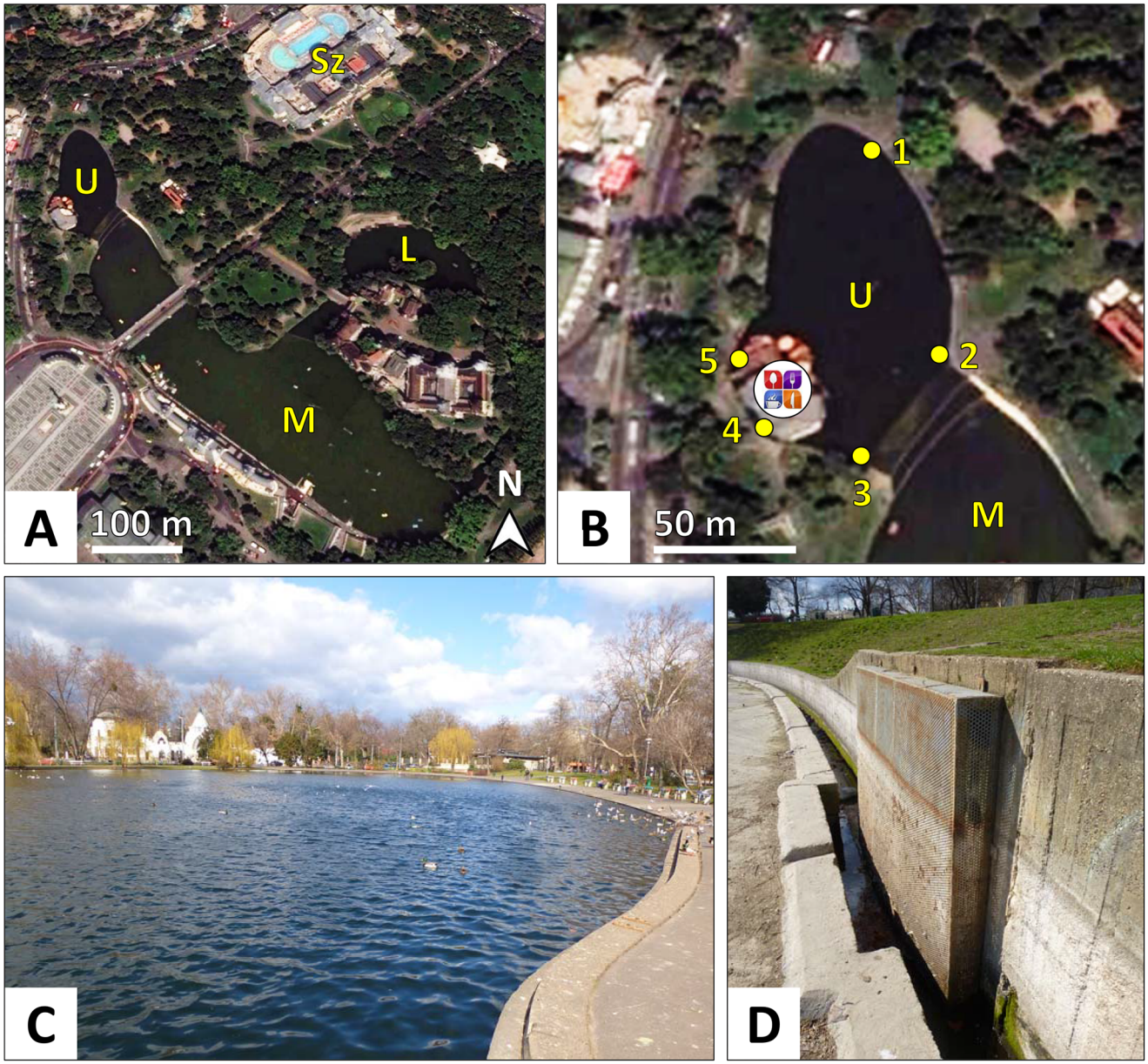

Fig. 1. (A) Three sections of the Városliget pond system in Budapest, Hungary; U: upper section, M: middle section, L: lower section, Sz: Széchényi thermal bath. (B) Detail of the upper section with sampling points indicated by numbers. The restaurant under which all *Cherax* individuals other than the redclaw were caught is located between sampling points 4 and 5. Maps taken from Google Earth. (C) The upper section of the thermal pond in early March 2020. (D) The iron grid on the outflow of the pond in January 2020. The stain on the grid and concrete wall indicates the water level in the middle section from the end of March until the end of October.

as indicated above. Detailed characteristics of the locality are given in Table 3.

2.3 Species identification

Samples from one Florida crayfish, one marbled crayfish (Gombás brook), and all *Cherax* specimens other than the redclaw (Városliget thermal pond) were molecularly analyzed. Genomic DNA extraction and PCR amplification were performed according to Bláha *et al.* (2016). Two mitochondrial genes, COI and 16S rRNA, were amplified with primers LCO-1490 and HCO-2198 (Folmer *et al.*, 1994), and 16S-1471 and 16S-1472 (Crandall and Fitzpatrick Jr, 1996), respectively. Only COI was amplified in the Florida crayfish and marbled crayfish specimens. Product purification and sequencing was performed by Macrogen Inc., South Korea. Obtained sequences were checked manually in GENEIOUS 8.0.5 (Kearse *et al.*, 2012). The most similar sequences were located using standard nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/) implemented in GENEIOUS.

The Bayesian implementation of the Poisson tree processes (PTP) model with non-ultrametric gene trees was performed using the bPTP web server (http://species.h-its.org/) for species delimitation (Zhang *et al.*, 2013). The tree was

Table 3. Városliget pond (Budapest, Hungary). Detailed characteristics of the site and sampling points, with the range of each parameter, during the survey period (August 2018–July 2020).

Environmental parameters	Upper section	Middle section	Lower section
Water temperature (°C)	19.9–38.4	5.3–38.2	2.6–32.1
Water depth (cm)	135–150	10–125	0.05–110
Distance from the bank (m)	0.1– 0.5	0.5– 3	0.1– 0.5
Water velocity (m/s)	0–0.1	0	0
Submerged vegetation (%)	0	0	0–0.02
Emergent vegetation (%)	0	0–0.01	0
Woody debris (%)	<10	<10	<20
Shading tree cover (%)	<15	<10	<30
Depth of sediment (m)	0.10–0.45	0.05	0.05–0.30
Type of bottom	concrete and mud[1,2]	concrete and mud[2]	concrete and mud[2]

[1]Covered with filamentous algae during vegetation period.
[2]Leaves observed in the autumn and winter.

assigned as rooted and the outgroup was excluded from the analysis. Although for species delimitation, the standalone PTP model generally outperforms the general mixed Yule coalescent (GMYC) method (Fujisawa and Barraclough, 2013), both of these approaches were used. The non-ultrametric tree was constructed in BEAST v2.5.1 (Drummond and Rambaut, 2007) under TrN+G+I model for COI dataset. The search was set to 50 million generations and was sampled every 5,000 generations, and the run was analyzed in TRACER (Rambaut *et al.*, 2014) to check for chain convergence. The posterior distributions of the topologies generated during the analyzes were synthesized using maximum clade credibility (MCC) trees in TreeAnnotator 1.8.1 (Drummond and Rambaut, 2007).

3 Results

3.1 Gombás brook

In total, 130 crayfish were caught during the monitoring period (Tab. 4). The dominant species was the spiny-cheek crayfish (99 individuals; 67 females and 32 males) at all sampling points. Three out of five spiny-cheek crayfish females caught had glair glands in September 2018 and one out of five females was carrying eggs in October 2018. The female caught in June 2019 had the remains of eggshells. Three out of 11 females also had eggs in October 2019 (Tab. 4).

The generally blue coloration of the captured individuals (Fig. 2) suggested that this species was the Florida crayfish, which was subsequently confirmed by the COI gene barcoding (GenBank accession number COI: MT832311) that had a 100% match with the sequence of Florida crayfish mitochondrion (KT074363). In total, 31 Florida crayfish (25 females and 6 males) were detected in the two upper sampling points, with just one female at sampling point 3. A single Florida crayfish was seen being consumed by a grey heron (*Ardea cinerea*) close to sampling point 5 (B. Tóth, pers. obs., April 2020; not included in data). Three out of 13 females had glair glands in September 2018, and three out of six females had eggs a month later. These successfully hatched in a home aquarium at room temperature in January 2019. Florida crayfish were detected throughout the monitoring period, which suggests that it successfully overwintered in at least two years. Sampling in early autumn was the most productive, while cold periods resulted predictably in few crayfish. The spiny-cheek crayfish population seemed to be normally distributed in terms of body sizes; Florida crayfish were on average larger with two more abundant classes (50–70 and 90–100 mm TL; Fig. 3). Juveniles of the dominant spiny-cheek crayfish (<25 mm TL) were regularly observed but were not counted. The smallest Florida crayfish was 42.2 mm TL (19.8 mm CL). Additionally, a single marbled crayfish was also detected at sampling point 5 at the end of monitoring campaign. COI gene barcoding (GenBank accession number COI: MT832312) revealed 100% similarity with several sequences of the marbled crayfish mitochondrion (HM358010, HM358011 – Martin *et al.*, 2010; KT074364 – Vogt *et al.*, 2015).

3.2 Városliget pond

In total, 2165 crayfish belonging to seven species were caught during the monitoring period at Városliget (Tab. 5). Red swamp and marbled crayfish dominated (1020 and 1113 individuals, respectively) and were regularly sampled throughout the whole monitoring period. Juvenile red swamp crayfish represented one third (32%) of all sampled individuals. Adults were dominated by males (68%). Adult and juvenile marbled crayfish were present in similar numbers (535 and 578, respectively).

Twenty-six (8 females and 18 males) redclaws were caught, with highest numbers recorded in the second half of 2019. Females and males ranged in size from 51.0 to 102.3 and 66.9 to 115.8 mm TL, respectively (corresponding to 22.2–42.0 and 26.8–48.3 mm CL). Neither glair glands, eggs, nor juveniles were detected.

A further five *Cherax* crayfish caught at the locality were not redclaws: all were trapped at sampling point 4 in the vicinity of a restaurant. The first individual (Fig. 4) noticed in January 2019 was a male measuring 38.1 mm CL (GenBank accession numbers COI: MT833298, 16SrRNA: MT833284). This individual was partly predated by the red swamp and marbled crayfish present in the exposed trap. Molecular

Table 4. Gombás brook (near Vác, Hungary). The dates (month and year) and number of sampling occasions within a given month at five sampling points, with the number of captured individuals of females/males (when applicable) of the Florida crayfish *Procambarus alleni* and spiny-cheek crayfish *Faxonius limosus*. Note that a marbled crayfish *P. virginalis* individual was also caught at sampling point 5 in July 2020.

Date/number of samplings per month	Sampling point				
	1	2	3	4	5
VIII. 2018/1	0/0 & 2/0	1/1 & 2/0	0/0 & 1/1	–	0/0 & 0/1
IX. 2018/4	8/1 & 0/0	5/1 & 0/0	0/0 & 1/2	0/0 & 3/1	0/0 & 1/0
X. 2018/4	3/0 & 0/0	2/0 & 1/3	1/0 & 1/2	0/0 & 2/0	0/0 & 1/0
XI. 2018/1	–	0/0 & 2/0	0/0 & 1/0	–	–
XII. 2018/2	0/0 & 1/0	0/0 & 1/0	–	–	–
I. 2019/1	–	–	–	–	–
II. 2019/1	–	–	–	–	–
III. 2019/1	0/0 & 1/0	–	–	–	–
IV. 2019/2	1/1 & 0/0	–	0/0 & 0/1	0/0 & 1/0	–
V. 2019/2	2/0 & 0/0	0/0 & 1/0	–	–	–
VI. 2019/2	–	–	0/0 & 0/1	0/0 & 1/0	–
VII. 2019/1	–	0/0 & 1/0	0/0 & 1/0	0/0 & 1/1	–
VIII. 2019/1	–	0/0 & 1/0	0/0 & 1/1	0/0 & 0/1	–
IX. 2019/4	1/0 & 2/3	0/0 & 2/2	0/0 & 2/0	–	–
X. 2019/4	–	0/0 & 8/2	0/0 & 3/1	–	–
XI. 2019/2	–	0/0 & 2/1	0/0 & 1/0	–	–
XII. 2019/1	–	–	–	–	–
I. 2020/1	–	–	–	–	–
II. 2020/1	0/0 & 0/1	0/0 & 0/1	–	–	–
III. 2020/1	–	0/0 & 1/0	–	–	–
IV. 2020/1	0/0 & 2/1	1/1 & 1/0	0/0 & 0/1	–	–
V. 2020/1	0/0 & 1/0	0/0 & 2/2	0/0 & 2/0	0/0 & 1/1	0/0 & 1/0
VI. 2020/1	0/0 & 1/0	0/0 & 2/0	0/0 & 1/1	–	–
VII. 2020/1	0/0 & 1/0	0/1 & 1/0	0/0 & 1/0	–	–
Total	**15/2 & 11/5**	**9/4 & 28/11**	**1/0 & 16/11**	**0/0 & 9/4**	**0/0 & 3/1**

Fig. 2. Florida crayfish *Procambarus alleni* female caught in the Gombás brook, near Vác, Hungary.

analysis revealed this crayfish to be identical to a hitherto undescribed species of *Cherax* (similarity of COI: 100% KU821416; 16S rRNA: 100% KU821431 – Bláha *et al.*, 2016). The closest related species is *C. pulcher*, with a similarity of 93.2% (COI) and 96.4% (16S rRNA).

The second unidentified crayfish (Fig. 4) caught in October 2019 was a female measuring 97.2 mm TL, 41.3 mm CL (GenBank accession numbers COI: MT833302, 16SrRNA: MT833288). Molecular analysis also revealed that this individual was similar to a scientifically undescribed species of *Cherax* (similarity of COI: 99.5% KJ950507; 16S rRNA: 100% KJ920783 – Eprilurahman, 2014). *Cherax pulcher* was again found to be the closest described species (similarity of COI: 96.6% KY654083; 16S rRNA: 98.1% KY654091 – Lukhaup *et al.*, 2017).

The third crayfish caught (Fig. 4) in November 2019 was a female measuring 73.0 mm TL, 33.0 mm CL (GenBank accession numbers COI: MT833301, 16SrRNA: MT833287). Given its general characteristics and overall orange coloration, it was identified as *C. holthuisi*, an identification later confirmed by molecular methods (similarity of COI: 98.9% KU821421 – 16S rRNA: 100% KU821433 – Bláha *et al.*, 2016; 16S rRNA: 100% KJ920801, KJ920804 – Eprilurahman, 2014).

The final two unidentified specimens caught in June 2020 were both females with body lengths of 94.9 and 100.2 mm (49.7 and 52.6 mm CL; GenBank accession numbers COI: MT833299, MT833300, 16SrRNA: MT833285, MT833286). Two other individuals of this species were recorded visually at the same time at sampling point 1 but not caught. Based on their general characteristics, morphology, and coloration, these

individuals were determined as *C. snowden* (jun. syn. *C. subterigneus*; Fig. 4). Using molecular methods, great similarity was found with *C. snowden* (COI: 98.8 and 98.5% KT626459 – Lukhaup, 2015; 16S rRNA: 99.4% KY654087 – Lukhaup *et al.*, 2017).

The GMYC and PTP analyses assigned individuals from this study to monophyletic clades corresponding in two cases (individuals from November 2019 and June 2020) to *C. snowden* and *C. holthuisi*, respectively, with moderate support (0.57 and 0.63, respectively). Two other individuals (January 2019 and October 2019) were clustered with scientifically undescribed species, albeit with strong support (0.84 and 0.95, respectively). The only disparity was the assigning of GenBank individual KU821426 as a different species using PTP, whereas GMYC identified it as a *C. warsamsonicus* (Fig. 4).

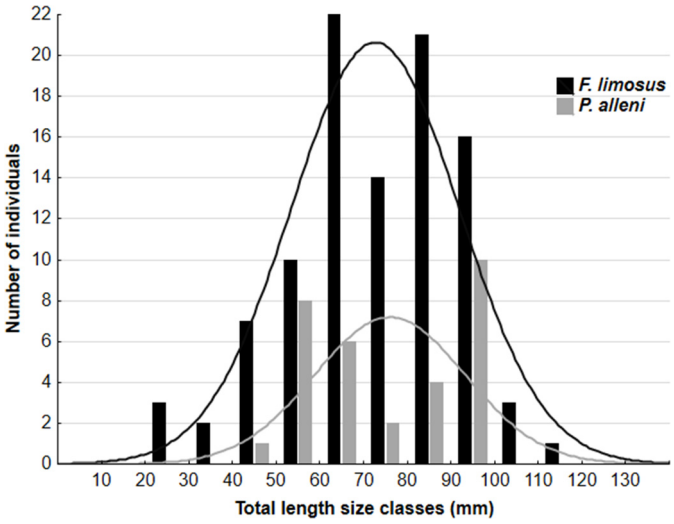

Fig. 3. Histogram of total body length size classes (mm) of the spiny-cheek crayfish *Faxonius limosus* and Florida crayfish *Procambarus alleni* at Gombás brook near Vác, Hungary, during the survey period (sampling points and sex merged).

4 Discussion

Thanks to their size, population density, omnivorous nature, and certain other biological features, crayfish have a great potential for colonizing and altering new environments, a trait that can give rise to biological invasions (Gherardi *et al.*, 2011; Lodge *et al.*, 2012). Recently, the pet trade (Faulkes, 2015a; Chucholl, 2013; Patoka *et al.*, 2014) and related intentional and non-intentional releases have accelerated their spread, especially in Europe (Hossain *et al.*, 2018; Jaklič and Vrezec, 2011; Patoka *et al.*, 2017; Weiperth *et al.*, 2017). This article provides evidence of the range of pet-traded crayfish species that can be detected in the wild and confirms that the pet trade represents an obvious introduction pathway that is highly difficult to control effectively (Patoka *et al.*, 2018).

4.1 Gombás brook

Florida crayfish, also known as the Everglades crayfish, electric blue crayfish, or blue crayfish in the pet trade, are native to Florida, where they are ubiquitous, above all in the

Table 5. Városliget thermal pond, Budapest, Hungary. The dates (month and year) with number of captured adult females, males, and juveniles (when applicable) of captured crayfish species: red swamp crayfish *Procambarus clarkii*, marbled crayfish *Procambarus virginalis*, redclaw *Cherax quadricarinatus,* and other *Cherax* spp. Note that only females are present in the marbled crayfish.

Date	*P. clarkii*	*P. virginalis*	*C. quadricarinatus*	other *Cherax* spp.
I. 2019	0/2/0	75/15	–	0/1
II. 2019	15/7/26	19/22	0/1	–
III. 2019	6/21/39	26/45	–	–
IV. 2019	10/22/8	42/11	–	–
V. 2019	2/16/2	16/3	–	–
VI. 2019	16/28/10	5/26	–	–
VII. 2019	5/16/27	11/16	–	–
VIII. 2019	23/8/41	11/22	0/2	–
IX. 2019	10/27/2	12/36	1/0	–
X. 2019	14/28/11	10/16	0/2	1/0
XI. 2019	35/159/85	120/85	2/9	1/0
XII. 2019	9/26/23	29/18	2/3	–
I. 2020	4/15/8	16/3	1/0	–
II. 2020	12/19/5	10/6	–	–
III. 2020	16/8/0	18/55	–	–
IV. 2020	15/11/1	16/49	–	–
V. 2020	11/20/0	20/39	–	–
VI. 2020	12/9/13	35/41	–	2/0
VII. 2020	29/23/0	41/70	2/1	*
Total	**244/465/311**	**535/578**	**8/18**	**4/1***

* Two *Cherax snowden* individuals identified visually.

