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Phylogeny of the genus *Caledonica* (Coleoptera: Cicindelidae)

Master thesis

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Master thesis

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Anotace

Phylogeny of the New Caledonian tiger beetle genus *Caledonica* is reconstructed using three phylogenetic trees based on one mitochondrial (COI), one nuclear (CAD) gene and their combination. Sequences were obtained from recent samples collected by the author in situ. Brief description of New Caledonian habitats, geography and geological history are presented and results are discussed. *Caledonica viridicollis laevioricollis* is synonymized with *C. rubicondosa*. Other synonymizations and splitting of variable species are prospective.

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Arnošt Kudrna

Phylogeny of the genus *Caledonica* (Coleoptera: Cicindelinae)

Abstract. Molecular phylogenetic relationships among members of the New Caledonian tiger beetle genus *Caledonica* were studied using COI mitochondrial, and CAD nuclear gene sequences. We demonstrate the valid species status of thirteen previously only morphologically determined species. One species (*C. viridicollis laevioricollis*) is synonymised with *C. rubicondosa* according to new morphological and molecular data. Both phylogenetic and haplotype network analyses available for the variable species *Caledonica mniszechii* suggest its splitting into two species. The biology of the species is given along with a brief description of New Caledonian biogeography and vegetation types.

Key words. Coleoptera, Cicindelinae, *Caledonica*, phylogeny

INTRODUCTION

The archipelago of New Caledonia, although small in size, is frequently referred to as an important biodiversity hotspot. Both the fauna and flora of New Caledonia are characterized by high endemism. For example, the species endemism rate in plants reaches 74% (with 108 endemic genera and 5 endemic families) (Jaffré *et al.* 1998), and 86% in reptiles, where 11 out of 23 genera are endemic (Bauer *et al.* 1993).

Concerning insects, about 4000 species were known in 1993 (Chazeau 1993) and many new species are described every year. Surveys of various specific groups of New Caledonian insects also indicate a very high rate of endemic species. For example, studies of darkling beetles (Coleoptera: Tenebrionidae) (Kaszab 1982, 1986) showed that 215 species out of 234 are endemic. In the family Tingidae (Heteroptera) there are 33 endemic species out of 41 present (Murienne *et al*. 2009). It is to be assumed that similar high endemism rate occurs in many other genera and families of insects in New Caledonia.

Four expeditions were organized to collect data and samples of local tiger beetles, and resulted in morphological revision of the most diverse New Caledonian tiger beetle genus *Caledonica* Chaudoir, 1860 (Kudrna 2016). This study was the most recent and most complete morphological revision of the genus, but it lacked any kind of molecular analysis.

The present study focuses primarily on molecular relationships within the genus *Caledonica*, and together with the previous morphological revision, summarises all available information on its phylogeny.

Geography of the archipelago

The New Caledonian archipelago belongs to the Melanesian region of the southwestern Pacific and is located about 1200 km east of Queensland, Australia, with a total area exceeding 18 000 km^2. It primarily consists of a main island, Loyalty Islands, Ile des Pins and Belep. The main island, called Grande Terre, is cigar shaped and runs in a northwest-southeast orientation. It is about 400 km long and 50–70 km wide with an area of 16372 km^2 (Turner 2008). The central mountain range divides nearly the entire island longitudinally into the wet windward eastern side and the much drier leeward west coast. Mont Panié is the highest peak (1628 m) of this range and also of all the archipelago.

The Loyalty Islands, lying about 100 km east of Grande Terre, are of limestone coral origin with three main (Maré, Lifou and Ouvéa) and few smaller islands and islets. The southernmost part of New Caledonia is composed of the small Isle of Pines (Île des Pins) that is only 152 km^2, but is very rich in fauna and flora.

Belep islands with two principal islands–Art Island and Pott Island – are located to the north of the main island. The New Caledonian archipelago also contains hundreds of other larger or smaller islands, including the very remote Chesterfield islands located 550 km northwest, or Matthew or Hunter Islands located 300 km east of Grande Terre.

Geological history of New Caledonia

Except the main island, the other islands of New Caledonian archipelago are of recent volcanic origin. These original volcanoes were progressively surrounded by coral reefs and when they sunk into the ocean, coral rings remained.

On the contrary, Grande Terre is much older, of Gondwanan origin. However, it is not simply a fragment of the Gondwana supercontinent, but also a mosaic of different terranes that were accreted to Gondwana in the Late Jurassic/Early Cretaceous as arc-associated material which accumulated in the pre-Pacific Ocean (Heads 2008). The eastern part of Gondwana´s continental crust (including New Caledonia) broke and rifted in a north-eastern direction about 80 million years ago forming Zealandia. Zealandia is a mostly submerged continent, with only 6% of its surface above sea level, comprising New Zealand, New Caledonia, the Lord Howe island group, Norfolk Island and Elizabeth and Middleton reefs.

In the past, the biota of New Caledonia was generally regarded as predominantly of Gondwanian origin. However, some more recent studies doubt this paradigm and their authors (Pole 1994, Pelletier 2006) have accepted the theory that New Caledonia (and also New Zealand) was completely submerged during some period, and that their biota is of more recent origin and is likely a result of long-distance dispersal. Thus, all this biota colonised the island after its emergence about 37 Mya with subsequent radiation and adaptation (Grandcolas *et al.* 2008). Another hypothesis doubts this complete submergence, believing in the existence of at least small volcanic islands that emerged in certain times and places, providing microrefugia (Lowry 1998, Morat *et. al* 1983, Heads 2011).