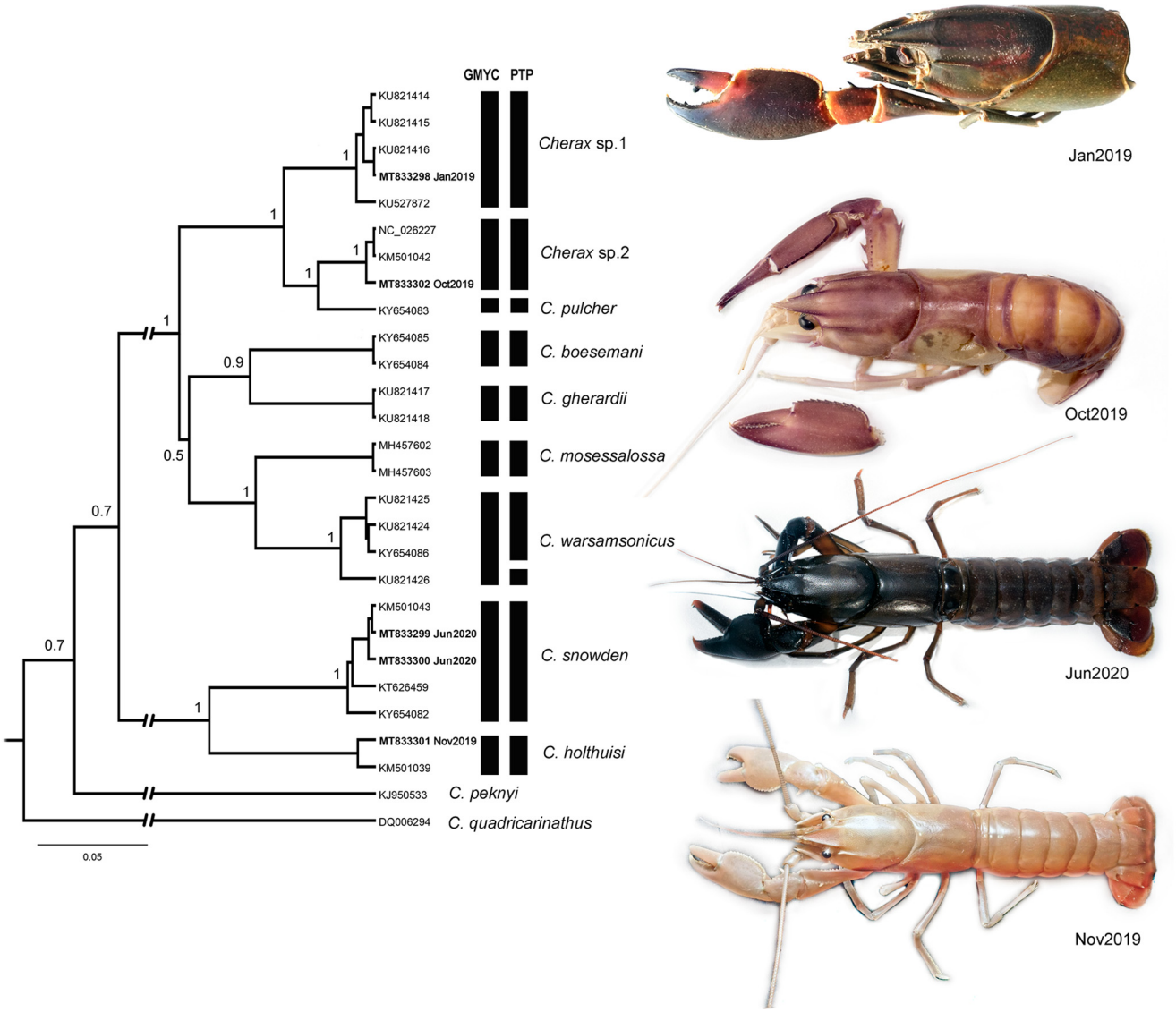

Fig. 4. Bayesian tree depicting the relationship between the *Cherax* species in the COI dataset. The species analyzed in this study are shown in bold text. Bayesian inference is displayed at each node. Each *Cherax* specimen is labelled with its GenBank code, including the specimens analyzed in this study. Full bars next to individuals indicate species status assigned by GMYC and PTP methods. The illustrations correspond to individuals found and analyzed in this study. Note that the second *Cherax* specimen from the top is ethanol-preserved and so does not fully show its natural coloration.

center and south of this state (Hobbs, 1942; Kushlan and Kushlan, 1979). To date, no population of this species has been recorded in any other US state (Taylor *et al.*, 2007) or elsewhere in the world. Given its attractive blue coloration, it is popular as a pet and populations may occur near crayfish farms, especially if crayfish are bred in ponds. This may be the case of certain ornamental crayfish-producing countries in southeast Asia such as Indonesia (Yonvitner *et al.*, 2020). To date, a single individual has been reported from France (Souty-Grosset *et al.*, 2006) and Groß et al. (2008) captured a single large male in the river Rhine in Germany in March 2013.

The Florida crayfish is a medium-sized species whose carapace rarely exceeds 45 mm in length (Dorn and Trexler, 2007; Vanarman, 2003). In our survey, several individuals reached this maximum size, although none exceeded 10 cm TL (Fig. 3). The lifespan of this species is three to four years (Acosta and Perry, 2002). It occurs in a broad range of ephemeral as well as permanent waterbodies (Hendrix and Loftus, 2000; Hobbs, 1942) but prefers temporary waters and those that are still or very sluggish, or even littoral zones that dry out seasonally (Dorn and Volin, 2009; Hendrix and Loftus, 2000; Hobbs, 1942). Potential habitats thus include ephemeral pools and flooded marshes with short hydroperiods (flooded for less than nine months of the year) and salinities as high as 18 ppt (Conover and Reid, 1972; Hendrix and Loftus, 2000). Thus, it is the typical inhabitant of seasonally flooded marl prairie wetlands in the Everglades National Park, Florida (Acosta and Perry, 2000). During summer and autumn

(the rainy seasons) it inhabits inundated shorelines with dense vegetation that reduces the threat of predation by fish and cannibalism. During the dry season (winter and spring), Florida crayfish move into existing burrows or construct new ones in diverse substrates, where it avoids desiccation and is able to reproduce. The young hatch in these burrows near the end of the dry season (April–May), where they remain with the adult females until the next flood, generally in June–July (Acosta and Perry, 2001; Dorn and Volin, 2009; Hendrix Jr *et al.* 1999; Hobbs, 1942; Jordan *et al.*, 1996). This species influences community structures, alter landscapes, and as an omnivore enhances nutrient cycling. It is a predator as well as prey for many other organisms. It is faster growing and more aggressive than the slough crayfish *Procambarus fallax* and may outcompete it for food and shelter under certain environmental conditions. The biology of these two species is often contrasted in the literature (Dorn and Trexler, 2007; VanArman, 2011).

The evaluation of the biology and population status of the Florida crayfish present in the Gombás brook is of great interest. The number of sampled individuals is likely to represent the largest documented wild stock outside its native range. Three out of 13 females caught in September 2018 had glair glands, while three out of six females had eggs in October 2019, which completed successful embryogenesis in a home aquarium at room temperature in January 2019 (Tab. 4). This indicates that hatching at this locality in early spring is possible. However, it is unclear whether or not the eggs would develop successfully under natural winter temperatures. Notwithstanding, pooled biometric data reveal two different but abundant size groups at this locality (Fig. 3), which possibly coincide with two different age classes. However, a direct comparison of this species' life history in its native range is difficult due to substantially differing environmental conditions as well as the limited information gathered at the study site. Nevertheless, overwintering in two years does seem to have occurred. The size (CL in mm) of caught individuals did not increase over time (order of month in the sampling campaign; slope -0.0042, data not shown). Given the relatively short lifespan of the species (Acosta and Perry, 2002), only once released animals would reach senescence and grow in size during the two-year monitoring period which was not the case. This suggests that reproduction has taken place at the locality or that there is ongoing propagule pressure. Florida crayfish were mainly detected in the upper two sampling points, but one was also seen being eaten by a grey heron in the Danube floodplain (sampling point 5). Despite the high mobility of this predator, we presume herons can manipulate crayfish with ease and so this crayfish was probably consumed where it was caught. Assuming that the temperature requirements of Florida crayfish are similar to those of the closely related marbled crayfish (cf. Weiperth *et al.*, 2019), it seems that even localities with natural or close-to-natural temperature regimes (non-thermal water bodies) are suitable for this species in this region. In light of the absence of juveniles, further monitoring is needed to clarify the status of this population, above all given that long-term propagule pressure cannot be entirely excluded.

Florida crayfish are a particularly popular crayfish species in the pet trade; its blue form, as seen in all the individuals from the Gombás brook (Fig. 2), is the most commonly marketed one. The species is available in markets in the United States (Faulkes, 2015b), Great Britain (Peay *et al.*, 2010), the Netherlands (Soes and Koese, 2010), Slovakia (Lipták and Vitázková, 2015), and Turkey (Turkmen and Karadal, 2012). Taking national crayfish pet trade surveys into account, along with risk evaluation of species present in Germany, the Czech Republic, Hungary, and Ukraine, Florida crayfish are rarely to very commonly available, and are placed in a medium risk category by the Freshwater Invertebrate Invasiveness Scoring Kit scheme (Chucholl, 2013; Kotovska *et al.*, 2016; Patoka *et al.*, 2014; Weiperth *et al.*, 2019). In terms of its potential environmental impact and management strategies, the Florida crayfish is included in the proposed Watch List for the Czech Republic (Pergl *et al.*, 2016). Its role in the dissemination of the crayfish plague pathogen (cf. Svoboda *et al.*, 2017) and its burrowing capacities (Dorn and Trexler, 2007; Hobbs, 1942) are two of the main potential impacts associated with the Florida crayfish.

The spiny-cheek crayfish that co-occurs with the Florida crayfish in Gombás is well known as an invasive species in Hungary (Györe *et al.*, 2013; Ludányi *et al.*, 2016). The data gathered in this study provide a possibly interesting insight into the reproduction biology of this relatively well-studied species (Buřič *et al.*, 2013; Pacioglu *et al.*, 2020; Pârvulescu *et al.*, 2015). Spiny-cheek crayfish are known to have two peaks of mating activity, first in September–October and then in early spring. Mating activity is suppressed to a certain degree in winter and ovulation take place shortly after spring mating. Juveniles hatch in May–June (Buřič *et al.*, 2013; Holdich, 2002; Kozák *et al.*, 2006; Kozák *et al.*, 2007). The presence of glair glands in September 2018 and the remains of eggshells in June 2019 agree with this generally known pattern of spiny-cheek crayfish reproduction. However, we have found no records of egg ovulation in the autumn in this species (noted in one out of five and three out of eleven females in October 2018 and 2019, respectively). The success of this alternative reproduction mode is unclear. Considering the overlap in the timing of Florida crayfish oviposition mentioned above, hatching could take place in early spring. However, there is no information on spiny-cheek crayfish embryogenesis in low winter temperatures. If it is possible, it may take place in combination with the so-called diapause known in cold-water species such as the signal crayfish and European astacids. If development is not disrupted and hatching occurs in late autumn/early winter, the overwintering of small juveniles is equally questionable. Taking into account available information, this reproduction mode is probably only exceptional and most likely to be unsuccessful. However, it might also have been overlooked, as exemplified by the facultative parthenogenesis documented by Buřič *et al.* (2011). An inlet with municipal waters from the nearby Vác affects the first sampling point and, in fact, dominates the water flow in the Gombás during drought periods, especially in summer. We are unaware and to what extent these municipal waters are cleaned as part of the local sewage treatment plan. However, these waters have higher average temperatures, especially during the winter, which will presumably enable eggs to develop or juveniles to survive. The coldest temperature observed in February 2020 was 7.2 °C at the first sampling point, which slowly fell to 3.2 °C at sampling point 5 (Tab. 2). It is unclear whether or not this temperature alteration alone is

enough to completely reverse the normal pattern of spiny-cheek crayfish ovulation in early spring. Furthermore, due to insufficient cleansing efficiency during water treatment, municipal waters also contain numerous pollutants including hormonally active compounds that could disrupt reproduction processes in aquatic organisms (Grabicova *et al.*, 2015; Kumar *et al.*, 2015), thereby potentially giving rise to the documented autumn oviposition in the spiny-cheek crayfish.

Given its parthenogenetic mode of reproduction (Martin *et al.*, 2007), the discovery of even a single marbled crayfish in the Danube floodplain downstream of the Gombás brook is enough to set alarm bells ringing as it would be evidence of a population spread of this invasive species (Vodovsky *et al.*, 2017 and reference therein). To our knowledge, this is the northernmost occurrence of the species in the Danube in Hungary (at km 1678). Numerous reports situate this species at km 1644, including records from the Városliget thermal pond, the thermal pond on Margaret Island, the Dera and Barát brooks including their confluences with the Danube, and several arms of the Danube in this region. Other locations are also known from Hungary (Lőkkös *et al.*, 2016; Szendőfi *et al.*, 2018; Weiperth *et al.*, 2015; Weiperth *et al.*, 2020). Bearing in mind the presumed spread of the marbled crayfish around Bratislava, Slovakia (Lipták *et al.*, 2017) and a recent report from Vienna, Austria (Moog *et al.*, 2018), the scenario of a potentially rapid spread of this species throughout the mid-course of the River Danube is now much more likely. This process might be also accelerated by further introductions elsewhere in the broader region (Pârvulescu *et al.*, 2017; Samardžić *et al.*, 2014).

4.2 Városliget pond

As the number of non-native crayfish species increases and their ranges expand, new and often surprising species assemblages appear. This provides ground for comparative research into the factors that could determine the success of particular species (Jackson *et al.*, 2014; Kouba *et al.*, 2016; Veselý *et al.*, 2015). However, studies that undertake simultaneous comparisons of multiple non-native crayfish species in natural settings are as yet few and far between (Herrmann *et al.*, 2018; Jackson, 2015). Therefore, places where various species coincide are of particular interest as they can provide a better understanding of such relationships. Given its array of non-native crayfish species, Városliget is such a locality. This site is apparently dominated be two notorious invasive species, the marbled and red swamp crayfish. Marbled crayfish first appeared in 2014, when several large individuals (~15 cm TL) were noted. A single male red swamp crayfish was first recorded in 2015, and 17 individuals were detected in 2016. Since then, both species have become numerous. Marbled crayfish do not exceed 10 cm TL and frequently inhabit algal surfaces on concrete walls (Weiperth *et al.*, 2015). It seems that the conditions are more suitable for red swamp crayfish, whose mean adult sizes are larger (data not shown). It is likely that this site is an important source of the red swamp crayfish that inhabit the adjacent River Danube. Numerous reports exist of this crayfish from other places in the country, including distant locations along the Danube and in the vicinity of Budapest, where it is found along 50 km of the brooks Barát, Dera, and Sulák and their confluences with the Danube. These reports thus most probably reveal the continuous occurrence of this species (Gál *et al.*, 2018; Szendőfi *et al.*, 2018; Weiperth *et al.*, 2015, 2020).

Redclaws have been reported from several places in Hungary (Weiperth *et al.*, 2019, 2020) and Városliget is where this species is most numerous. Despite the relatively low sampling effort and the undoubtably enormous competitive pressure exerted by the dominant marbled and red swamp crayfish, 26 redclaws were recorded at Városliget, which suggests that hundreds of this crayfish are present here. Nevertheless, its population status remains unclear given that no documented females with glair glands, eggs, or juveniles have ever been found. If breeding does not in fact take place, the propagule pressure must be enormous in this locality. The high susceptibility to the crayfish plague might prevent the establishment of *Cherax* species at this site (Marino *et al.*, 2014; Svoboda *et al.*, 2017; Unestam, 1975). However, if the original marbled and red swamp crayfish stocks were plague-free, this consideration would be irrelevant. High temperatures (Oidtmann *et al.*, 2002; Svoboda *et al.*, 2020) and the specific water chemistry (Svoboda *et al.*, 2014; Unestam, 1969) might also hinder the proliferation of disease at this locality; however, more research in this field is still needed in order to gather more relevant data.

Although the BLAST search helped identify the most similar sequences/species of all the other *Cherax* individuals captured at Városliget, only individuals caught in November 2019 and June 2020 could be assigned to scientifically described species, namely *C. holthuisi* and *C. snowden*, respectively. The habitus of these individuals was distinctive and was confirmed by molecular analysis. The remaining individuals shared the habitus of species resembling *C. boesemani* and *C. pulcher* and the use of molecular tools confirmed their identification. None of these individuals matched the sequences of *C. boesemani* from the type locality (similarity: COI: 90%; 16S rRNA: 94.5% for both individuals) recently published by Lukhaup *et al.* (2017). Although both were most similar to *C. pulcher*, according to species delimitation analysis (Fig. 4) and molecular divergences (January 2019: 6.5% and October 2019: 3.5%) it is likely that neither of these crayfish belongs to this species. Thus, based on Bayesian inference and species delimitation methods, each belongs to different, as yet scientifically undescribed species. New Guinean *Cherax* species diversity is much higher than expected and distinct morphological characteristics are not sufficient for distinguishing this diversity (Bláha *et al.*, 2016). The fact that numerous species descriptions in the past were performed before the molecular definition of type specimens was possible further complicates species assignment. Here, we identified individuals of two different yet hitherto undescribed species, which underlines the need to revise New Guinean *Cherax* species biodiversity and define new suitable morphological characteristics that could be helpful for species identification (Patoka, 2020).

Given their proximity to *C. pulcher*, the native range of the two undescribed species will probably be around the Bird's Head Peninsula, West Papua Province, Indonesia, which is also where *C. holthuisi* and *C. snowden* are found (Bláha *et al.*, 2016; Patoka, 2020). Thanks to their coloration, these endemic *Cherax* species are particularly attractive for the pet trade and are usually wild-caught in their native ranges

(Faulkes, 2015a; Patoka, 2020; Patoka *et al.*, 2015a). While the indirect environmental impact of these species cannot be entirely ruled out (as exemplified by the introduction of the crayfish plague from North America into Europe in the past; Svoboda *et al.*, 2017), these species should not be considered to be problematical in continental climates (like Hungary's) as their temperature requirements prevent their spread beyond thermal waters and their fecundity is thought to be low. However, a more detailed assessment of their situation in Europe is hampered by a lack of information regarding their life histories. To our knowledge, this is the first ever published report of releases of New Guinean endemic crayfish species (other than the redclaw). This circumstance is probably uncommon as the retail prices of even small specimens of these highly valued species often exceed 15 EUR per individual (Chucholl, 2013; Patoka *et al.*, 2015b).

5 Conclusion

We report here the monitoring of two localities in Hungary with three and seven co-occurring non-native crayfish species, which is evidence that non-native crayfish introductions into Europe are apparently on the increase. Urban and especially thermal waters have become hotspots of crayfish allodiversity and even high market prices do not completely curb such releases. These sites are worth closer investigation as they can provide us with contextual information on how particular species will coexist, which will help gather information for predicting future changes once they become more widespread. Furthermore, these localities are places from where these species disperse. Given that the eradication methods for established non-native crayfish populations are only feasible for a very narrow range of specific conditions, prevention remains the best measure for halting any increase in non-native crayfish species in Europe. Besides, research into insufficiently known biodiversity of New Guinean *Cherax* spp. is still needed.

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Supplement 9

Patoka, J., **Bláha, M.**, Kouba, A., 2017. *Cherax acherontis* (Decapoda: Parastacidae), the first cave crayfish from the Southern Hemisphere (Papua Province, Indonesia). Zootaxa 4363 (1): 137–144.

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Cherax acherontis (Decapoda: Parastacidae), the first cave crayfish from the Southern Hemisphere (Papua Province, Indonesia)

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Abstract

Cherax acherontis **n. sp.**, is a crayfish endemic to the submerged river Yumugima in Hagepma/Jugurama cave in the New Guinea Highlands, Jayawijaya Regency, Papua Province, Indonesia. This species is the first cave crayfish from the Southern Hemisphere. The new species is most similar to *Cherax monticola*. Both species can be easily distinguished by certain morphological characteristics, which easily demonstrate *C. acherontis* **n. sp.** is a valid species.

Key words: Yumugima crayfish, New Guinea, troglobiont, endemism, morphology

Introduction

Crayfish from the genus *Cherax* (Decapoda: Parastacidae) naturally occur in Australia and New Guinea (Munasinghe *et al*. 2004; Bláha *et al*. 2016; Crandall & De Grave 2017). In the case of New Guinea, 23 native and mostly endemic species are known. Some of them have been described recently due to their exploitation for ornamental purposes in the international pet trade (e.g. Lukhaup 2015; Patoka *et al*. 2015a, b; Lukhaup *et al*. 2017).

About 45 described crayfish species and subspecies are known to be cave-dwelling (troglobitic) (Crandall & De Grave 2017; Stern *et al*. 2017). All of them belong to the family Cambaridae with native occurrence confined to North America and Cuba (Hobbs & Barr 1960). Stern *et al*. (2017) suggested that the speciation rate in cave crayfish lineages is higher than the extinction rate and crayfish are able to spread across subterranean environments with speciation occurring via subsequent gene flow restriction. Since this highland (in New Guinea) is a karst landscape with many partially flooded cave systems, we hypothesized that there is a potential to find more crayfish adapted to this highly specific environment. Here we present the first cave crayfish species occurring on the Southern Hemisphere.

Material and methods

All specimen morphometric measurements were taken with a digital caliper with an accuracy of 0.1 mm. The following abbreviations are used below: TL, total body length; TCL, total carapace length; PCL, postorbital carapace length; RW, rostral width; RL, rostral length; AW, areola width; AL, areola length; ChW, chela width; ChL, chela length; ChD, chela depth.

Specimen and tissue collection. During a collecting trip from 11 to 12 July 2017 in Baliem Valley (Papua Province, Indonesia), we captured crayfish in a cave situated north-east of the city of Wamena. Altogether, we collected 41 individuals (24 adult males, 8 adult females, and 9 juveniles). Type specimens were photographed and sampled for further DNA analysis. Juveniles were released alive immediately after this procedure at their original

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locality. Adult specimens were preserved in formaldehyde. One male was designated as holotype, one female as allotype, and the other adults as paratypes.

Systematics

Cherax acherontis Patoka, Bláha and Kouba, n. sp.

Figs 1–3, 6

Diagnosis. Carapace surface smooth except for numerous small, blunt, indistinct, tubercles posterior and ventrolateral to the cervical groove. Eyes small, reduced, and pigmented with eyestalk prominently broader than cornea. Rostrum lanceolate in shape, setose with lateral margins elevated. Rostral length/width ratio range 2.0–3.5. Rostral margins with 2–4 prominent teeth. Rostral carinae developed. Postorbital ridges prominent with one very short acute tubercle at anterior terminus. Median carina present but very indistinct. Very broad semicircular scaphocerite broadest at midlength with a single distinct spine at terminus of apex. Antennular peduncle more or less reaching behind acumen, antennal peduncle reaching behind apex of scaphocerite. Antennae longer than body. Lateral margins of epistome separated by narrow row of small tubercles. Third maxillipeds long and covered by dense long setae. Areola length/width ratio range 2.2–3.7. Uncalcified patch on lateral margin of chelae of adult male absents. Chelae length/width ratio range 2.3–5.3, width/depth ratio range 1.3–2.5. Cutting edge of dactyl with row of very small granules, while cutting edge of opposite propodus with one prominent tubercle. Fingers slightly gaping, in distal part with hooked tips, cutting edges setose. Entire inner lateral margin of palm covered with two or three rows of numerous bluntly topped teeth with short setose hairs. Outer margin of palm with small teeth near its basal part. Colouration of body and appendages mostly ochre and body surface slightly marbled. Gonopores of both sexes normal in shape and position. Terminal half of caudal fan soft and membranous.

Description of holotypic male. (Fig 1–3). Eyes pigmented and reduced. Body subovate, slightly compressed laterally, pigment reduced. Cephalothorax 1.1 times broader than pleon. Rostrum (Fig. 3A, B) 2.2 times as long as wide, reaching beyond the end of second segment of antennular peduncle. Upper rostral surface smooth and setose. Acumen with anteriorly oriented spine at terminus. Median carina present but only slightly prominent on the cephalon, on the rostrum absents. Lateral rostral margins elevated, anteriorly convergent throughout length to acumen, posteriorly continuing in prominent carinae ending indistinctly behind the level of postorbital ridge spines. Lateral rostral margins bearing three pairs of teeth distally, distal part and upper surface of rostrum covered with dense setose hairs. Postorbital ridges (Fig. 3A, B) prominent, strongly elevated, posteriorly fading and in the last third of the cephalon indistinct. Anterior terminus of postorbital ridges with slightly upturned short spiniform tubercle. Eyes (Fig. 3A) very small; cornea globular, darkly pigmented, eyestalk prominently broader than cornea. Antennulae and antennae normal in shape. Antennae longer than body. Antennular peduncle reaching behind acumen, antennal peduncle reaching behind apex of scaphocerite. Coxicerite of antennal peduncle with blunt tubercle anteriorly. Scaphocerite (Fig. 3F) flat and horizontal, broad semicircular in shape, narrower in basal part, with lamina 1.8 times as long as broad, broadest at midlength; not reaching behind antennular peduncle; short prominent acute spiniform tubercle at apex reaching distinctly beyond the lamina; rounded inner margin strongly covered by setae. Third maxillipeds reaching 0.7 of CTL, covered by dense and long setae (Fig. 3G); epistome (Fig. 3E) with subcordiform cephalis lobe anteriorly bearing rounded cephalomedian projection constricted at base; each side covered with two groups of small tubercles separated by a smooth area; central part smooth with fovea, not pitted; lateral margins separated by narrow row of small tubercles; posterior margin of cephalomedian projection with short setose hairs, not pitted; epistomal zygoma prominent and thick, moderately arched with oblique arms.

Dorsal surface of carapace smooth, with exception of numerous small blunt and indistinct tubercles posterior and ventrolateral cervical groove. Areola three times as long as wide at the narrowest part. Length of areola 39% of TCL; surface smooth, slightly pitted, formed by two parts in first third. Cervical groove distinct, non-setose.

Chelipeds and chelae (Figs. 3C, D) equal in form and size; 4.3 times as long as broad and 6.7 times as long as deep; chela surface smooth, pitted; fingers 1.3 times longer than palm; chela equally long as carapace; fingers slightly gaping; slightly convex dactyl tapering to the tip; cutting edge setose with five rather small granular teeth over the full length, one larger tooth at basal third. Fixed finger narrow and 1.4 times broader than dactyl at base;

cutting edge setose with 7 to 11 rather small granular teeth in two rows. Tips of fingers with acute, hooked spines slightly crossing when fingers clasp. Entire inner lateral margin of palm covered with two or three rows of numerous bluntly topped teeth with short setose hairs. Outer margin of palm with eight small teeth near its basal part. Carpus smooth and pitted; with one well-developed acute and hooked spiniform tubercle in the middle of dorsolateral inner margin (mentioned tubercle is characteristic for genus *Cherax*) and numerous smaller tubercles; terminated with one spiniform tubercle oriented anteriorly. Ventral carpal surface non-setose, with fovea. Merus strongly laterally depressed in basal part; surface smooth and pitted; one prominent anteriorly oriented tooth and row of small anteriorly oriented tubercles (serrate carina) present on dorsal surface; one prominent anteriorly oriented tooth and row of small tubercles present on ventral surface; two prominent anteriorly oriented teeth and row of small tubercles on entire inner ventrolateral margin. Second pereiopod reaching behind apex of scaphocerite. Palm equally long as fingers; fingers and palm sparsely setose; tip of dactylus hooked; cutting edges of both fingers densely setose. Carpus 1.4 times longer than palm. Merus 1.6 times longer than carpus and 2.3 times longer than ischium. Third pereiopod 1.3 times longer than second pereiopod, palm 1.2 times longer than fingers; fingers and palm sparsely setose; tip of dactylus hooked; cutting edges of both fingers densely setose. Carpus 1.1 times longer than palm. Merus 1.6 times longer than carpus and 2.6 times longer than ischium. Fourth pereiopod reaching behind the end of antennal peduncle. Propodus and dactyl strongly setose. Dactyl slightly hooked and densely setose on cutting edge. Propodus 1.4 times longer than carpus. Merus 2.2 times longer than carpus and 2.4 times longer than ischium. Fifth pereiopod reaching midlength of scaphocerite. Propodus and dactyl strongly setose. Dactyl slightly hooked and densely setose on cutting edge. Propodus 1.7 times longer than carpus. Merus 2.1 times longer than carpus and 2.6 times longer than ischium.

Dorsal surface of pleon smooth in median region and sparsely setose; pleura smooth, pitted. Each pleomere strongly setose with short hairs on ventral posterior margin. Telson with two posteriorly directed spiniform tubercles in caudolateral corners. Protopod of uropod with single posteriorly directed spiniform tubercle on distal margin. Endopod of uropod with two posteriorly directed spiniform tubercles in the middle and outer margin of mesial lobe. Exopod of uropods with transverse row of posteriorly directed diminutive spiniform tubercles ending in posteriorly directed spiniform tubercles on outer margin of mesial lobe. Terminal half of caudal fan soft, membranous, setose on the posterior margin.

FIGURE 1. *Cherax acherontis* **n. sp.**, holotypic male, side view.

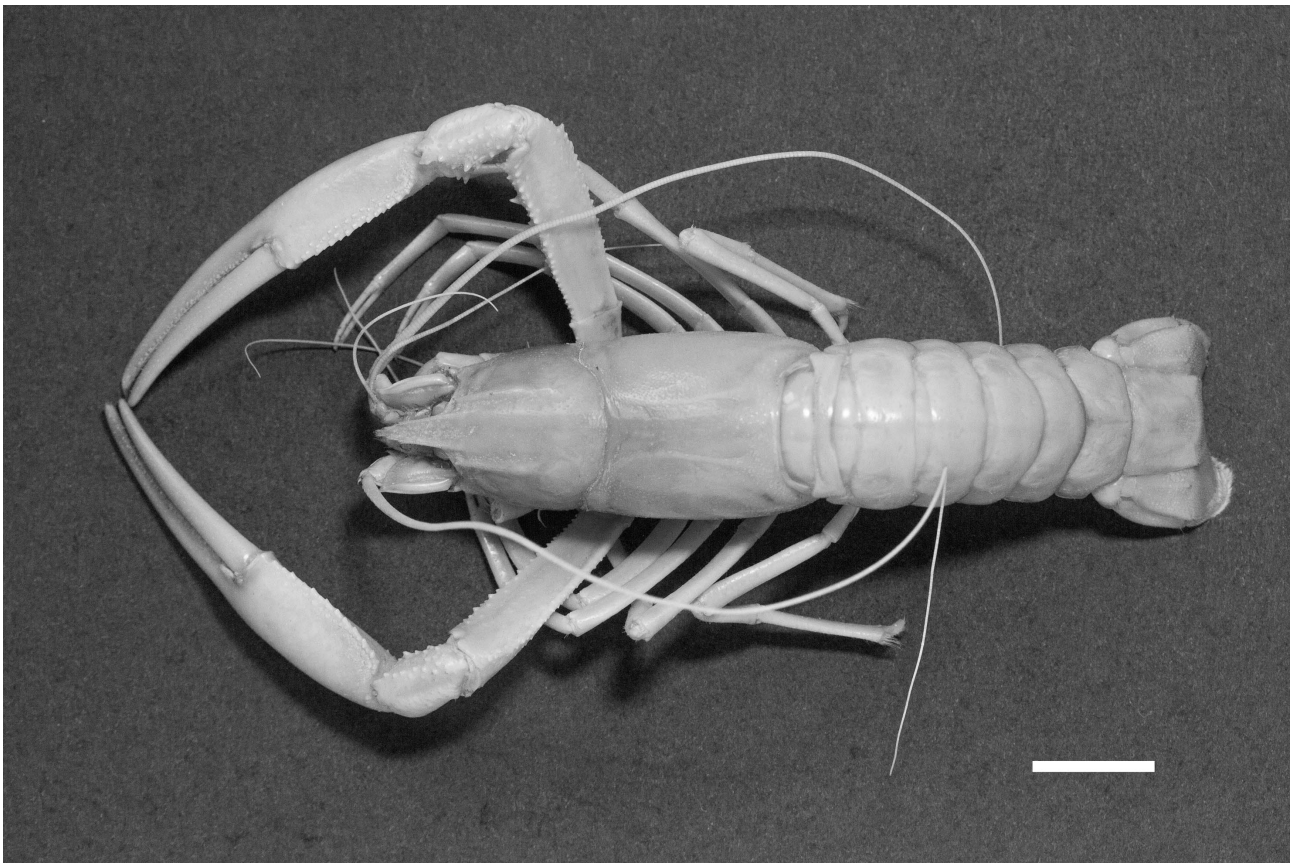

FIGURE 2. *Cherax acherontis* **n. sp.**, holotypic male, dorsal view. Scale bar: 20 mm.

Description of allotypic female. (Fig 4). Differing from the holotype in the following respects: the chelae 5.0 times as long as broad and 8.3 times as long as deep; carapace 1.4 times longer than chela. Pleon equally broad as cephalothorax; areola 2.2 times long as wide at the narrowest part.

Remarks. The single, well-developed, acute and hooked spiniform tubercle in the middle of the dorsolateral inner carpus margin is characteristic of *Cherax*. Right cheliped of holotype was autotomized during the transport. The allotype was without any visible damages.

Size. Holotype: ♂, TL = 128 mm, TCL = 62 mm, PCL = 48 mm, RW = 5 mm, RL = 14 mm, AW = 8 mm, AL = 24 mm, ChW = 14 mm, ChL = 60 mm, ChD = 9 mm. Allotype: ♀, TL = 81 mm, TCL = 36 mm, PCL = 27 mm, RW = 4 mm, RL = 9 mm, AW = 6 mm, AL = 13 mm, ChW = 5 mm, ChL = 25 mm, ChD = 3 mm. Paratypes: ♂ (n = 23), TL = 50–140 mm ($\bar{x}$ = 78 mm, SD = 21.6 mm), TCL = 24–66 mm ($\bar{x}$ = 36 mm, SD = 10.2 mm), PCL = 18–52 mm ($\bar{x}$ = 28 mm, SD = 8.3 mm), RW = 2–6 mm ($\bar{x}$ = 3 mm, SD = 0.9 mm), RL = 6–14 mm ($\bar{x}$ = 8 mm, SD = 3.0 mm), AW = 3–8 mm ($\bar{x}$ = 5 mm, SD = 1.3 mm), AL = 9–27 mm ($\bar{x}$ = 14 mm, SD = 4.3 mm), ChW = 3–19 mm ($\bar{x}$ = 7 mm, SD = 3.7 mm), ChL = 16–58 mm ($\bar{x}$ = 28 mm, SD = 12.0 mm), ChD = 2–11 mm ($\bar{x}$ = 4 mm, SD = 2.2 mm); ♀ (n = 7), TL = 63–105 mm ($\bar{x}$ = 78 mm, SD = 21.6 mm), TCL = 29–49 mm ($\bar{x}$ = 36 mm, SD = 10.2 mm), PCL = 22–38 mm ($\bar{x}$ = 28 mm, SD = 8.3 mm), RW = 3–5 mm ($\bar{x}$ = 3 mm, SD = 1.0 mm), RL = 7–11 mm ($\bar{x}$ = 8 mm, SD = 3.0 mm), AW = 4–6 mm ($\bar{x}$ = 5 mm, SD = 1.3 mm), AL = 11–19 mm ($\bar{x}$ = 14 mm, SD = 4.3 mm), ChW = 4–7 mm ($\bar{x}$ = 5.75 mm, SD = 1.1 mm), ChL = 20–25 mm ($\bar{x}$ = 23 mm, SD = 1.9 mm), ChD = 3–4 mm ($\bar{x}$ = 3.5 mm, SD = 0.5 mm).

Colouration of live specimens. Colouration of body and appendages mostly ochre, rarely pink or blueish. Body surface slightly marbled, legs paler than body. Ventral side of palm of chelae coloured as dorsal side. Fingers and joints of the dactyls rarely blueish.

Deposition of types. Holotype (MZB Cru 4678), allotype (MZB Cru 4679) and seven paratypes (MZB Cru 4680) deposited in The Zoological Museum of Bogor (Museum Zoologicum Bogoriense, Indonesia); 10 paratypes (RMNH.CRUS.D.57252 until RMNH.CRUS.D.57261) are deposited in Naturalis Biodiversity Center, Leiden, the Netherlands; 13 paratypes (Y2017JP/01 until Y2017JP/13) deposited in the Collection of Aquatic Crustaceans at the Department of Zoology and Fisheries, Czech University of Life Sciences Prague, Czech Republic.

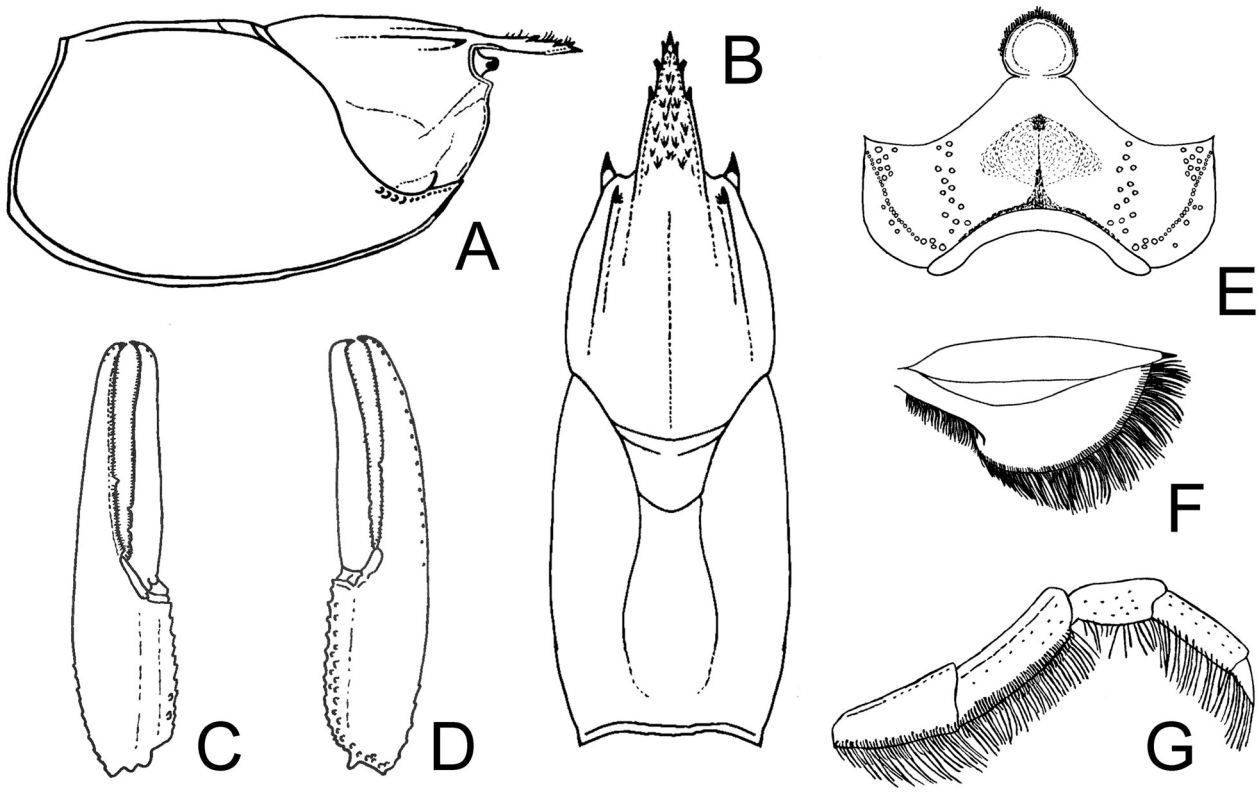

FIGURE 3. *Cherax acherontis* **n. sp.**, from holotype. A, lateral view of carapace; B, dorsal view of carapace; C, ventral view of right chela; D, dorsal view of right chela; E, epistome; F, dorsal view of right scaphocerite; G, third maxilliped.

FIGURE 4. *Cherax acherontis* **n. sp.**, allotypic female, dorsal view.

Systematic position. In regard to energy economy, low food availability and due to the lack of photostimuli, the troglobitic crayfish are characterised by morphological traits such as long and slender claws, reduced eyes, loss of body pigmentation, and long antennae. We found these characteristics also in *C. acherontis*. A special feeding adaptation is known in one of the North American crayfish, *Troglocambarus maclanei*, which has long and thickened third maxillipeds provided with dense and usually filtrating setae (Hobbs *et al*. 1977). The same characteristic was found in *C. acherontis*. It is obvious that the environmental pressures created by cave habitats influenced the convergent evolution of such species despite their phylogenetic and zoogeographical origin and divergence of epigean congeners.

There are various characteristics such as shape of scaphocerite and absence of soft uncalcified patch on outer margin of adult male claws which class the new species to subgenus *Cherax;* nevertheless, there are some other characteristics such as shape of rostrum, presence of prominent rostral teeth and well developed rostral and postorbital carinae which class the new species to the subgenus *Astaconephrops*. In accordance to a recent phylogenetic study (Bláha *et al*. 2016), the validity of the subgenera suggested by Holthuis (1950) is controversial and therefore we do not use any subgenus to describe the new species.

In comparison to all New-Guinean species from the genus *Cherax*, the new species is most similar to *C. monticola* and differs from this species in the following characters: body and chelae colouration of live individuals, length and width of rostrum, lateral carapace surface behind the cervical groove, longer and narrower chelae, absence of soft uncalcified margin of chela in adult males, and the longer third maxilliped and second pereiopod. Rostrum reaches the end of antennular peduncle in *C. monticola* while this peduncle more or less terminates behind the acumen in the new species. Rostrum of adult individuals 1.8 to 1.9 times as long as broad at base in *C. monticola* while it is 2.25 to 2.8 in the new species. Third maxillipeds covered by longer setae and 1.2 times longer in *C. acherontis* than in *C. monticola*. Numerous rather widely separated tubercles are present on the lateral carapace behind the cervical groove in *C. monticola* while the carapace is smooth in the new species. Row of small blunt tubercles posteriorly cervical groove laterally in *C. monticola* while ventrolaterally in the new species. Chela of adult males 2.3 to 2.7 times as long as broad in *C. monticola* while 2.3 to 5.3 times in the new species. The fingers are longer in the new species. One or two decalcified spots present on the outer margin of chela of adult males and sometimes also females of *C. monticola* while always absent in the new species. Base of outer margin of chela without tubercles in *C. monticola* while small teeth are present in the new species. The legs of second pair reach to the end of the scaphocerite in *C. monticola* while reaching behind the end of the scaphocerite in the new species.

Although we conducted a preliminary study of the phylogenetic position of the new species, the results were ambiguous and require further analysis. Nevertheless, the results showed a close relationship with *C. monticola*.

Etymology. The specific name corresponds to the Latin genitive singular of Acherōn, one of the mystic rivers in the underworld in ancient Greek mythology.

Common name. Since the crayfish were captured in the submerged river Yumugima, we propose the name Yumugima Crayfish as a common name for the new species, *Cherax acherontis* **n. sp.**

Distribution. The karst cave with rich dripstone decoration is located in the New Guinea Highlands, Jayawijaya Regency, Papua Province, GPS coordinates: S 04°01.933’ E 139°00.221’ (Fig 5, 6). There are three entrances to the cave, Hagepma and two others are both named Jugurama. Close to the Hagepma entrance is situated Palimoro village inhabited by one family of local Dani people. The cave was formed by the submerged Yumugima river. The river flows out from the mountains in Baliem Valley as a tributary of Baliem river. There are both fast flowing shallow riffles (depth about 0.5 m) and pools (deeper than 1 m), water temperature was 15.0 °C and pH 7–8. Based on information from local people, the water level increases up to two meters during the rainy season. On the bottom of the river, there are large and small boulders, rock, clay, sand deposits, and sporadically wood debris (Fig 6B) inhabited with a few benthic invertebrates such as mayfly larvae and oligochaetes. No crayfish burrows were recorded. Since there was some garbage observed, such as plastic bags, we assumed that the river flows on the surface somewhere upstream, but local Dani people have no information in this regard.

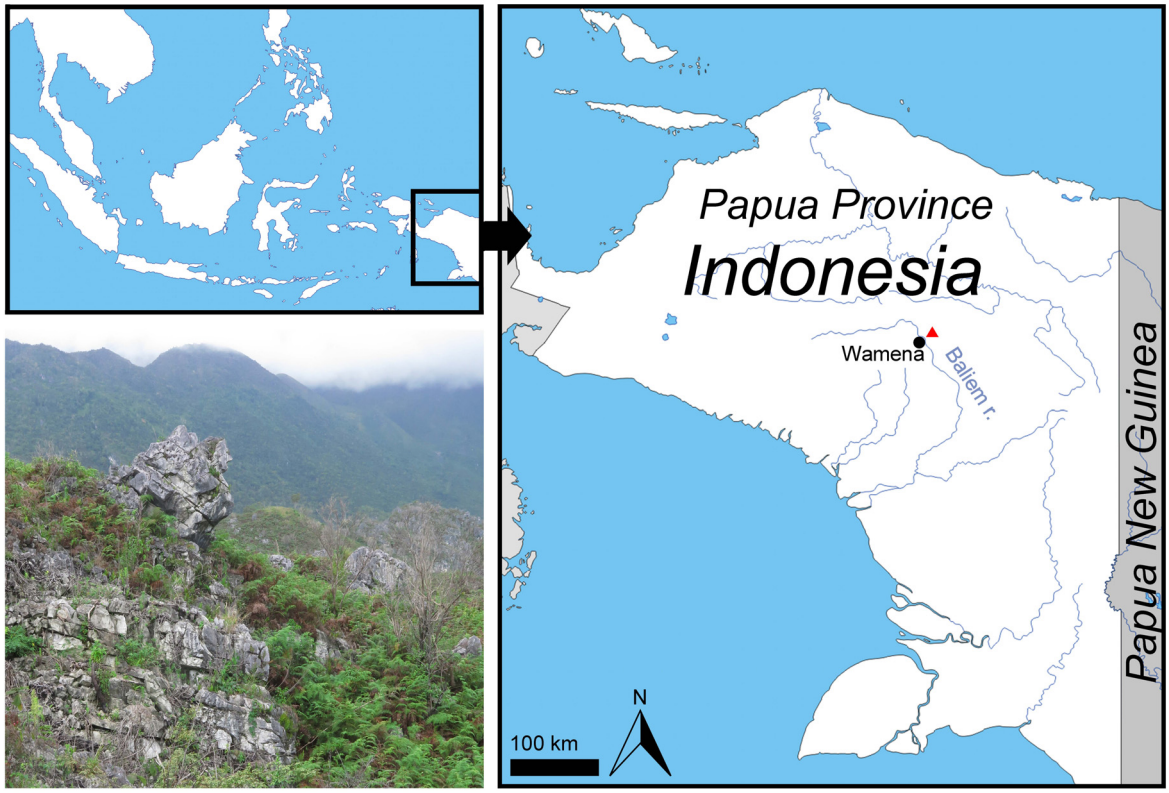

FIGURE 5. Map of Papua Province, Indonesia. The position of the cave near city of Wamena indicated by red triangle. The photo shows a typical terrain in the vicinity of the cave.

FIGURE 6. A, Hagepma entrance into the cave; B, wood debris in the river in the cave; C, dripstone decoration in the cave.

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Supplement 10

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RESEARCH ARTICLE

WILEY

Procambarus clarkii (Girard, 1852) and crayfish plague as new threats for biodiversity in Indonesia

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Abstract

1. Numerous freshwater crayfish species are known to become successful invaders when introduced to new territories. One of the most invasive species in this group is the red swamp crayfish, *Procambarus clarkii* (Girard, 1852). In addition to other characteristics facilitating its invasiveness, it is also a vector of crayfish plague, a disease mostly lethal to crayfish of non-North American origin.
2. *Procambarus clarkii*, at present the most widespread crayfish species globally, is popular in many countries both for human consumption and as an ornamental animal. An established population of this species was documented for the first time within Indonesia, representing the first record for maritime Southeast Asia. The species is also common in the local ornamental pet trade.
3. Infection by the crayfish plague pathogen *Aphanomyces astaci* Schikora was confirmed both in the outdoor population of *P. clarkii* and in one of the surveyed pet shops. Furthermore, *A. astaci* was detected in specimens of freshwater crab and shrimp species coexisting with *P. clarkii*.
4. Local climatic conditions and the species temperature requirements suggest a high probability of the establishment of *P. clarkii* in Indonesia. Its further spread could irreversibly damage populations of many native endemic crustaceans in the country (as well as elsewhere in the region), and the thriving local aquacultures of the redclaw crayfish *Cherax quadricarinatus* (von Martens, 1868) may also be threatened.
5. The total ban of *P. clarkii* and other crayfish species of North American origin in Indonesia is strongly recommended, especially considering that aquaculture and trade with Australasian species is a viable alternative option.

KEYWORDS

Aphanomyces astaci, aquaculture, biological invasion, Cambaridae, Java, pet trade, red swamp crayfish

1 | INTRODUCTION

In the era of globalization, human activities contribute immensely to the spread of numerous species worldwide. Non-indigenous freshwater crayfish species introduced outside their native ranges often become invaders. Their impact on native biota and ecosystems is frequently severe, with irreparable changes in community structure and ecosystem functions, resulting also in economic losses (Gherardi, Aquiloni, Diéguez-Uribeondo, & Tricarico, 2011; Havel, Kovalenko, Thomaz, Amalfitano, & Kats, 2015). As freshwater animals, including crayfish, can be hard to detect in the early stage of an invasion, their effective mitigation or eradication is extremely difficult without having devastating impacts on the entire ecosystem (Gherardi et al., 2011).

The pet trade is at present one of the main pathways for introductions of non-indigenous crayfish species globally (Chucholl, 2013; Faulkes, 2015a). Trade with crayfish as ornamental species began expanding in the mid-1990s (Chucholl, 2013; Faulkes, 2010), and at present about 30 species are sold relatively frequently and kept in aquaria in various countries (e.g. Chucholl & Wendler, 2017; Faulkes, 2015b; Patoka, Kalous, & Kopecký, 2014; Vodovsky, Patoka, & Kouba, 2017). The wide list of presently traded species includes species harvested from the wild, such as *Cherax* crayfish from New Guinea (Lukhaup, Eprilurahman, & von Rintelen, 2017; Lukhaup, Panteleit, & Schrimpf, 2015; Patoka, Bláha, & Kouba, 2015; Patoka, Kalous, & Kopecký, 2015), as well as species cultured exclusively for aquaria, such as the marbled crayfish *Procambarus virginalis* Lyko, 2017 (Faulkes, 2015b). However, certain crayfish exploited for human consumption are also rapidly growing in popularity as ornamental species, such as the common yabby *Cherax destructor* Clark, 1936, the redclaw *Cherax quadricarinatus* (von Martens, 1868), and the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Patoka et al., 2016; Souty-Grosset et al., 2016). The crayfish pet trade has helped to popularize these species, with positive impacts such as the education of the general public, and economic profit for producers and sellers. On the other hand, numerous intentional or unintentional releases of non-indigenous crayfish species from aquaria have been recorded, resulting in established populations in many regions of the world (e.g. Chucholl, Morawetz, & Groß, 2012; Lipták et al., 2017; Patoka et al., 2016; Weiperth et al., 2017). Invasive crayfish generally affect native biota through direct competition, predation, and disease transmission, and indirectly affect native biota through habitat alteration and changes to food webs (Gherardi, 2007). Moreover, intensive capture for the pet trade can be a potential threat for endemic crayfish species with small ranges, such as New Guinean *Cherax* species, the population statuses and trends of which are in many cases unknown (Bláha, Patoka, Kozák, & Kouba, 2016).

Indonesia has previously been identified as the leading supplier of ornamental crayfish (Faulkes, 2015b; Patoka, Kalous, & Kopecký, 2015), which are exported mainly to Europe, East Asia, and North America. The territory of Indonesia also includes the western part of New Guinea, with various native endemic *Cherax* species, such as the blue-legged crayfish *Cherax gherardii* Patoka, Bláha, & Kouba, 2015, *Cherax holthuisi* Lukhaup and Pekny, 2006, and *Cherax pulcher* Lukhaup, 2015 (Bláha et al., 2016). These crayfish are not cultured for the ornamental trade but are exclusively harvested from the wild. A notable exception is *Cherax quadricarinatus*, native to the southern part of New Guinea, which is stocked and bred in lakes, rivers, ponds, and reservoirs in Indonesia for consumption as well as for further sale for ornamental purposes (Patoka et al., 2018, 2016). Moreover, Indonesia is also known as an exporter of the North American *P. clarkii*, especially its full red and white colouration morphs (Patoka, Kalous, & Kopecký, 2015). As the traded morphs differ from the wild type, it is assumed that these crayfish are produced intentionally as ornamentals, but detailed data are not available.

The uncontrolled culture of non-indigenous crayfish in Indonesia is alarming, especially because of the generally favourable climatic conditions across the entire country, which may facilitate the establishment of many alien crayfish species. Some of these are then likely to become invasive, as predicted for example for *C. quadricarinatus* (Patoka et al., 2016). Moreover, *P. clarkii* is known to be a non-symptomatic vector of crayfish plague, a disease caused by the oomycete *Aphanomyces astaci* Schikora that is lethal to crayfish of non-North American origin (Diéguez-Uribeondo & Söderhäll, 1993). Other freshwater decapods have also been reported as potential hosts of this pathogen (Svoboda, Mrugała, et al. 2014; Svoboda, Strand, et al., 2014), but its impact on their populations remains unknown. As there are dozens of native and, in many cases, endemic freshwater shrimps and crabs in Indonesia (Ng, Schubart, & Lukhaup, 2015; von Rintelen & Cai, 2009), the introduction of crayfish plague into this country may be potentially very harmful for native biota. Moreover, *P. clarkii* may also represent a significant risk to amphibians owing to its potential role in the transmission of the chytrid fungus *Batrachochytrium dendrobatidis* Longcore, Pessier, & Nichols, the pathogenic agent of amphibian chytridiomycosis (Brannelly, McMahon, Hinton, Lenger, & Richards-Zawacki, 2015; McMahon et al., 2013).

Procambarus clarkii is a fast growing, adaptable, and very popular crayfish, both for human consumption and aquaria (Souty-Grosset et al., 2016). It is a very successful invader, and is the most widespread crayfish species globally: apart from its native North American range, it has become established in southern and western Europe (Kouba, Petrusek, & Kozák, 2014), South America (Loureiro et al., 2015), Africa (Nunes, Hoffman, Zengeya, Measey, & Weyl, 2017), China (Yue, Li, Bai, Wang, & Feng, 2010), and Japan (Kawai & Kobayashi, 2005). This crayfish has been assessed as a high-risk species in every region for which a risk assessment has been performed (Chucholl, 2013; Patoka et al., 2014; Vodovsky et al., 2017; Weiperth et al., 2018). Furthermore, it has been recently demonstrated that its introduced populations host the crayfish plague pathogen in Japan (Mrugała, Kawai, Kozubíková-Balcarová, & Petrusek, 2017) and Brazil (Peiró et al., 2016). Infected *P. clarkii* may be purchased through the European pet trade (Mrugała et al., 2015), although individuals from one batch of this species imported from Indonesia did not carry *A. astaci* (Mrugała et al., 2017).

Although the import of *P. clarkii* to Indonesia is banned by Regulation No. 41/PERMEN-KP/2014, which prohibits the import of hazardous fish species into the territory of the Republic of Indonesia, its culture and transport within the country are legal. The likelihood of escape or release of *P. clarkii* to the wild increases because of the general ignorance of the threat of biological invasions

by Indonesian policymakers: at present, there is no regulation of the breeding, handling, or release of *P. clarkii* by farmers, hobbyists, or the general public.

The aim of this study was to highlight the potential threat that *P. clarkii* may pose to Indonesian freshwater diversity. The availability of *P. clarkii* in Indonesian pet shops and aquaculture was assessed, and individuals obtained during the survey were tested for the presence of the crayfish plague pathogen.

2 | MATERIALS AND METHODS

2.1 | Availability in pet shops

The main pet shops in Jakarta, which can be considered the hub of the ornamental pet trade in Java (and Indonesia in general), and in the nearby large city of Bogor, were surveyed for the sale of North American crayfish, and for *P. clarkii* in particular. Six shops were inspected personally, including a shop in the public freshwater aquarium Dunia Air Tawar in Jakarta. In addition, three street markets were visited. If *P. clarkii* was found, the colouration morph(s) were recorded. In some shops, several specimens were purchased for *A. astaci* screening. Sellers were interviewed for information on the origin of the advertised crayfish.

2.2 | Outdoor population

Crayfish were captured by local fishermen in a system of ponds used for fish and crayfish farming. These ponds, connected with the Cilegok brook by outflow bamboo tubes and open outflow drainage (Figure 1), are situated near Pasir Angin village, Cisaat Subdistrict, Java (6°53′21″S, 106°51′43″E). The area harbours many interconnected water bodies such as paddy fields, drainages, and brooks.

In the same ponds as crayfish, the shrimp *Macrobrachium lanchesteri* (de Man, 1911) and the crab *Parathelphusa convexa* de Man, 1879 occur together with ornamental fish, such as the three spot gourami *Trichopodus trichopterus* (Pallas, 1770), the goldfish *Carassius auratus* (Linnaeus, 1758), the guppy *Poecilia reticulata* Peters, 1859, and the southern platyfish *Xiphophorus maculatus* (Günther, 1866). Environmental parameters, measured around 14:00 h on 28 April 2018 in six ponds and three places in the brook downstream from the outflow, were as follows: (i) ponds, pH 7.35–9.03, water temperature 26.8–30.0 °C, and dissolved oxygen 3.0–7.4 mg L^{-1}; (ii) brook,

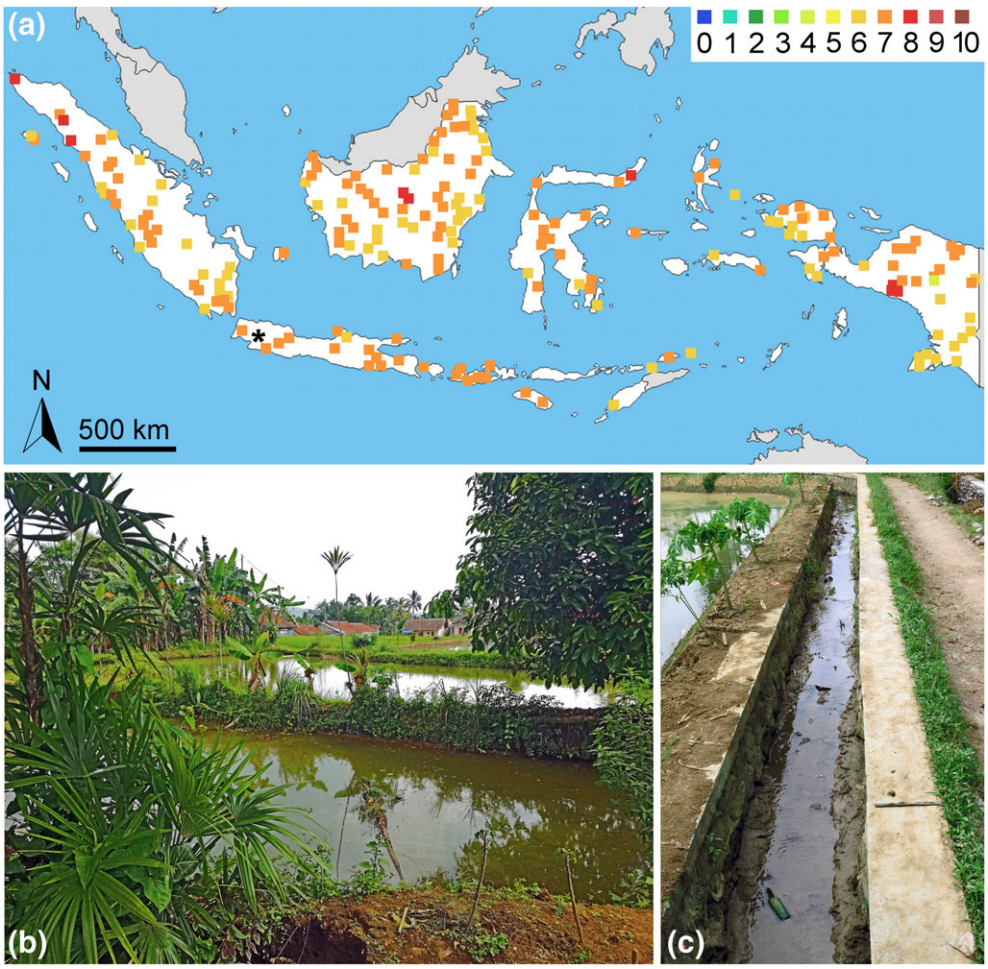

FIGURE 1 (a) Climate match map of Indonesia showing colour-coded regions with different probabilities for the establishment of the red swamp crayfish *Procambarus clarkii*, based on its temperature requirements. Scores of ≥7 indicate no climatic barrier to survival. The outdoor population of *P. clarkii* in Cisaat Subdistrict, Java, is marked with a black asterisk. (b) Outdoor ponds where *P. clarkii* is cultured. (c) Outflow drainage connecting the ponds with the Cilegok brook

pH 8.05–8.11, water temperature 25.2–28.3 °C, and dissolved oxygen 4.0–6.8 mg L^{-1}.

Crayfish were captured from one of the ponds by a baited lift net, a tool commonly used by local people for crayfishing. Furthermore, specimens of other freshwater decapod species present at this locality were also collected for *A. astaci* screening.

2.3 | Testing for the presence of the crayfish plague pathogen

Individuals of *P. clarkii* obtained from pet shops as well as from the outdoor ponds, and the shrimp and crab species coexisting with *P. clarkii*, were analysed for the presence of DNA from *A. astaci*, following the protocols described by Mrugała et al. (2015). The soft abdominal cuticle and telson of each crayfish individual were dissected, and DNA was isolated from a mixed subsample of these tissues (approx. 50 mg per individual) using a DNeasy tissue kit (Qiagen, Hilden, Germany) to a volume of 200 µL. Shrimp and crab specimens were processed similarly, but owing to their small size the ventral part of the cephalothorax with basal pereopod joints and eyestalks were also included in the processed tissue of shrimps, and the whole carapace and pereopods were used from crabs. Then, a TaqMan minor groove binder quantitative polymerase chain reaction (qPCR) assay modified from Vrålstad, Knutsen, Tengs, and Holst-Jensen (2009), which specifically and with high sensitivity detects *A. astaci* DNA, was run on an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA), using 5 µL of the DNA isolate in a 25−µL reaction. The quantity of *A. astaci* DNA in the reaction was calculated in PCR forming units, and the infection status of the analysed individuals was then expressed in semiquantitative agent levels (Kozubíková, Vrålstad, Filipová, & Petrusek, 2011; Vrålstad et al., 2009). Further details of the protocol are provided in Mrugała et al. (2015) and Svoboda, Strand, et al. (2014).

2.4 | Climate matching

To evaluate the potential climatic limits of the spread of *P. clarkii* within Indonesia, particularly temperature suitability, a climate match between source and target areas was computed using CLIMATCH 1.0 (Invasive Animals Cooperative Research Centre, Bureau of Rural Sciences). This approach has been used in previous climate matching of non-indigenous crayfish species (Chucholl, 2013; Patoka et al., 2016). Climatic data were obtained from the database of the WorldClim project (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005). The region of the native geographic range of *P. clarkii* in North America (111 meteorological stations; Crandall, Fetzner, & Hobbs, 2001) was used as the source area. The target area covered the entire territory of Indonesia (211 meteorological stations). Temperatures during the wettest and driest quarters of the year were used as climatic variables. In accordance with previous studies (Britton, Cucherousset, Davies, Godard, & Copp, 2010; Kalous, Patoka, & Kopecký, 2015; Uderbayev et al., 2017; Vodovsky et al., 2017), if the values of a climate match between the source area and the climatic station in the target area reached or exceeded 7.0, this was interpreted as no evidence for climatic constraints to the survival of *P. clarkii*.

3 | RESULTS

Individuals of *P. clarkii* advertised for sale were found in all pet shops visited and in the public aquarium, as well as in one of the three surveyed street markets. Crayfish were usually sold as 'merah lobster air tawar' (red freshwater crayfish); in one case they were correctly labelled as *P. clarkii*, but they were also misnamed as '*Cherax* sp. from Papua' in a shop and in the description presented at the Jakarta public aquarium Dunia Air Tawar. Various colouration morphs, including wild, full red, orange, blue, and white, were found in the ornamental trade. The crayfish were stored in tanks separately from other animals, but occasionally together with *C. quadricarinatus* and *Cherax peknyi* Lukhaup & Herbert, 2008. Based on answers from the shop owners, all crayfish were produced locally.

The semi-intensive culture of *P. clarkii* in ponds near Pasir Angin village started in 2007, and can be considered as well developed, with an estimated yield of approximately 1 t ha^{-1}. Many captured adult females were ovigerous. Wild, full red, and blue colouration morphs were found among the captured crayfish. There are no barriers between the ponds and the brook, and crayfish may easily spread in the vicinity, and according to information provided by local people, crayfish are occasionally captured in the brook. At the same locality, other decapods were also sampled: the invasive shrimp *M. lanchesteri*, native to Brunei, Malaysia, and Thailand, and the native crab *P. convexa*.

Altogether, 38 *P. clarkii* individuals from four sources (10 from the AJM pet shop, eight from the Brayamustika pet shop, 10 from the Toko Terang pet shop, and 10 from the outdoor culture near Cilegok brook) were tested for the presence of *A. astaci* DNA. The pathogen was unambiguously confirmed in individuals from two sources. In the Toko Terang pet shop in Bogor, two out of 10 tested individuals had a very low level of infection (agent level A2, according to Vrålstad et al., 2009). In the outdoor population in ponds connected with Cilegok brook, three out of 10 tested individuals had a very low to low level of infection (one with A2; two with A3). *Aphanomyces astaci* DNA was also detected in both freshwater decapod species coexisting with *P. clarkii*; specifically, one of four tested individuals of the crab *P. convexa* and one of nine individuals of the shrimp *M. lanchesteri* tested positively (agent levels A2 and A3, respectively).

Climatic conditions, here represented by temperature, apparently do not constrain the spread of *P. clarkii* in Indonesia. In total, 130 meteorological stations had a climate match score of $\geq$7.0; only one station (in the central highlands of Papua Province) had a score lower than 6 (Figure 1a).

4 | DISCUSSION

As *P. clarkii* was found to be frequently traded in Indonesian pet shops (at least in Java), it is probable that this crayfish will be released by hobbyists and farmers intentionally for further exploitation at new localities. This is well demonstrated by the first confirmed Indonesian population of the species, which was recorded in outdoor ponds connected with the Cilegok brook in Java. This population seems well established, with naturally occurring ovigerous females, and there are apparently no barriers preventing dispersal to local water bodies, as

evidenced by captures of crayfish in the connected Cilegok brook. Furthermore, major geographical barriers (such as the sea) that prevent the spontaneous spread of the species across Indonesia can be circumvented by legal transport within the country. Therefore, further new introductions of *P. clarkii* on various islands of the archipelago may be expected, if they have not already happened.

Endemic species of *Cherax* crayfish from New Guinea (Bláha et al., 2016) should be considered seriously threatened, because the western part of this island belongs to the territory of Indonesia. It must be noted that the Indonesian territory is relatively close to Australia, a very important hot spot of more than 150 described crayfish species from the family Parastacidae (Crandall & De Grave, 2017), which are probably all threatened by crayfish plague (Svoboda, Mrugała, Kozubíková-Balcarová, & Petrusek, 2017).

Globally, *P. clarkii* is one of the most popular pet-traded crayfish. It has been found in all of the surveyed markets with ornamental decapod crustaceans, and is commonly available (Chucholl & Wendler, 2017; Kotovska, Khrystenko, Patoka, & Kouba, 2016; Patoka, Kalous, & Kopecký, 2015). In contrast with *C. quadricarinatus*, which is also consumed locally (Patoka et al., 2016), the production of *P. clarkii* in Indonesia seems restricted to the pet trade. In contrast to previous assumptions (Patoka, Kalous, & Kopecký, 2015), shipments of ornamental crayfish exported from Indonesia do not consist mostly of New Guinean *Cherax* species, but also include large numbers of non-indigenous *P. clarkii*. In addition, part of the production is also sold in the local Indonesian pet market, and it is likely that other exotic crayfish species are also cultured in Indonesian waters. The presence of crayfish in several other localities in Java was checked in our preliminary surveys, especially those close to large settlements where substantial propagule pressure may be expected (e.g. lakes Cilala, Kemang, and Lido in Bogor). Although no *P. clarkii* were found during single visits, a systematic screening of natural habitats with much more intensive sampling effort is needed to obtain better knowledge of the present status and distribution of exotic crayfish in Indonesia.

When released or escaping to new localities, climatic conditions apparently present no environmental obstacles to the successful establishment and further spread of *P. clarkii* across the Indonesian territory (Figure 1a). There are many water bodies at the same altitudes as the ponds surveyed here, and the red swamp crayfish could probably be successfully cultured or established throughout the area, considering its success in various regions of the world (from tropical to temperate regions, including survival in Central European winter conditions; Chucholl, 2011; Veselý, Buřič, & Kouba, 2015). Therefore, the successful survival of *P. clarkii* in Indonesian mountain regions with lower temperatures cannot be ruled out. Its spread may have serious impacts on local ecosystems and biodiversity, as well as cause economic losses. This species is known to be a digger that can damage dams and water drainages, an agricultural pest that consumes rice plants, an invader that can outcompete native species, and, as already mentioned, an important vector of crayfish plague (Souty-Grosset et al., 2016).

The crayfish plague pathogen was confirmed in specimens from one pet shop as well as in the outdoor population of *P. clarkii* in Java; this is the first report of this disease in Indonesia, which is the second country in Asia (after Japan; Mrugała et al., 2017; Martín-Torrijos et al., 2018) where established populations of North American invasive crayfish have been confirmed to carry this pathogen. The spread of the disease along with escaping crayfish, and possibly also with waste water from aquaria or ponds, is probable. *Aphanomyces astaci* DNA was also confirmed in other decapods from the outdoor locality, namely in native *P. convexa* and non-indigenous *M. lanchesteri*; these, and probably also shrimps and crabs too, may thus serve as vectors of this pathogen (Svoboda, Mrugała, et al., 2014; Svoboda, Strand, et al., 2014). Their sensitivity to crayfish plague is so far unknown, but the possibility that increasing levels of this pathogen in the environment may result in mortalities of crabs or shrimps cannot be excluded.

Astaciculture (aquaculture focused on freshwater crayfish production) has shown a growing trend in Indonesia over the past decade (Edgerton, 2005; Patoka et al., 2016). The main target species is *C. quadricarinatus*, which is farmed and harvested both in natural lakes and rivers, and in artificial ponds and reservoirs (Patoka et al., 2018). This crayfish is produced both for human consumption and for ornamental purposes (Patoka et al., 2016). *Cherax quadricarinatus* is susceptible to crayfish plague (Svoboda et al., 2017), and therefore its culture could be dramatically affected by the spread of the pathogen. Indeed, crayfish plague outbreaks with severe impacts on *C. quadricarinatus* aquacultures, probably transmitted directly or indirectly from *P. clarkii*, have recently been reported from Sicily (Marino et al., 2014) and Taiwan (Hsieh, Huang, & Pan, 2016).

The crayfish plague pathogen is considered to be one of the most serious invasive pathogens affecting invertebrates (Lowe, Browne, Boudjelas, & De Poorter, 2000), and the spread of its vector *P. clarkii* within Indonesia deserves the attention of wildlife managers, conservationists, policymakers, and other stakeholders, not only locally, but also from the perspective of Australia and New Zealand. Moreover, *P. clarkii* may have direct adverse effects on native biota and aquaculture facilities in Indonesia. Therefore, intensive education of the general public in this regard is strongly recommended. Considering that aquaculture and trade with Australasian species (including *C. quadricarinatus*), both for food production and the ornamental pet trade, is a viable alternative option that poses lower environmental risks, the local authorities should consider a total ban of *P. clarkii* and other crayfish species of North American origin in Indonesia. This should be a feasible way of protecting the rich Indonesian biota, particularly its indigenous freshwater crustaceans. Further monitoring of non-indigenous crayfish species and analyses of the susceptibility of indigenous freshwater decapods to crayfish plague is also strongly recommended.

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Supplement 11

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Rapid Communication

Redclaw crayfish, *Cherax quadricarinatus* (von Martens, 1868), widespread throughout Indonesia

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Abstract

The redclaw crayfish, *Cherax quadricarinatus*, is a freshwater crayfish native to north-eastern Australia and southern New Guinea. In 2016, the species was found for the first time west of the Wallace Line in Java, Indonesia and, based on climate matching, its further spread within Indonesian territory was predicted. In this study, surveys of selected localities within Indonesia were performed to examine the species occurrence. Redclaw crayfish were found throughout Indonesia, in numerous rivers, lakes, ponds and reservoirs in Batam and Bintan Islands (Riau Archipelago), Java, Kalimantan (Borneo), Sulawesi and Sumatra. Some stocks were apparently well established, providing a food source for local people and sustaining capture for pet trade purposes. Because there are no effective regulations of introductions and exploitation of this crayfish in Indonesia, its further spread to new localities is expected. Increased attention to this issue, especially regarding crayfish management and policy implementation, is urgently needed.

Key words: biological invasion, non-indigenous species, Parastacidae, aquaculture, pet trade, Java

Introduction

The redclaw crayfish, *Cherax quadricarinatus* (von Martens, 1868) (Decapoda: Parastacidae), is globally one of the most widely exploited freshwater crayfish species, cultivated mostly in extensive aquaculture systems (Saoud et al. 2013). It belongs to the northern group of *Cherax* crayfish, with a native range that includes northern parts of the Northern Territory and far north Queensland in Australia and the southern part of New Guinea (Munasinghe et al. 2004; Bláha et al. 2016). It has been previously introduced for aquaculture into numerous countries, especially in tropical or subtropical regions (Ahyong and Yeo 2007; Lodge et al. 2012; Kouba et al. 2015). In Indonesia, *C. quadricarinatus* is native only to the Papua province but, in 2016, it was recorded as non-indigenous and established at two localities west of the Wallace Line in Java, Indonesia (Patoka et al. 2016).

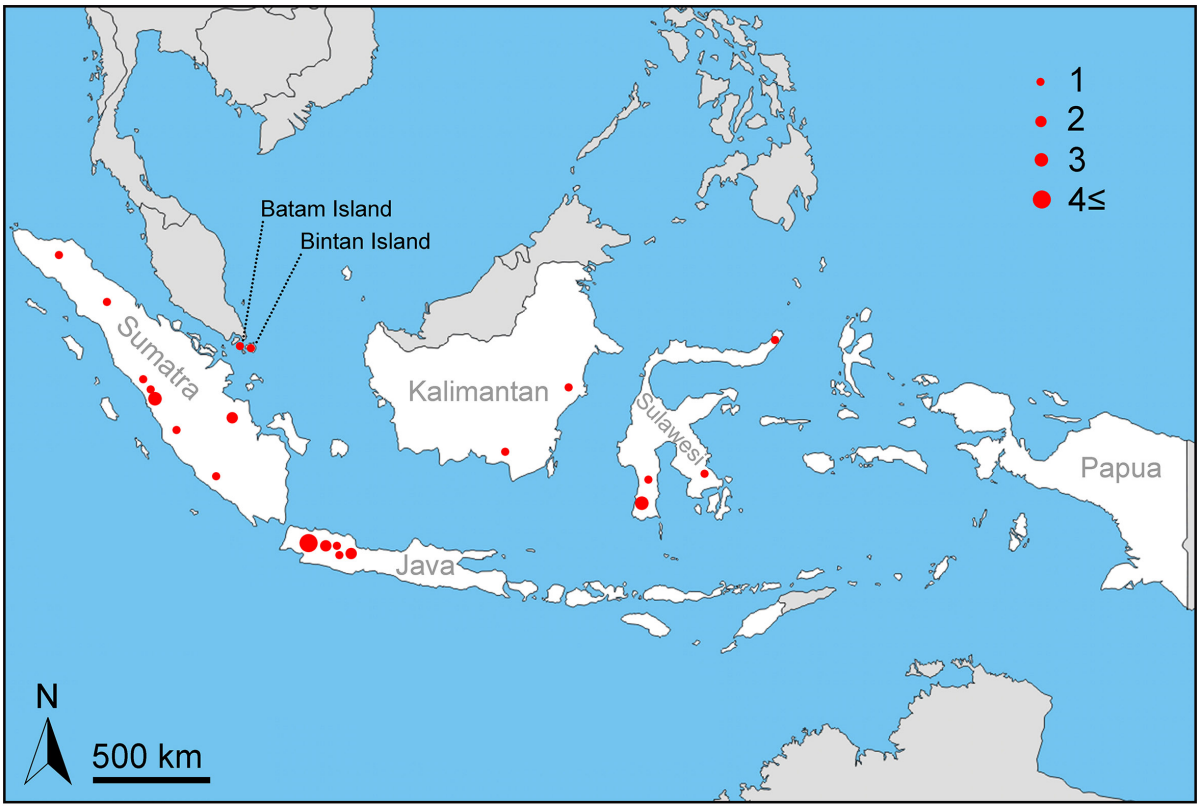

Figure 1. Map of Indonesia showing localities where *Cherax quadricarinatus* was recorded. Circles of different sizes represent different number of sites sampled at each locality.

The introduction history of *C. quadricarinatus* in Indonesia is not well known. Edgerton (2005) noted that *C. quadricarinatus* was imported into Indonesia for the establishment of aquaculture industries in 2003. The species performed well and has increased in popularity for human consumption in the country (Patoka et al. 2016). Moreover, Indonesia has been identified as one of the leading exporters of ornamental crayfish, especially of the genus *Cherax* (Patoka et al. 2015). The production of *C. quadricarinatus* for ornamental purposes has also been recorded there (Patoka et al. 2016). Nevertheless, information about the methods of farming and harvesting in this region remains anecdotal and detailed monitoring is lacking.

Based on climate matching between its native range and the entire Indonesian territory, this species has been predicted to have a high potential to become established when introduced to different parts of Indonesia (Patoka et al. 2016). *Cherax quadricarinatus* is a relatively large and highly fecund species (Jones et al. 2000), known to be a successful invader in warm climatic conditions. Its spread and associated potential negative consequences to native Indonesian crustaceans including shrimps, crabs and other freshwater biota can be expected. A possible reflection of such negative impact may already be observed at Lido Lake, Java, where the occurrence of *C. quadricarinatus*, together with the invasive shrimp *Macrobrachium lanchesteri* (de Man, 1911), has resulted in the decline and eventual extirpation of the native shrimp *M. sintangense* (de Man, 1898) (Aprila, Wowor and Farajallah, unpubl. data).

Because there are no effective legislative measures against non-native crayfish introductions in Indonesia (Jerikho R., unpubl. data), *C. quadricarinatus* is probably already found in different areas of the country and further spread to new localities is likely. This is a highly alarming scenario, given the region contains prominent global biodiversity hotspots, such as Sundaland and Wallacea (Myers et al. 2000). This study aimed to investigate the current distribution of *C. quadricarinatus* in several Indonesian islands, with the associated goal of providing a starting point for future management actions to be implemented.

Figure 2. Net cages used for culture of ornamental fish and crayfish in Kemang Lake, Java. Photo by Martin Bláha

Methods

From June 2016 to August 2017, selected localities within Indonesia were surveyed to ascertain the presence of *C. quadricarinatus*. Sampling localities were selected based on information provided by local people about crayfish occurrence and cultivation: sampling was only performed at localities where crayfish presence was suspected (Supplementary material Table S1). Crayfish were collected at each locality during one night sampling events, with use of bamboo and/or foldable net traps, baited with fish and gastropod meat. The number of traps varied between collecting events, but up to ten traps were used per locality within one night captures. Identification of captured crayfish was based on morphological characteristics according to Holthuis (1949) and Souty-Grosset et al. (2006).

Results

Populations of *C. quadricarinatus* were found in all surveyed waterbodies in Batam and Bintan Islands (Riau Archipelago), Java, Kalimantan (Borneo),

Figure 3. Ovigerous female of *Cherax quadricarinatus* captured in Cirata Reservoir, Java. Photo by Yusli Wardiatno.

Sulawesi, and Sumatra (Figure 1). *Cherax quadricarinatus* was recorded from one locality in Batam Island, one locality in Bintan Island, 13 localities in Java, two localities in Kalimantan, six localities in Sulawesi and 12 localities in Sumatra. Crayfish were captured in many different habitat types, including natural lakes and rivers, and also artificial ponds and reservoirs (Table S1, Figure 2). Crayfish were found in very high abundances at all sampling localities, usually in numbers well above hundreds of adults. Subadult individuals, as well as ovigerous females (Figure 3), the latter often caught by locals using submerged bamboo traps, were found in all populations.

Discussion

In addition to previous records from Cilala and Lido Lakes, Java (Patoka et al. 2016), we here report the presence of 35 populations of *C. quadricarinatus* widespread within the Indonesian territory, outside its native range. These populations are assumed to be established in the area given the presence of subadults, ovigerous females and the long-term cultivation history of redclaws in the surveyed localities. This validates the prediction (Patoka et al. 2016), of high potential for this crayfish to establish and spread in Indonesian territory.

Edgerton (2005) noted that *C. quadricarinatus* was imported into Indonesia in 2003 for the establishment of aquaculture industries. However, based on information obtained from local people, the crayfish in Kemang Lake, Java, were already cultivated for the pet trade in net cages in 2002 (Figure 2), having escaped from the facility the same year.

Cherax quadricarinatus is also commonly advertised for trade as an ornamental animal in local pet shops in Jakarta and Bogor, Java, being offered under the general local name "lobster air tawar" (freshwater crayfish) and, in one case, under the misnomer *C. albertisii*. Based on information from pet shop owners, the traded crayfish originates from Kemang Lake.

The only national regulation on aquatic non-native species in Indonesia is Regulation No. 41/PERMEN-KP/2014, which bans the import of 152 selected non-native fish. In this law, fishes are defined as "all types of organisms in which all or part of their life cycle is in an aquatic environment". Only four species of crayfish are listed and banned: *Faxonius rusticus* (Girard, 1852) [previously known as *Orconectes rusticus*], *F. virilis* (Hagen, 1870) [previously known as *Orconectes virilis*], *Pacifastacus leniusculus* (Dana, 1852) and *Procambarus clarkii* (Girard, 1852).

It is clear that *C. quadricarinatus* is popular for exploitation in Indonesia and, because release of this crayfish species into the wild is not illegal in the country, more and more waterbodies will probably be used for its culture. As a consequence, more unintentional escapes can be expected. Local people have very poor knowledge of the risks of invasive species (Patoka J., pers. obs. 2017). As eradication of established crayfish populations is practically impossible, further education of the general public seems crucial for prevention of new introductions of redclaws and other exotic crayfish species in the area. Therefore, active measures implemented by wildlife managers and national policymakers are urgently and strongly recommended to address this crayfish invasion.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Populations of *Cherax quadricarinatus* sampled in Indonesia.

This material is available as part of online article from:
http://www.reabic.net/journals/bir/2018/Supplements/BIR_2018_Patoka_etal_Table_S1.xlsx

Supplement 12

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WILEY

RESEARCH ARTICLE

Irresponsible vendors: Non-native, invasive and threatened animals offered for garden pond stocking

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Abstract

1. The pet trade has been responsible for many introductions of non-native species. Freshwater ornamental plants and animals originating from the pet trade are stocked to garden ponds. The present survey focused on awareness and responsible behaviour related to biological invasion risks of companies that designed, built, and stocked garden ponds.
2. A representative number (n = 124) of companies (commercial garden pond architects and builders) in the Czech Republic were surveyed regarding the offer of non-native, invasive and threatened native species. The survey was conducted over the entire warm period (from 1 May 2015 to 31 September 2015) while using personal visits, correspondence and interviews to list the species on offer.
3. 'Traditional' ornamental species were offered by 39.1% of surveyed vendors, non-traditional 'marginal' species by 5.6%, common native species by 6.5% and threatened native species by 2.4%.
4. The findings of this study support the hypothesis that 'garden pond' vendors offer non-native species with a risk of biological invasion; moreover, threatened native animals were also on sale. It is probable that a similar situation exists in other countries. It is important for the conservation of native aquatic biota to raise awareness of the need to reduce risk through responsible behaviour of those involved in the pet trade. It is also essential to prohibit stocking of potential invaders and to enforce the illegal capture and sale of native species.

KEYWORDS

alien species, aquaculture, endangered species, fish, introduction, invertebrates, pond, urban development

1 | INTRODUCTION

The development and growth of global markets for non-native species is considered the dominant proximate cause of the increase in biological invasions (Perrings, Williamson, & Dalmazzone, 2000). The pet trade has been particularly responsible for introductions and, in some cases, subsequent establishment of numerous non-native aquatic species and can constitute a threat for species in their native areas (Arndt, Gessner, & Raymakers, 2002; Lodge, Taylor, Holdich, & Skurdal, 2000; Nunes, Tricarico, Panov, Cardoso, & Katsanevakis, 2015; Padilla & Williams, 2004; Patoka et al., 2016; Rixon, Duggan, Bergeron, Ricciardi, & Macisaac, 2005). This phenomenon has been brought about by the fact that many people around the world are involved in the keeping of aquatic species for ornamental purposes (Duggan, 2010; Maceda-Veiga, Domínguez-Domínguez, Escribano-Alacid, & Lyons, 2016; Padilla & Williams, 2004). Moreover, the trend has been for this hobby to accelerate in recent decades within Europe (Patoka, Kalous, & Kopecký, 2015; Peay, 2009; Van der Velde, Nagelkerken, Rajagopal, & de Vaate, 2002). Despite positive effects such as economic profit for producers and vendors, education of hobbyists, and popularization of the species, this increase provides opportunities for geographically isolated species to become established in new but suitable localities. This occurs because of parallel increases in species quantity and availability on the market, and propagule pressure (Duggan, Rixon, & MacIsaac, 2006). The present situation has stimulated several studies evaluating the risks associated with various aquatic invertebrate and vertebrate species involved in the pet trade (Chucholl, 2013; Kalous, Patoka, & Kopecký,

2015; Kopecký, Kalous, & Patoka, 2013; Magalhães & Jacobi, 2013; Mazza et al., 2015; Patoka, Kalous, & Kopecký, 2014; Perdikaris et al., 2015). Although the risks associated with non-native species have been widely discussed, certain threatened and native species are included in the pet trade and are affected by over-exploitation (Raghavan et al., 2013; Schlaepfer, Hoover, & Dodd, 2005).

Freshwater ornamental plants and animals are usually stocked in indoor tanks, but small, isolated outdoor water reservoirs, generally described as garden ponds, are stocked also with individuals from the pet trade (Nunes et al., 2015). Moreover, animals stocked in garden ponds are likely to be better adapted to the local environment than those kept indoors, so they can be expected to represent a greater potential risk of biological invasions. The popularity of garden ponds and the potential associated risks of biological invasions have been noted in previous studies (Lodge et al., 2000; Patoka, Petrtýl, & Kalous, 2014; Peay, 2009; Stloukal, 2009), but the relationship with the pet trade has been insufficiently explored. Most garden ponds are designed, built, and stocked by specialized companies. The present survey focused on garden pond vendors, and especially on their awareness and responsible behaviour, investigating the hypothesis that these vendors constitute a risk associated with the introduction of non-native invasive species.

2 | METHODS

One-quarter (n = 124) of the nearly 500 vendors (commercial garden pond architects and builders) in the Czech Republic were surveyed. Vendors were chosen at random among those identified using the internet search engine at www.seznam.cz; the Czech equivalent of the key term 'garden ponds' was used in the search category 'companies'. Online pond accessories shops which do not design or build garden ponds were excluded from the analysis. Lists of animal species were examined and all non-native, invasive and also threatened native species were recorded. The degree of native species vulnerability was assessed in accordance with respective national Red Lists of threatened species (Farkač, Král, & Škorpík, 2005; Lusk, Lusková, Hanel, Lojkásek, & Hartvich, 2011; Plesník, Hanzal, & Brejšková, 2003). To ensure correct identification, all 49 vendors who offered these categories of species for sale were visited anonymously. Additional explanation that helped to clarify specific inquiries or provided detailed information on animal stocking, origin and availability in garden ponds was obtained by e-mail correspondence (44 vendors) and/or by personal interviews (five vendors). The survey was conducted over the entire warm period (from 1 May 2015 to 31 September 2015) when outdoor ponds are usually stocked.

3 | RESULTS

Among the animal species offered by vendors for keeping in garden ponds, 26 were vertebrates (24 fishes, two frogs) and nine were invertebrates (three crayfishes, three bivalves, and three gastropods) (Table 1). In total, 39.1% of vendors surveyed offered ornamental fish species. The offered species included: (i) 'traditional' ornamental species that, despite the vulnerability of some of them, have a long-documented ornamental history (10 species); (ii) 'marginal' ornamental species that are offered in wild forms only and not in large quantities (seven species); (iii) common native species (three species); and (iv) threatened native species (four species) (Table 1). All vendors who offered fish, offered traditional ornamental species, marginal species were offered by 5.6% of vendors surveyed, common native species by 6.5%, and threatened native species by 2.4%. The most frequently offered ornamental fish were nishikigoi, also known as koi (*Cyprinus rubrofuscus* Lacépède), goldfish (*Carassius auratus*, L.) and their colour morphs.

Crayfish were offered by 2.4% of the vendors surveyed. Two of the crayfish belong to 'old non-indigenous crayfish species' (Holdich, Reynolds, Souty-Grosset, & Sibley, 2009): spiny-cheek crayfish (*Orconectes limosus* Rafinesque) and red swamp crayfish (*Procambarus clarkii* Girard). Only one species, the parthenogenetic form of the marbled crayfish (*Procambarus* fallax f. *virginalis* Martin et al.), is classified as 'new non-indigenous crayfish species' (Holdich et al., 2009). The threatened native bivalve species, swan mussel (*Anodonta cygnea*, L.), was offered by 3.2% of the vendors surveyed, and the same vendors also sold a common native bivalve species, the duck mussel (*Anodonta anatina*, L.). The non-native bivalve species, Chinese pond mussel (*Sinanodonta woodiana*, Lea), was offered by 1.6% of the vendors surveyed. All gastropods were common native species: great pond snail (*Lymnaea stagnalis*, L.), great ramshorn (*Planorbarius corneus*, L.), and one of the river snails, *Viviparus viviparus* (L.). As found during a personal visit, one threatened native species of frog, the European fire-bellied toad (*Bombina bombina*, L.) and one common native species, the European common frog (*Rana temporaria* L.), were sold by 0.8% of the vendors surveyed.

According to information given by vendors, most species were bred in captivity, but some species had been captured in the field, including eight fish species, one crayfish species, and all bivalves, gastropods and frogs (Table 1). Only 4.8% of all the vendors surveyed warned their customers of the potential environmental risks related to non-native species and the illegal practice of keeping threatened native species.

4 | DISCUSSION

Although the introduction (i.e. release) of non-native species is banned in the Czech Republic either by local law (Act No. 114/1992 Coll, n.d.), or European Union legislation (Regulation No. 1143/2014; Council of the European Communities, 2014), certain non-native invasive species were offered by architects and builders of garden ponds together with traditional ornamental species. This situation is similar to findings reported from Brazil, where restrictions on the non-native crayfish *Procambarus clarkii* decreased market availability, but the species continued to be available in the pet trade within the country (Magalhães & Andrade, 2014). In some cases, species native to a particular country can become invasive and threaten native biota when introduced to a part of the country outside their native range. An example can be seen in the common nase (*Chondrostoma nasus*, L.), which is locally threatened but outcompetes and eliminates native fishes in drainages where it has been introduced (Kottelat & Freyhof, 2007).

TABLE 1 Animal species offered for keeping in garden ponds within the Czech Republic; their nativeness (native at least in part of the territory, or non-native); origin (C: captivity breeding, F: field capture); vulnerability of native species (LC: Least Concern, NT: Near Threatened, VU: Vulnerable, EN: Endangered, CE: Critically Endangered, non-native species not evaluated); colouration (offered morphs); offered (proportion stated as percentage of all vendors surveyed offering the species); fish species category (TO: traditional species with long ornamental history, MO: marginal ornamental species, CN: common native species, TN: threatened native species)

Species	Nativeness	Origin	Vulnerability	Colouration	Offered	Category
Fishes						
Acipenser baerii	non-native	C		wild	3.2	TO
Acipenser gueldenstaedtii	non-native	C		wild	8.8	TO
Acipenser ruthenus	native	C	CE	wild, albino	17.7	TO
Acipenser stellatus	non-native	C		wild	3.2	TO
Ameiurus melas	non-native	F		wild	1.6	MO
Barbatula barbatula	native	C	LC	wild	0.8	CN
Carassius auratus	non-native	C		many morphs	38.7	TO
Carassius carassius	native	F	CE	wild	0.8	TN
Cyprinus rubrofuscus	non-native	C		many morphs	39.1	TO
Chondrostoma nasus	native	C	VU	wild	5.6	TN
Gasterosteus aculeatus	non-native	C		wild	1.6	MO
Gobio gobio	native	F	LC	wild	4.8	CN
Lepomis gibbosus	non-native	C		wild	4.0	MO
Leucaspius delineatus	native	F	CE	wild	0.8	TN
Leuciscus idus	native	C	NT	gold, albino	32.3	TO
Myxocyprinus asiaticus	non-native	C		wild	1.6	MO
Notropis chrosomus	non-native	C		wild	0.8	MO
Perca fluviatilis	native	F	LC	wild	4.0	CN
Phoxinus phoxinus	native	F	EN	wild	0.8	TN
Polyodon spathula	non-native	C		wild	1.6	MO
Rhodeus amarus	non-native	F		wild	0.8	MO
Scardinius erythrophthalmus	native	C	LC	gold	33.1	TO
Silurus glanis	native	C	LC	albino	4.8	TO
Tinca tinca	native	C	LC	gold, blue	14.5	TO
Crayfishes						
Orconectes limosus	non-native	F		wild	1.6	
Procambarus clarkii	non-native	C		full red, albino	2.4	
Procambarus fallax f. *virginalis*	non-native	C		wild	2.4	
Bivalves						
Anodonta anatina	native	F	LC	wild	3.2	
Anodonta cygnea	native	F	VU	wild	3.2	
Sinanodonta woodiana	non-native	F		wild	1.6	
Gastropods						
Lymnaea stagnalis	native	F	LC	wild	2.4	
Planorbarius corneus	native	F	LC	wild	2.4	
Viviparus viviparus	native	F	NT	wild	2.4	
Frogs						
Bombina bombina	native	F	EN	wild	0.8	
Rana temporaria	native	F	LC	wild	0.8	

Both vertebrates and invertebrates are available in the Czech Republic for keeping in garden ponds (Table 1). With the exception of three sturgeons, all fishes classified as 'traditional' ornamental species have been artificially selected and differ from their wild conspecifics in many traits. These species are offered by vendors in fancy bright red, gold, yellow, blue or albino colouration and/or in various body and fin shape morphs (Hanel et al., 2011; Komiyama et al., 2009; Rylková, Kalous, Šlechtová, & Bohlen, 2010). These morphs are usually rare in the wild because of their increased vulnerability to predation (Slavík, Horký, & Maciak, 2015). Consequently, establishment of traditional ornamental fish species in the wild is improbable. On the other hand, *C. auratus* has been evaluated as the species with the highest invasive potential in the EU of all aquarium fishes (Kalous et al., 2015). Moreover, Arndt et al. (2002) have suggested that increasing the availability of non-native sturgeons in the pet trade and subsequent higher frequency of escapes or releases into the wild

can lead to a decline in native sturgeons caused by various genetic impacts (Havelka, Kašpar, Hulák, & Flajšhans, 2011). Thus, offering the three non-native sturgeon species risks endangering wild sterlet (*Acipenser ruthenus*, L.) in the Czech Republic. A further risk is that ornamental fish and invertebrates may serve as vectors of non-native pathogens and parasites (Jeffery et al., 2007; Mrugała et al., 2015; Ondračková, Dávidová, Přikrylová, & Pečínková, 2011).

Compared with traditional ornamental fishes, the species classified as 'marginal' are naturally coloured and without abnormal body and fin shape. Two of them, the black bullhead (*Ameiurus melas*, Rafinesque) and pumpkinseed (*Lepomis gibbosus*, L.), are established in the Czech Republic and both are considered as invaders (Musil, Jurajda, Adámek, Horký, & Slavík, 2010). Two other species, the three-spined stickleback (*Gasterosteus aculeatus* L.) and European bitterling (*Rhodeus amarus*, Bloch), are classified as 'naturalized' in the Czech Republic (Musil et al., 2010). Even though the Asian hi-fin shark (*Myxocyprinus asiaticus*, Bleeker) and rainbow shiner (*Notropis chrosomus*, Jordan) have not been recorded in the wild in the Czech Republic, they are able to tolerate low temperatures (below 5°C) (Funnell, Heaton, MacDonald, & Brownson, 2009; Holder & Powers, 2010) and therefore may become established. The last species in this category, the American paddlefish (*Polyodon spathula*, Walbaum), had been classified as capable of acclimatization in the Czech Republic (Musil et al., 2010). Except for one subadult captured in a fish pond near Třeboň (Vladimír Vrabec, unpublished data), no records are known from the wild in the country. This species nevertheless has a wide range in the USA that includes northern areas where winter temperatures are low and it also regularly engages in upstream migratory behaviour (Scarnecchia et al., 2007). Therefore, although it is capable of surviving, establishment is unlikely because of the many river obstacles such as dams and weirs in the Czech Republic (Musil, Horký, Slavík, Zbořil, & Horká, 2012). A similar situation was observed for native fish species that migrated to the Czech Republic in connection with reproduction in the past and are today evaluated as extinct in the wild (Lusk, 1996).

Despite protective legislation (Act No. 114/1992 Coll, n.d.), native fish species captured from wild stocks are advertised for sale. Three species, the stone loach (*Barbatula barbatula*, L.), gudgeon (*Gobio gobio*, L.) and European perch (*Perca fluviatilis* L.), are common in the Czech Republic. Two species, the crucian carp (*Carassius carassius*, L.), and sunbleak (*Leucaspius delineatus*, Heckel), are considered as critically endangered and two others, the common nase and common minnow (*Phoxinus phoxinus*, L.), as vulnerable in the national Red List of threatened species (Lusk et al., 2011). The capture of threatened species (Table 1) is obviously endangering the wellbeing of wild stocks.

All offered crayfish species (*O. limosus*, *P. clarkii* and *P. fallax* f. *virginalis*) are North American cambarids, and they carry and transmit crayfish plague (Diéguez-Uribeondo & Söderhäll, 1993; Keller, Pfeiffer, Roessink, Schulz, & Schrimpf, 2014; Kozubíková, Viljamaa-Dirks, Heinikainen, & Petrusek, 2011; Mrugała et al., 2015). These species have high invasive potential and have been evaluated as very dangerous from this perspective for the territory of the Czech Republic, as well as for Europe more generally (Chucholl, 2013; Patoka, Kalous et al., 2014). Only *O. limosus* occurs in the wild in the country (Kouba, Petrusek, & Kozák, 2014) and it is probably supplied from field capture. *Procambarus clarkii* originates from both import and domestic production, while *P. fallax* f. *virginalis* is produced only domestically (Patoka et al., 2015). Moreover, *P. clarkii* and *P. fallax* f. *virginalis* are the most widely available and lowest-priced crayfish species in the pet market (Faulkes, 2015; Lipták & Vitázková, 2015; Patoka et al., 2015) and both exhibit substantial resistance to low winter temperatures (Veselý, Buřič, & Kouba, 2015).

All three species of bivalves offered for sale belong to the family Unionidae. The non-native *S. woodiana* was first-recorded in the Czech Republic in 1996 and is present in rivers, oxbow lakes and ponds. It probably competes with indigenous unionids (Beran, 2008; Douda, Vrtílek, Slavík, & Reichard, 2012). The two other unionids offered, *A. anatina* and *A. cygnea*, are native and so are being sold illegally (Act No. 114/1992 Coll, n.d.). The former is common in the Czech Republic, while the other is classified as Vulnerable in the national Red List of threatened species (Farkač et al., 2005).

The offered gastropods are native to the Czech Republic. Only one of them, *V. viviparus*, is considered Near Threatened (Farkač et al., 2005). The other two species, *L. stagnalis* and *P. corneus*, are common and widespread. The environmental risks of these gastropods may be associated with the transmission of pathogens: at least *L. stagnalis* is an intermediate vector for the liver fluke (*Fasciola hepatica* L.), a common parasite of ruminants and humans (Kendall, 1949).

The only amphibian species being sold which is common in the Czech Republic is *R. temporaria*. Even so, capture in the field is banned and its sale by vendors is illegal. The other species, *B. bombina*, is also offered illegally (Act No. 114/1992 Coll, n.d.) because it is considered endangered (Plesník et al., 2003).

The problems described in this paper seem to be much greater than has been reported previously (Patoka, Petrtýl et al., 2014; Peay, 2009) because many vendors probably do not publicize that they offer 'problematic' species. Moreover, pet retailers may misidentify some mollusc and crayfish species (Patoka, Kalous et al., 2014). We agree with Lodge et al. (2000) who recommended that all sales to garden ponds should be accompanied by educational material and warnings to hobbyists about the dangers of releasing non-native species. Inasmuch as only 4.8% of the vendors surveyed provided customers with this information, most customers may be unaware of the environmental threat. Although this study was confined to the Czech Republic, it is likely that similar situations exist in other countries. Given that escape from garden ponds is considered one of the main pathways for introducing freshwater organisms globally (Copp, Templeton, & Gozlan, 2007; Leuven et al., 2009; Lodge et al., 2000), banning the stocking of potential invaders in outdoor reservoirs, including garden ponds, and strict enforcement of laws prohibiting the illegal capture and sale of native species, are essential for the conservation of native aquatic biota.

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Supplement 13

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Predictions of marbled crayfish establishment in conurbations fulfilled: Evidences from the Czech Republic

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Abstract: The marbled crayfish (*Procambarus fallax* f. *virginalis*) has become one of the potentially most dangerous non-indigenous crayfish species spreading in European countries and elsewhere. This taxon reproduces parthenogenetically and recently has been verified as a vector of the crayfish plague pathogen. Here, we report on two established populations of marbled crayfish in the Czech Republic. The marbled crayfish was observed during autumn 2015 in an urban pond connected by sewer piping with the Rokytka brook near its mouth to the Vltava River in Prague. Subsequently, three adult females, two of them having well-developed glair glands and oocytes, were captured in this pond during spring 2016, suggesting successful overwintering of the local population. Furthermore, four adult females were captured in an artificial pond at the Radovesická lignite spoil heap in the vicinity to the industrial conurbation of Bílina in summer 2016; one of them carried eggs. We tested these for the presence of the crayfish plague pathogen *Aphanomyces astaci*, with negative results. The introduction pathway for both populations is most likely a release from private aquaria, as these sites are popular for recreation activities. Our findings substantiate previous predictions that conurbations are likely to be the primary areas for marbled crayfish introductions.

Key words: *Procambarus fallax* f. *virginalis*; biological invasion; first record; pet trade; Marmorkrebs; urban pond; post-mining site

Introduction

The introduction, establishment, and subsequent spread of non-indigenous crayfish species (NICS) are known to constitute one of the main factors seriously affecting abundance of European indigenous crayfish species (Peay 2009; Gherardi et al. 2011; Perdikaris et al. 2012; Chucholl 2014). The trade for ornamental purposes (pet trade) has been considered an important source of new alien species introductions worldwide (Padilla & Williams 2004; Duggan 2010; Chucholl 2013; Patoka et al. 2015a, 2016). The pet trade in imported crayfish started to expand around 1995, and consequently production of crayfish for ornamental purposes developed in various countries (Vogt et al. 2004; Faulkes 2010; Patoka et al. 2015b). Germany, currently with 28 NICS available for sale (Chucholl & Wendler 2016), and the Czech Republic, with 27 NICS (Patoka et al. 2014a, b), have been identified as the leading European countries in this regard.

The marbled crayfish (Marmorkrebs in German) has been identified as one of the most dangerous of ornamental NICS from a European perspective (Scholtz et al. 2003; Chucholl 2014; Patoka et al. 2014a; Kotovska et al. 2016; Souty-Grosset et al. 2016). Although it cannot be excluded that the origin of this triploid parthenogenetic crayfish could have been a hybridization between *Procambarus fallax* (Hagen, 1870) and some other species of the genus *Procambarus* (Martin et al. 2016), it is usually regarded as a parthenogenetic form of the former (*P. fallax* forma *virginalis* Martin et al., 2010). Although the native geographical distribution of this form, if at all present in the wild, is still unknown (Martin et al. 2010, 2016; Kouba et al. 2014), the sexually reproducing *P. fallax* occurs in Florida and Georgia (Taylor et al. 2007).

Populations of the marbled crayfish are exclusively composed of females, which reproduce by obligatory apomictic parthenogenesis. Thus, a single female can theoretically be sufficient to establish a viable popula-

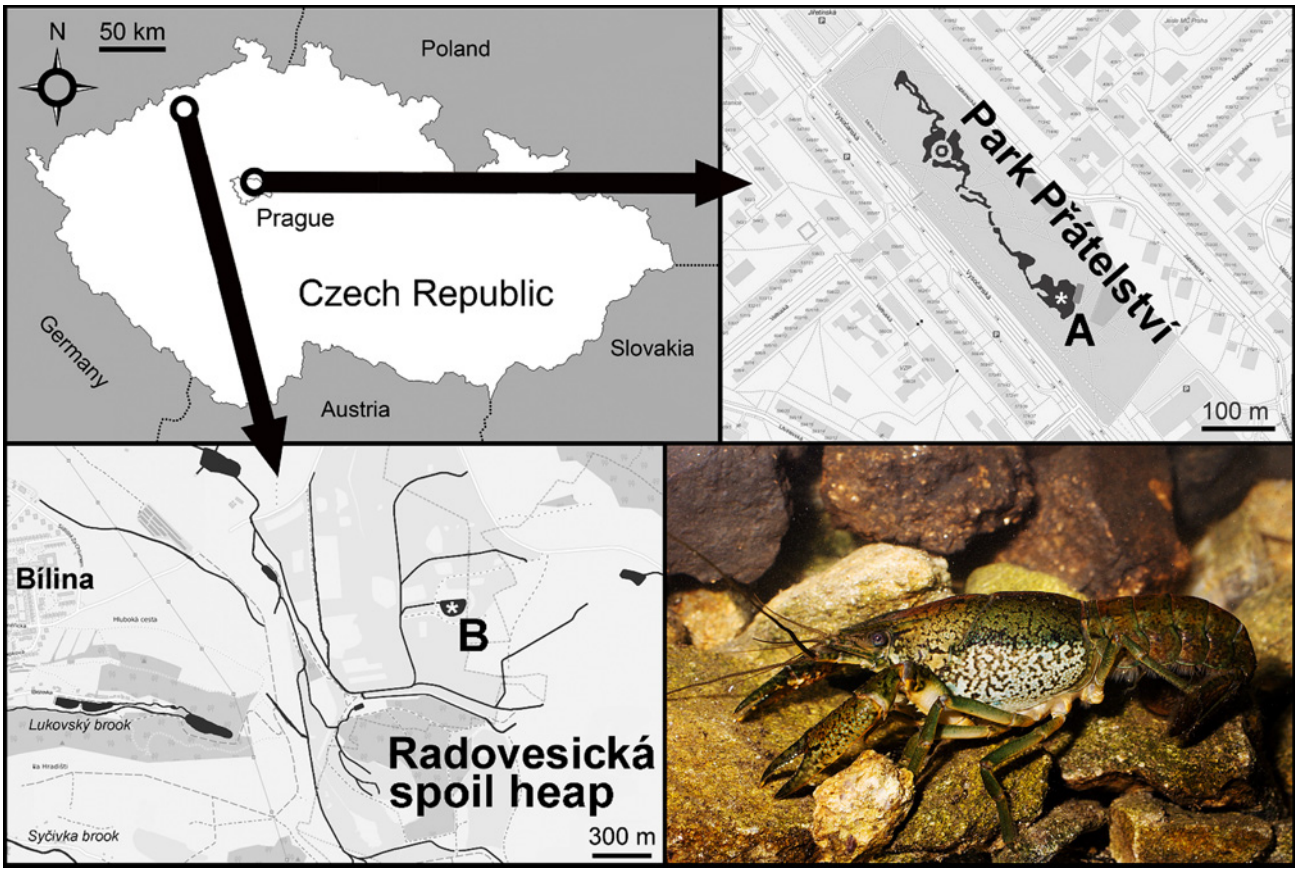

Fig. 1. Map showing the location of the Přátelství Park in Prague (A), and the Jiřina pond (B) at the Radovesická spoil heap, Czech Republic; both indicated by white asterisks. The crayfish female on the photo was captured by funnel trapping in the Jiřina pond. The basis for the maps are available under the Open Database License (www.openstreetmap.org).

tion (Scholtz et al. 2003). This reproduction strategy, together with low intraspecific aggressiveness, a generation time of only 6 months, and high fecundity, frequently lead to population explosion in a very short time (Scholtz et al. 2003; Vogt et al. 2004).

This crayfish has been recorded in the wild in several European countries, including Croatia, Germany, Hungary, Italy, the Netherlands, Slovakia, Sweden, and Ukraine (Nonnis Marzano et al. 2009; Chucholl & Pfeiffer 2010; Janský & Mutkovič 2010; Soes & Koese 2010; Bohman et al. 2013; Samardžić et al. 2014; Vojkovská et al. 2014; Weiperth et al. 2015; Lipták et al. 2016; Lőkkös et al. 2016; Novitsky & Son 2016), although the establishment success in some of those countries is not clear. As one of the negative effects to native biodiversity, ornamental animals can serve as important hosts and vectors of exotic commensals (Patoka et al. 2015a) and pathogens (e.g., Martinez-Murcia et al. 2008; Kalous et al. 2015). Indeed, marbled crayfish has been recently confirmed as a vector of the crayfish plague pathogen *Aphanomyces astaci* Schikora (Keller et al. 2014; Mrugała et al. 2015), which still threatens European indigenous crayfish species (Holdich et al. 2009). Due to its invasion potential as well as disease carrier status, marbled crayfish has been recently included, together with four other invasive crayfish of North American origin, in the list of 37 Invasive Alien Species of European Union Concern (EU Regulation No. 1143/2014; Commission Implementing Regulation No. 2016/1141).

We present here the first records of marbled crayfish from open waters in the Czech Republic from two different, geographically distant sites, in which it seems to have established populations. In an urban pond located in the city of Prague, we could confirm a successful overwintering of the population. In a pond on a post-mining spoil heap close to Bílina, several adult animals were captured, one of which carried eggs in late summer. Perdikaris et al. (2012), Chucholl (2014), and Souty-Grosset et al. (2016) had noted that the occurrence of some NICS in European waters, including the marbled crayfish, is entirely driven by propagule pressure in relation to the pet trade, and that crayfish are usually released into the nearest ponds or streams in the vicinity of conurbations. Our observations support this view.

Material and methods

Study sites

The first site, Přátelství Park, is situated in the town district Prosek, Prague, Czech Republic (GPS 50°07′21″ N, 14°29′43″ E) at the altitude 288–295 m a.s.l. (Fig. 1A). The park was founded for recreational purposes of local inhabitants. The park contains a system of small ponds connected with cascades and channels. This system, which was renovated in 2008, has a total length of 450 m and volume of

1800 m^3. The water is circulated through the vegetation season and kept at a low level. The majority of the system is completely drained at the end of vegetation season, with the exception of a pond (denoted as A in Fig. 1). In late autumn, the water is discharged into the water sewer connected with the Rokytka brook close to its confluence with the Vltava River (Elbe basin). The bottom and banks of ponds and channels are made from concrete, and the bottom is covered by a thin layer of mud and detritus, mainly formed by leaf litter. Aquatic macrophytes are represented at the site by three patches of water lilies (*Nymphaea alba* L.). These plants are taken out of the water over winter.

The second site, Radovesická spoil heap (Fig. 1B), is situated in the North Bohemian lignite basin. The whole area is heavily affected by opencast lignite mining, resulting also in numerous larger heaps of spoil consisted mainly of tertiary clays. The Radovesická spoil heap is a relatively large heap (ca 12.5 km^2) formed between 1964 and 2003 in a vicinity to the Bílina conurbation. Its majority has been technically reclaimed with artificial creation of several pools and ponds supplemented by numerous pools formed spontaneously in the terrain depressions (Harabiš et al. 2013). The Jiřina pond (0.6 ha; GPS 50°33′08″ N, 13°48′53″ E; altitude 340 m a.s.l.; denoted as B in Fig. 1) is a middle-sized artificial pond created in the early 1990s for the future recreation of Bílina inhabitants. Although still officially inaccessible, it is already relatively popular for fishing and swimming. It has no affluent, but there is an outlet conducting water after strong rains or snowmelt to a drainage channel system flowing into the Bílina River (Elbe basin). The pond banks and bottom are formed by clays and are regularly rounded and highly homogeneous. Its littoral zone is very narrow (max. 1 m) because the bottom steeply descends to the depth of over 2 m; it is dominated by dense common reed [*Phragmites australis* (Cav.) Trin. ex Steud.] and much sparser bladderwort (*Utricularia* sp.).

Data collection

Marbled crayfish was first observed to occur in open waters in the Czech Republic during October 2015, when three individuals were captured in the Přátelství Park by Jakub Friedl, a student of the Czech University of Life Sciences Prague. These crayfish were photographed and subsequently released to the pond. On 16 March 2016, we intensively sampled the pond by electrofishing, using a backpack electroshocker (Bednář; www.r-bednar.cz). Supplementary sampling included small seine-netting and sweep-netting in detritus.

In the Radovesická spoil heap, the first marbled crayfish was recorded on 30 July 2016. A female entered one of five funnel traps (80 × 27.5 × 27.5 cm, 0.5 cm green mesh nylon, 3.5 cm of an entrance diameter) exposed for 24 hours in the littoral zone for the purpose of aquatic insect monitoring. In the night of 3 September 2016, we exposed eight funnel traps in the littoral zone close to the first record. We also actively searched for crayfish by snorkelling, by sweep-netting of detritus and littoral vegetation, and exploring potential shelters.

All animals captured in both surveyed sites were identified immediately after their capture and subsequently, with exception of crayfish, were returned to the pond.

Identification

The identity of crayfish specimens was verified by both morphological and genetic analyses. Morphological analyses followed Martin et al. (2010). The mitochondrial gene for the cytochrome c oxidase subunit I (COI) from one captured female per each site was sequenced using the universal primer pair LCO1490/HCO2198 (Folmer et al. 1994) and following the protocols described in Mrugała et al. (2015).

Testing for the presence of the crayfish plague pathogen

As the marbled crayfish may host the crayfish plague pathogen *Aphanomyces astaci* (Keller et al. 2014; Mrugała et al. 2015), we analysed individuals captured at the Radovesická spoil heap for the presence of its DNA, following the protocols in Mrugała et al. (2015). We dissected the soft abdominal cuticle and telson of each individual, and isolated DNA from the mixed subsample of these tissues (ca 50 mg per individual) by DNeasy tissue kit (Qiagen) to the volume of 200 µl. Then, TaqMan minor groove binder quantitative PCR (qPCR) assay modified from Vrålstad et al. (2009), which specifically and with high sensitivity detects *A. astaci* DNA, was run on iQ5 real-time PCR detection system (Bio-Rad), using 5 µl of the DNA isolate in 25 µl reaction. Further details of the protocol are provided in Mrugała et al. (2015) and Svoboda et al. (2014).

Results

Three females were captured in the pond A of the Přátelství Park (Fig. 1A) in March 2016. The first individual, with cephalothorax length (CL, measured from tip of rostrum to posterior end of cephalothorax) of 35 mm and total body length (BL, measured from tip of rostrum to posterior edge of telson) of 73 mm was captured by electrofishing and had a carapace surface densely covered by periphyton. The other two females (CL/BL of 33/70 and 32/70 mm) were captured by sweep-netting in detritus and had well-developed glair glands and oocytes.

Four females were captured in the Jiřina pond at the Radovesická spoil heap (Fig. 1B). In July 2016 one individual (CL/BL of 56/115 mm) was captured by funnel trapping. In September 2016, one female entered a funnel trap at the same location as the first one, and two females were found in shelters under a stone and a sunken concrete block close to the pond bank (CL/BL of 38/83, 42/90, and 42/90 mm). One of the two larger individuals carried three eggs.

Morphological characteristics of the crayfish corresponded with the description given in Martin et al. (2010). The DNA barcoding confirmed the morphological identification of the captured crayfish as *P. fallax* f. *virginalis*. The obtained COI fragments matched completely each other and the reference sequences for marbled crayfish publicly available from GenBank (acc. nos. KC107813, HM358011, JF438007; Filipová et al. 2011; Martin et al. 2010; Shen et al. 2013). The qPCR assay did not detect DNA of *A. astaci* in any of the tested individuals.

Fish stock captured by electroshocking and seine-netting in the pond A of the Přáteství Park included the following potential crayfish predators: catfish *Silurus glanis* L., 1758 (2 subadults), perch *Perca fluviatilis* L., 1758 (2 adults), carp *Cyprinus carpio* L., 1758 (1 adult). Also, five pairs of mallard ducks (*Anas platyrhynchos* L., 1758) were observed feeding at the site. In the Jiřina

pond at the Radovesická spoil heap, we have observed several (sub)adults of eel *Anguilla anguilla* (L., 1758).

Discussion

We discovered and subsequently surveyed two populations of marbled crayfish occurring within an urban pond system in Prague and at a larger artificial pond on a reclaimed spoil heap. Based on our results, we consider both populations to be established. The repeated findings of marbled crayfish in the Přátelství Park suggest that the local population successfully overwintered in the pond that had not been drained at the onset of winter. Although the marbled crayfish has been considered a warm water taxon (Chucholl & Pfeiffer 2010), its established populations occur in European temperate zone (Chucholl et al. 2012; Lipták et al. 2016) and tolerance to winter water temperatures was confirmed also experimentally (Veselý et al. 2015). However, an alternative scenario that animals found in the Přátelství Park after winter originated from repeated introductions of marbled crayfish from aquaria cannot be entirely ruled out without a continuous monitoring or a capture-recapture study. The reproductive potential of the local population is indicated by the fact that the captured females had well-developed glair glands.

All females from the Radovesická spoil heap were large adults, which are likely to reproduce *in situ*. One was observed to carry a few eggs at the end of summer, when no other crayfish species in Central Europe breed (Reynolds 2002). We presume this was a remainder of originally larger clutch, considering that the typical size at maturity of this species (at least in laboratory conditions) is less than half of the size of captured females, female fecundity increases with body size, and brood sizes even for very small females usually exceed 45 eggs and may reach up to hundreds of eggs (Seitz et al. 2005; Kouba et al. 2015).

At present, marbled crayfish apparently does not reach high density in either of the studied sites. We hypothesise that the low number of captured individuals in the Přátelství Park could be caused by draining of the channels, and concentration of water only into the pond A over the winter season. The low water level can be very harmful for crayfish due to intensive predation pressure from mallard ducks (Malone 1965) as well as by fish (Syväranta et al. 2010), which are abundant at the location. In the Radovesická spoil heap, we presume a strong predation pressure by the relatively abundant European eel, which has a strong potential to decrease the crayfish population density (Aquiloni et al. 2010). Furthermore, the presence of these predators likely drives the crayfish into little accessible shelters, making the manual surveys less effective.

However, it must be underscored that without rapid management activity focusing on extirpation or control of these populations, we expect a substantial potential of the spread of marbled crayfish from both sites. In the Přátelství Park, crayfish may drift through the water outlet to the nearby adjacent water bodies. Although the Jiřina pond at the Radovesická spoil heap is not permanently connected with any other waters, its outlet is filled by water after strong rains or during a spring melting when superfluous water flows into the drainage system, including more artificial ponds and finally flowing into the Bílina River. Thus, the establishment of marbled crayfish in some adjoining water bodies is also possible. At the spoil heap, both stagnant (Harabiš et al. 2013; Vojar et al. 2016) and flowing (Tichanek & Tropek 2015, 2016) waters are known to harbour unusually rich freshwater biodiversity, which can be potentially threatened by the expanding marbled crayfish. In particular, some of the local ponds have been recently stocked by the native noble crayfish *Astacus astacus* (L., 1758) after a rescue transfer, as the area has been considered free of alien species that may directly affect this protected species vulnerable to crayfish plague. Although we did not detect *A. astaci* in captured crayfish, the low number of tested crayfish does not guarantee absence of the pathogen in the studied site; furthermore, even if the population is pathogen-free at the moment, infection by *A. astaci* sometimes in the future cannot be entirely ruled out.

Both aquarium and garden fishkeeping are traditionally popular and widespread in the Czech Republic. Although the release of non-native organisms is illegal, irresponsible or uninformed hobbyists frequently release their pets, including crayfish, into the wild (Patoka et al. 2014b). Hence, the propagule pressure (*sensu* Duggan et al. 2006) is high. As noted by Patoka et al. (2015b), marbled crayfish originate almost exclusively from domestic production in quantities estimated to be as high as 100,000 individuals annually in the Czech Republic. In comparison with recorded retail prices of other traded crayfish species (€ 2.0 to 25.92), the marbled crayfish is very inexpensive and thus widely accessible; the lowest recorded price was € 0.55 per individual (Patoka et al. 2015b). In addition to its low price, the species is popular, is easy to keep and breed, reproduces rapidly and asexually, and has a short generation time (Faulkes 2015). These facts support the assumption that the discovered population originated due to intentional release from aquaria.

Chucholl et al. (2012) and Chucholl (2014) have predicted that the number of established populations within European territory will further increase due to release by hobbyists of surplus and unwanted crayfish at new sites near conurbations. This suggestion is supported by our both findings from Prague and the vicinity of Bílina, together with other recent records of marbled crayfish occurrence, such as in Budapest, Hungary (Weiperth et al. 2015), and Dnepropetrovsk and Odessa, Ukraine (Novitsky & Son 2016). Populations established in conurbations may serve as the source for further, spontaneous spread. The North American NICS have been evaluated as more dangerous for native European astacofauna than traded crayfish species originating from elsewhere in the world (Patoka et al. 2014a). In agreement with other authors (Peay 2009; Magalhães & Andrade 2014), we recommend further

education of the general public and sharing of information which will improve knowledge as to how dangerous are NICS and how important is conservation of indigenous crayfish species. As both discovered Czech populations of marbled crayfish are in isolated water bodies, their eradication before the further expansion of crayfish should be attempted.

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SHORT COMMUNICATION OPEN ACCESS

Slovak section of the Danube has its well-established breeding ground of marbled crayfish *Procambarus fallax* f. *virginalis*

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Abstract – Established populations of the non-indigenous parthenogenetically reproducing marbled crayfish *Procambarus fallax* f. *virginalis* have been recently reported from various European countries. The colonised sites are usually lentic and relatively isolated from major watercourses and in such cases the immediate threat of the spread of this taxon is limited. Here we report on a marbled crayfish population that is likely to become a seed for colonisation of the Danube in Slovakia. It is located in a channel within the Slovak capital Bratislava in the immediate vicinity of a pumping station that occasionally releases significant amounts of water into the side arm of the Danube. The population is well established with a high growth potential: numerous adult marbled crayfish individuals were observed at the site in September and October 2016 and the progeny (eggs or first two developmental stages) of 27 berried females exceeded 11 000 individuals. The maximum observed fecundity per female reached 647 juveniles in the second developmental stage. The Danube side arm downstream of the pumping station harbours a population of spiny-cheek crayfish *Orconectes limosus* infected with the crayfish plague pathogen *Aphanomyces astaci*. We presume that marbled crayfish is already present below the pumping station and it is just a matter of effort and time until it is discovered. The investigated specimens of marbled crayfish were found free of *A. astaci*, but horizontal transmission from infected spiny-cheek crayfish may be expected, as well as further spread of marbled crayfish in the Danube.

Keywords: pet trade / aquatic invasion / fecundity / asexual reproduction / Slovakia

Résumé – Une portion slovaque du Danube est un site de reproduction bien établie d'écrevisse marbrée *Procambarus fallax* f.*virginalis*. Des populations établies d'écrevisses marbrées non-indigènes à reproduction parthénogénétique *Procambarus fallax* f. *virginalis* ont récemment été signalées dans différents pays européens. Les sites colonisés sont généralement lentiques et relativement isolés des grands cours d'eau et, dans de tels cas, la menace immédiate de propagation de ce taxon est limitée. Nous rapportons ici sur une population d'écrevisses marbrées qui risque de devenir une source pour la colonisation du Danube en Slovaquie. Elle est localisée dans un canal situé dans la capitale slovaque Bratislava, à proximité immédiate d'une station de pompage qui libère occasionnellement d'importantes quantités d'eau dans le bras latéral du Danube. La population est bien établie avec un fort potentiel de croissance: de nombreux adultes d'écrevisse marbrée ont été observés sur le site en septembre et octobre 2016 et la progéniture (œufs ou deux premiers stades de développement) de 27 femelles grainées dépasse 11 000 individus. La fécondité maximale observée par femelle a atteint 647 juvéniles au deuxième stade de développement. Le bras latéral du Danube en aval de la station de pompage abrite une population d'écrevisses américaines *Orconectes limosus* infectées par l'agent de la peste de l'écrevisse *Aphanomyces astaci*. Nous supposons que les écrevisses marbrées sont déjà présentes au-dessous de la station de pompage et c'est juste une question de prospection et de temps jusqu'à ce qu'elles soient découvertes. Les spécimens étudiés d'écrevisses marbrées ont été trouvés exempts d'*A. astaci*, mais on peut s'attendre à une

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transmission horizontale à partir d'écrevisses infectées et à une propagation accrue des écrevisses marbrées dans le Danube.

Mots-clés : commerce d'animaux de compagnie / invasion aquatique / fécondité / reproduction asexuée / Slovaquie

1 Introduction

Biological invasions have devastating consequences on the native biota, which is particularly apparent in freshwater ecosystems (Richman *et al.*, 2015). Introduced non-indigenous crayfish species affect the invaded biotopes, with negative community-level impacts (Moorhouse *et al.*, 2014; Roukonen *et al.*, 2016). Among alien crayfish, the marbled crayfish *Procambarus fallax* f. *virginalis* is an emerging threat, particularly in Europe. It is the only known crayfish reproducing via obligate apomictic parthenogenesis, producing genetically uniform offspring (Martin *et al.*, 2010). This species is characterised by early maturation (Seitz *et al.*, 2005), reproduces throughout the whole year under favourable conditions (Vogt *et al.*, 2004; Seitz *et al.*, 2005), and its high competitiveness for food and shelters has been documented (Jimenez and Faulkes, 2011). Its survival under low temperatures was proven both in the laboratory and the field (Veselý *et al.*, 2015; Lipták *et al.*, 2016). The marbled crayfish was first discovered in the German aquarium trade in the mid-1990s, from where it further dispersed (Scholtz *et al.*, 2003). Its availability at the pet markets is usually high (*e.g.* Kotovska *et al.*, 2016; Vodovsky *et al.*, 2017). At the beginning of the new millennium, reports on occurrence of single specimens from the wild appeared, followed by confirmation of established populations in Germany and Slovakia in 2010; since then, the number of invaded European countries has steadily increased (see Patoka *et al.*, 2016 and references cited therein), and the ability of marbled crayfish to carry the crayfish plague pathogen has been confirmed both in aquarium trade (Mrugała *et al.*, 2015) and in the field (Keller *et al.*, 2014). Due to all these characteristics, the marbled crayfish became listed among the invasive alien species of European Union concern according to recent legislation (EU Regulation No. 1143/2014 and Commission Implementing Regulation No. 2016/1141). Here we report an established marbled crayfish population in Bratislava, Slovakia, which has presumably initiated the colonisation of the Danube.

2 Material and methods

The marbled crayfish was discovered by a chance during research focused on the ecology of another alien species, the yellow-bellied slider *Trachemys scripta scripta* and the red-eared slider *T. s. elegans*, both native to North America. Two marbled crayfish were caught in turtle traps on August 25, 2016, in front of the pumping station in the Chorvátske rameno in Bratislava. Chorvátske rameno is a dead-end artificial canal within the town district Petržalka, which ends at a pumping station (48.0996 N, 17.1306 E) next to a side arm of the Danube (Jarovecké rameno) directly connected to the river (Fig. 1A, B). The canal is approx. 5 km long and 20 m wide with a depth of 2–3 m in its centre. Submerged macrophytes are present in some sections of the canal, and its banks are usually lined with emergent macrophytes. The canal bed is formed by fine gravel mixed with organic detritus.

Two installed pumps at the station in Chorvátske rameno have a capacity of 260 $l \cdot s^{-1}$ and are activated mainly during elevated flow rates (floods) in the Danube and during extensive rainfalls in the area in order to regulate ground waters in this highly populated town district. They are also occasionally activated when being checked for functionality. The pumping activity will transfer any biota in the immediate vicinity of the station into the side arm of the Danube, with no further barriers to dispersal to the river itself.

After accidental finding of marbled crayfish, two additional field samplings followed, the first on September 11, and the second on October 24, 2016. Both samplings focused on the areas just above and below the pumping station, *i.e.*, places where the presumed chance of successful capture of crayfish was highest. The first survey of the Chorvátske rameno canal was performed by a single researcher, who explored 2 m long stony section of the shore for 30 min. The second survey was performed by three researchers on a 10 m stretch. The sampling lasted for 40 min. Thanks to the high abundance of the marbled crayfish and easy access to the site, no crayfish trapping was needed. The Jarovecké rameno side arm is stabilised by heavy stones forming several layers. Thus manual search (ineffective in such conditions) was combined with trapping, using six baited traps exposed overnight during the first survey and 25 traps in the second survey.

Carapace length of sampled crayfish was measured to the nearest 0.1 mm. The eggs and juveniles in the first two developmental stages were counted if present. Juveniles in the third developmental stage become gradually independent and their quantification would be inaccurate. All captured crayfish individuals were preserved in 96% ethanol. Screening of the presence of the crayfish plague pathogen *Aphanomyces astaci*, using the quantitative PCR-based methods of Vrålstad *et al.* (2009), was conducted on all adult crayfish captured at both investigated sites (Chorvátske rameno and Jarovecké rameno). Details of the laboratory protocols are described in Mrugała *et al.* (2015) and Lipták *et al.* (2016).

3 Results

During the two field sampling events, altogether 39 adult marbled crayfish (11 + 28 females) and 9 spiny-cheek crayfish *Orconectes limosus* (7 + 2 individuals of both sexes) were captured. All marbled crayfish were caught above the pumping station in the Chorvátske rameno canal, while all spiny-cheek crayfish individuals were caught into traps below the pumping station in the Jarovecké rameno side arm (Fig. 1B).

The carapace length (totalling *ca.* 50% of the body size) of marbled crayfish specimens ranged from 21.8 to 48.1 mm, with a mean of 39.2 mm (Fig. 1C). In total, 27 marbled crayfish (69% of the catch) carried eggs or juveniles. The quantity of the offspring ranged between 147 and 647 (on average 420) eggs or juveniles per female, with a positive correlation with

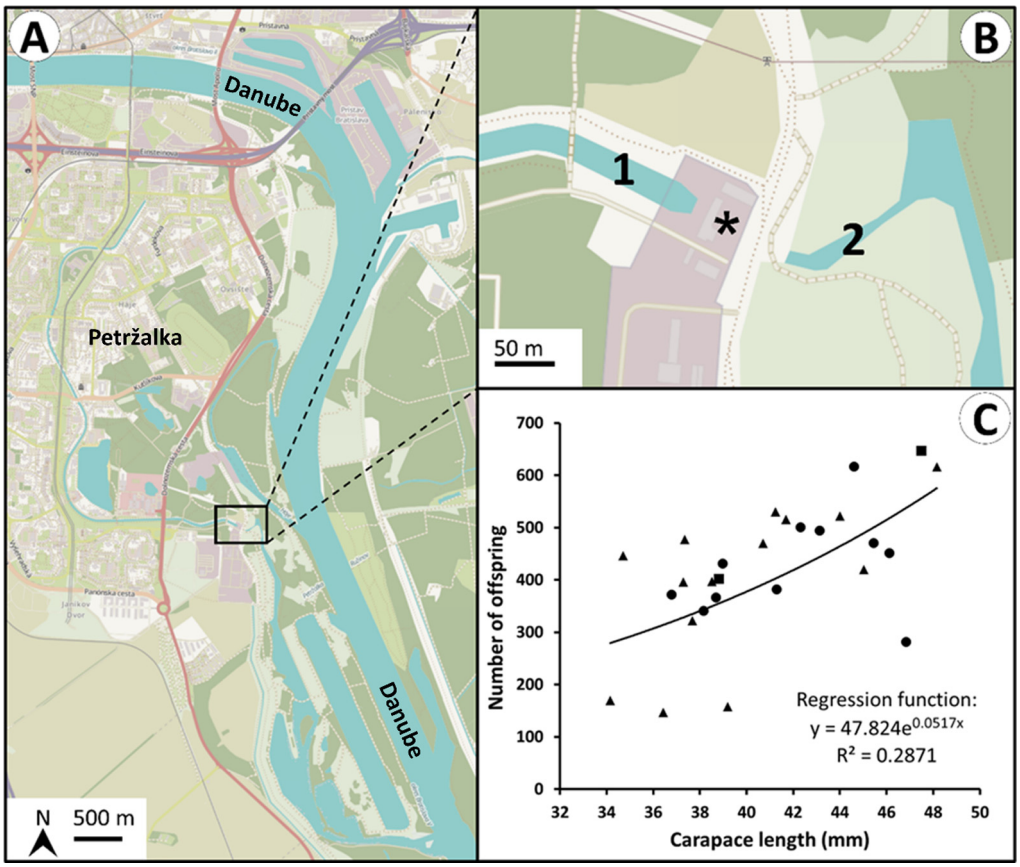

Fig. 1. Map showing the marbled crayfish *Procambarus fallax* f. *virginalis* occurrence in Bratislava, Slovakia – general view (A) and detailed location (B). Asterisk refers to the pumping station, while 1 and 2 to the locations in the Chorvátske rameno canal and the Jarovecke rameno side arm where marbled crayfish and spiny-cheek crayfish *Orconectes limosus* were found, respectively. Fecundity of marbled crayfish females (C) expressed as the number of eggs (circles), stage 1 (triangles) and stage 2 juveniles (squares), respectively, and the relationship between female carapace size and the number of offspring (exponential regression, with all three age categories pooled). The basis for the maps is available under the Open Database License (www.openstreetmap.org).

the size of the mother (Fig. 1C). Altogether, the 27 captured berried females carried 11 348 offspring. No trace of *A. astaci* DNA was detected in any analysed marbled crayfish.

Of the spiny-cheek crayfish (6 males, 3 females, carapace length 25.0–52.1 mm, mean 42.6 mm), one specimen was confirmed as being infected with *A. astaci* (agent level A3, according to the method of Vrålstad *et al.*, 2009).

4 Discussion

Due to irresponsible or uninformed hobby breeders, marbled crayfish are intentionally released into the wild and become established, as documented across Europe (Chucholl *et al.*, 2012; Kouba *et al.*, 2014). Most of the sites with well-documented established populations are lentic habitats relatively isolated from the main watercourses. However, records from some sizeable rivers (*e.g.* the Rhine in Germany or the Po delta in Italy) were also reported, although their recent population status remains unclear (Chucholl *et al.*, 2012; Vojkovská *et al.*, 2014; Patoka *et al.*, 2016 and literature cited therein). Weiperth *et al.* (2015) refer to several specimens of various sizes detected in thermal ponds and their outflows including adjacent Danube in Budapest, Hungary. Evaluation of the population status in the river is an issue of on going research (Weiperth A., pers. comm., 2017).

The newest discovered site with the marbled crayfish in Bratislava, Slovakia, also occurs in the immediate vicinity of the Danube, separated only by a pumping station that occasionally releases its waters to one of the river arms. This section of the Danube is already colonised by the non-indigenous spiny-cheek crayfish which invaded this river section in the last two decades and, recently, also by signal crayfish *Pacifastacus leniusculus* (Lipták and Vitázková, 2014). We have not confirmed syntopic occurrence of marbled crayfish with these species yet, but we consider that confirmation of marbled crayfish in the side arm of the Danube is just a matter of time and search effort. Water pumping, intentional translocation of marbled crayfish by humans, or active migrations of marbled crayfish, are factors that can transfer (or may have already transferred) the species into the Danube (Chucholl *et al.*, 2012; Lipták *et al.*, 2016).

The conditions in the side arm of the Danube, Jarovecké rameno, are favourable for crayfish, as indicated by the locally present spiny-cheek crayfish population. The documented

presence of *A. astaci* in that species corresponds to its infection status elsewhere in the Danube (Kozubíková *et al.*, 2010; Pârvulescu *et al.*, 2012). Upon contact of marbled crayfish with infected spiny-cheek crayfish, we may expect a horizontal transmission of *A. astaci* between the two host species (see James *et al.*, 2017). This means that thereafter the marbled crayfish expansion in the Danube catchment will be very likely accompanied by the expansion of the crayfish plague pathogen, which causes mass mortalities of indigenous crayfish stocks in Europe (Holdich *et al.*, 2009).

Any attempts to eradicate this marbled crayfish population are likely to be ineffective because of its obligate parthenogenetic reproduction mode, when even a single survivor may re-establish the whole population. Its remarkable reproductive capacity and extremely high fecundity, low-temperature tolerance and high competitiveness (Vodovsky *et al.*, 2017 and literature cited therein) all suggest that the marbled crayfish will become a permanent part of the Danube ecosystem, with great potential for an extension of its range, with largely unknown consequences so far. Some of its life history characteristics (*e.g.* higher fecundity, earlier maturation, supposedly faster growth and more reproduction events per year) provide significant advantages, even compared to other non-indigenous crayfish species already present in this section of the Danube, the spiny-cheek crayfish and the signal crayfish (Lipták and Vitázková, 2014).

To conclude, we expect that marbled crayfish might be already present in a side arm of the Danube, where horizontal infection with crayfish plague pathogen originating from spiny-cheek crayfish will occur. We presume that the marbled crayfish will spread actively further (mainly downstream), but its range extension may be accelerated by the occasional floods where successful reproduction of even single dispersed specimens is not limited. A competition with this new invader might have severe consequences for remaining stocks of the indigenous narrow-clawed crayfish *Astacus leptodactylus*, already under pressure of spiny-cheek crayfish (*cf.* Pârvulescu *et al.*, 2012, 2015). Successful competition of marbled crayfish with other non-indigenous crayfish species already present in the Danube may also be expected. However, given the role of crayfish in ecosystems in general and characteristics of marbled crayfish in particular, the spread of marbled crayfish has the potential for significant consequences for much broader range of taxa. This is a serious issue since the Danube possesses habitats for diverse biota, being a unique ecosystem of European importance. Future monitoring of marbled crayfish in the Danube is warranted, but at early phases of establishment may be methodologically challenging in such large river course. Utilisation of eDNA methods might be an useful tool in this regard.

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Supplement 15

Weiperth, A., Gál, B., Kuříková, P., **Bláha, M.**, Kouba, A., Patoka, J., 2017. *Cambarellus patzcuarensis* in Hungary: The first dwarf crayfish established outside of North America. Biologia 72: 1529-1532.

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Short Communication

Cambarellus patzcuarensis in Hungary: The first dwarf crayfish established outside of North America

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Abstract: In 2017, a new non-indigenous crayfish species was found established in Europe. The captured individuals were identified as an orange morph of the Mexican dwarf crayfish *Cambarellus patzcuarensis* Villalobos, 1943. Fifteen adults (including three ovigerous females) and 26 juveniles were collected in a thermal pond in Budapest, Hungary. Two additional adults were caught below the pond's outflow in the adjacent Danube River. To our knowledge, this is the first record of a *C. patzcuarensis* population outside North America, which is also true for the rest of dwarf crayfish (family Cambaridae, subfamily Cambarellinae). With this finding, indigenous crayfish species in Europe are now more than two-fold outnumbered by non-indigenous species. An analysis of the probability of establishment of *C. patzcuarensis* in continental Europe revealed that specific regions in the south of the continent are suitable areas for the establishment of the species. Moreover, as a confirmed carrier of the crayfish plague pathogen, this species should be treated with caution and eradicated if possible.

Key words: biological invasion; climate match; thermal water; dwarf crayfish

Introduction

The international trade in live ornamental animals is a well-known source of indigenous species worldwide (Padilla & Williams 2004). Contrary to commercial aquaculture where only a limited number of stakeholders possess large quantities of animals, the pet trade is characterised by limited numbers of exotic species kept by many hobbyists and accordingly higher risk of release in multiple locations. This trend was recently debated in relation to crayfish (Chucholl 2013; Faulkes 2015a).

Around 30 crayfish species are available on the market in countries with long history of trade in aquatic animals for the pet industry, such as the USA (Faulkes 2015b), Germany (Chucholl & Wendler 2017), and the Czech Republic (Patoka et al. 2015); certain species have also been detected as trade animals in Greece (Papavlasopoulou et al. 2014), Hungary (Weiperth et al. 2018), Kazakhstan (Uderbayev et al. 2017), the Russian Federation (Vodovsky et al. 2017), Slovakia (Lipták & Vitázková 2015), Turkey (Turkmen & Karadal 2012), and Ukraine (Kotovska et al. 2016). It is obvious that the propagule pressure of species under trade has increased. Moreover, crayfish are kept not only in indoor aquaria but also in garden ponds (Patoka et al. 2014b, 2017), and in outdoor tanks and ponds close to the restaurant which advertise crayfish as a delicacy (Chucholl & Daudey 2008; Perdikaris et al. 2017). It is not surprising that released or escaped crayfish have been consequently recorded in many countries. The majority of crayfish species under trade belong to the North-American cambarids which often established in the wild (e.g., Chucholl & Daudey 2008; Novitsky & Son 2016; Patoka et al. 2016a).

Material and methods

During two field surveys in Budapest, Hungary (May 19 and 30, 2017), crayfish were collected using nine (5+4) plastic bottle traps baited with halibut pellets and cyprinid fish meat and left in the pond for 24 hours.

Captured individuals were preserved for later identification in pure (96%) ethanol, and a single walking leg from four adult individuals was collected for genetic analysis. The initial morphological species identification was confirmed

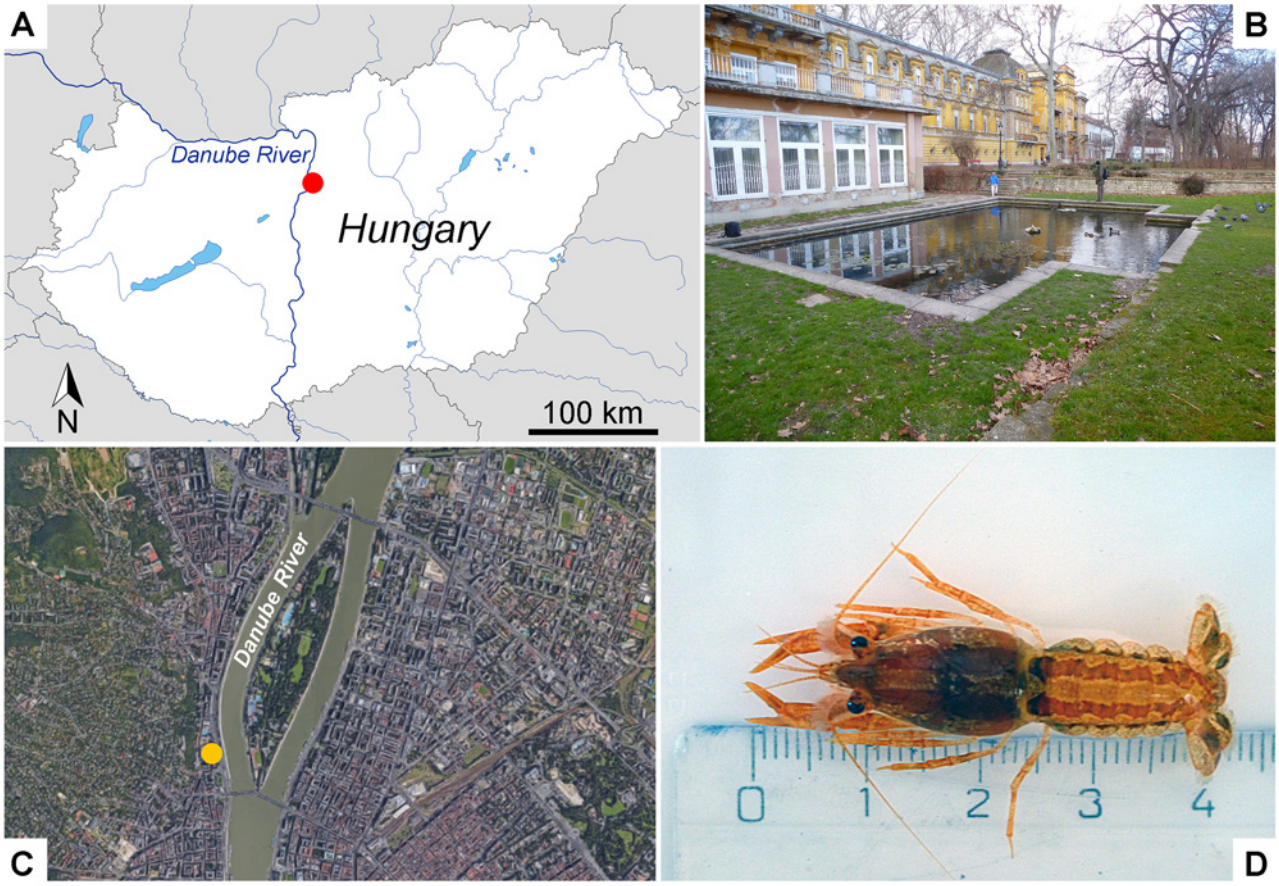

Fig. 1. Map showing the location of the thermal pond in Budapest, Hungary (indicated as coloured dots) (A, C) with the view on the locality (B) and an example of a captured adult *Cambarellus patzcuarensis* female (D).

by a molecular marker amplified by polymerase chain reaction. A primer pair 1471 (5'– CCTGTTTANCAAAAACAT-3') and 1472 (5'AGATAGAAACCAACCTGG-3') was used for amplification of the 16S gene (Crandall & Fitzpatrick 1996). The DNA extraction and amplification was processed according to Patoka et al. (2016b). The samples were sequenced using the Macrogen sequencing service (www.macrogen.com).

The probability of the establishment of captured crayfish throughout the entire European continent was evaluated using the Climatch tool (v.1.0; Invasive Animals Cooperative Research Centre, Bureau of Rural Sciences, Australia, http://data.daff.gov.au:8080/Climatch/climatch.jsp). Climatic conditions were represented by temperature during the coldest quarter of the year in the analysis. The region which is the native geographic range of the evaluated species was used as the source area. The target area was defined as the territory of Europe containing 1117 climatic stations from the database of the WorldClim project (Hijmans et al. 2005). Where the climate match between the source area and the climatic station in the target area reached a score of $\geq$7.0, this was interpreted as there is no environmental barrier to survival in accordance with previous studies (e.g., Kotovska et al. 2016; Patoka et al. 2016b).

Results and discussion

We captured 26 juveniles (2+24, total body length $<$ 9 mm, not sexed) and 15 adults (4+11, carapace length 11–17 mm, total body length 29–38 mm, ten males and five females, three of them ovigerous on May 30) were collected in a thermal pond (Fig. 1; 47°31′3.72″ N, 19°2′16.11″ E). The pond belongs to the complex of the Lukács Thermal Baths and is approximately rectangular in shape, ca. 8 × 14 m. The water temperature in the pond fluctuates from 31 to 37 ℃ during the year. The second survey was associated with monitoring a 400 m long section of shoreline of the adjacent Danube River (47°31′6.30″ N, 19°2′21.93″ E), which resulted in two adult males caught close to the mouth of the outflow. Subsequently, the species was identified as an orange morph of the Mexican dwarf crayfish *Cambarellus patzcuarensis* Villalobos, 1943 (Fig. 1). We identified one haplotype, which was a match with already known and available haplotypes in GenBank (Accession Numbers MF449471, MF449472, MF449473 and MF449474).

This is the first record of the species established as an outdoor population outside North America, which is also true for the rest of dwarf crayfish (subfamily Cambarellinae). *Cambarellus patzcuarensis* is an endangered endemic species having only a restricted native range in Mexico (Pedraza-Lara et al. 2012; Faulkes 2015b). Based on available information, we consider the population in the thermal pond established. In light of this finding, the indigenous crayfish species in Europe are now outnumbered by non-indigenous species more than two-fold (cf. Holdich et al. 2009).

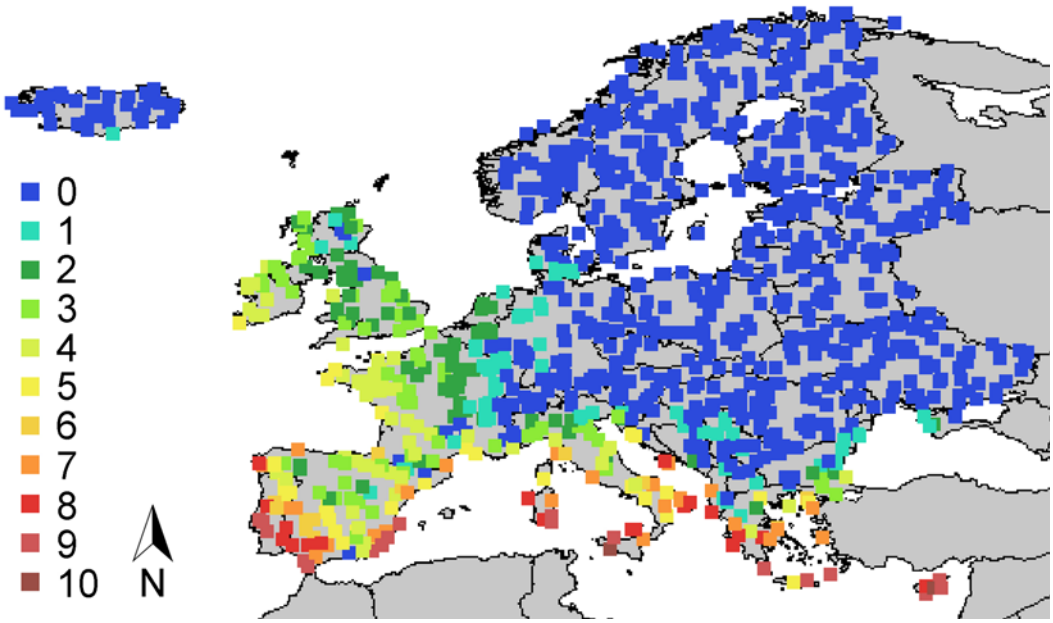

Fig. 2. Climate match map of Europe showing colour-coded regions with a different probability of establishment of *Cambarellus patzcuarensis*; scores of ≥ 7.0 were interpreted as there is no environmental barrier to survival.

Although the availability of this crayfish species in the Hungarian market was previously assessed as "rare" (species available occasionally in small quantities; Weiperth et al. 2018), this colour morph also called "CPO" is a very attractive and popular strain among hobby keepers (Patoka et al. 2014b; Faulkes 2015b; Chucholl & Wendler 2017). Because this species is not exploited in commercial aquaculture due to its tiny size (adult total body length is ca. 3.5 cm), we assume that it was intentionally released from aquaria. Since the climate matching for *C. patzcuarensis* in this region was low (Weiperth et al. 2018), its extensive spread outside the thermal pond is not expected. On the other hand, the overwintering ability of several ornamental crayfish initially considered to be "warm-water" species, has been also proved (Veselý et al. 2015).

Climate matching of native range of *C. patzcuarensis* and target area of Europe shows that the score of ≥7 was reached in 65 meteorological stations. All of these stations were located in the southern Europe, with the highest probabilities to establish wild populations predicted for Greece, Italy, Portugal and Spain (Fig. 2). Moreover, there are various examples of the occurrence of non-indigenous crayfish species in thermal waters in regions where climatic conditions are unsuitable (von Petutschnig et al. 2008; Jaklič & Vrezec 2011).

Similar to other crayfish of North-American origin, *C. patzcuarensis* can serve as a vector of the crayfish plague pathogen, an oomycete *Aphanomyces astaci* Schikora, which is a fatal disease for all crayfish species not originating from the North American continent (Mrugała et al. 2015; Svoboda et al. 2017). *Cambarellus patzcuarensis* is, compared to the Hungarian trade, more frequently available in the market of freshwater ornamental animals in other countries: e.g. in USA (Faulkes 2015b), Germany (Chucholl & Wendler 2017), the Czech Republic (Patoka et al. 2014a), and Ukraine (Kotovska et al. 2016). Moreover, the abundance of *C. patzcuarensis* in aquaria may increase in the future because it is usually proposed by pet shop owners to replace recently banned, and previously the most traded and kept crayfish *Procambarus clarkii* (Girard, 1852) and *P. fallax* f. *virginalis* Martin et al., 2010 in European Union (Regulation No. 1143/2014). Even if the bright orange colouration disadvantages this morph in the wild due the higher visibility to predators (Faulkes 2015b), the risk of crayfish plague exists. Since there are no available data on the crayfish pet trade in most regions of southern Europe, we propose this species to the attention of conservationists, wildlife managers and policymakers of European countries. We also recommend further surveys of the aquaria pet market and conducting a risk assessment of invasiveness based on this finding.

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