Habitats and vegetation of New Caledonia

New Caledonia, despite its small size, exhibits three distinct habitatforms with very different conditions:

Evergreen rain forest (Fig. 1). Mostly located on the eastern windward coast with the highest precipitation (up to 3000 mm) and in the central mountain range.

Fig. 1.Evergreen rain forest on the eastern coast in the vicinity of Kanala.

Originally, about two thirds of the archipelago was covered with this rain forest, however human activities (logging, mining, settlement, agriculture or intentional fires) have reduced it to one third of its original area. Evergreen rain forest is the richest biotope type.

Fig. 2.Dry forest remnant in Ouen-Toro, Noumea.

Dry sclerophyllous forest (Fig. 2). This vegetation type originally covered most of the lowland on the leeward west coast, where the average precipitation is about 1000 mm per year. Currently this is the most endangered vegetation type, with only about 2% (totally 10000 ha) of its original area remaining, and this reduced into small fragments (Bouchet *et al.* 1994).

Macchia (*maquis*). This dry vegetation type (Fig. 3), usually associated with ultramafic soils, dominates the southern area of Grande Terre. With the boom of mining, this macchia (often referred to as *maquis minier*) expanded to many areas of the main island, originally covered with forest. The ultramafic obligate flora is remarkable for its extremely high endemism, reaching nearly 97% (Isnard *et al.* 2016).

Fig. 3.Macchia in the south of Grande Terre, Port Boise.

One third of the archipelago´s area (6000 square kilometres) is occupied by savannah, often dominated by niaouli tree (*Melaleuca quinquenervia*) which, due to its multiple bark layers, is able to survive repeated fires. This savannah is not an original biotope, but a result of human activity.

Synonymization of *Caledonica viridicollis laevioricollis*

Caledonica viridicollis laevioricollis was originally described as a subspecies of *C. rivalieri* (Deuve 2006). The description was based on two specimens, male (holotype) and female (paratype), from Mandjelia. In the same publication Deuve also described *C. viridicollis rubicondosa*, based on seven female specimens.

According to Deuve (2006), both taxa are morphologically very similar, however as both occur together in the same locality (Mandjelia), he decided to describe each as a

subspecies under a different species, as he accepted the idea that two subspecies of one species should not occur together. Later on, he raised the rank of *C. viridicollis rubicondosa* to the species level and reconsidered *C. laevioricollis* a subspecies of *C. viridicollis* (Deuve 2015). In the same study he stated that the male holotype is the only known specimen of the taxon, as the female paratype belongs to an "other, not yet described species, morphologically close to *C. rivalieri*".

During his last field trip to New Caledonia, the author discovered a large population of *C. rubicondosa* in the forest of Haut Coulna situated in the central mountain chain, only about 20 kilometres from the type locality. This population proved to be key for clarification of the two above mentioned taxa. While females show their distinct and enlarged orange maculation, males possess much smaller maculation of ivory coloration. Such distinct sexual dimorphism is unique within the genus *Caledonica*. Subsequent morphological study confirmed that the holotypes of *C. viridicollis laevioricollis* (male) and *C. rubicondosa* (female) are just opposite sexes of the same species. This opinion is confirmed by molecular evidence presented in this study. Therefore, *C. rivalieri laevioricollis* Deuve, 2006 **syn. nov.** is hereby synonymized with *C. rubicondosa* Deuve, 2006, which is confirmed as a distinct species following the First reviser principle (ICZN 1999). According to the International Code of Zoological Nomenclature (ICZN 1999), the existence of the female paratype is irrelevant and has no effect on this act.

Remarks: This thesis is not issued for public and permanent scientific record, thus the taxonomic act of synonymization proposed here must not be considered as validly published in the sense of the International Code of Zoological Nomenclature.

This act of synonymization is placed already here and is not included into the "results" chapter to adjust the systematics of the genus properly for all the thesis.

Tiger beetles of New Caledonia

Currently, the tiger beetle fauna of New Caledonia comprises 24 species belonging to five genera. Two of them are non-endemic- *Myriochila* Motschulsky, 1862 and *Oceanella* Rivalier, 1963, each represented in the archipelago by a single species. *Myriochila (Myriochila) semicincta* (Brullé, 1834) has a wide distribution in the Pacific area from southern Australia in the south up to central Indonesia in the north (Wiesner 1994). *Oceanella vitiensis* (Blanchard, 1853) is the only member of its monotypic genus, previously considered to be restricted to Fiji and the Samoa Islands (Cassola 2009), but recently (Deuve 2015) also discovered on the eastern coast of main island of New Caledonia (Grande Terre).

Adults of both species are ground-dwelling and prefer areas of bare soil, paths or sandy beaches.

The remaining three tiger beetle genera are endemic- *Vata* Fauvel, 1903, *Manautea* Deuve, 2006 and *Caledonica* Chaudoir, 1860. The genus *Vata* has two known species: *Vata gracilipalpis* W. Horn, 1909 and *V. thomsoni* (Perroud, 1864). Nothing is known about their bionomy or behaviour, but adults seem to be nocturnal and attached to fresh water, as all specimens discovered by the author were collected using light traps placed close to small brooks or rivers. The recently discovered genus *Manautea* Deuve, 2006 comprises four species: *M. gracilior* Deuve, 2006, *M. millei* Deuve, 2006, *M. minimior* Deuve, 2006 and *M. tripotini* Deuve, 2006 (Deuve 2006a, 2006b). All known specimens were accidentally collected in Malaise traps set up in forested areas primarily for Hymenoptera research. Nothing is known about their behaviour or bionomy. The most diverse genus is *Caledonica* with 16 taxa (plates 1 and 2).

List of species of the genus *Caledonica*

The following 16 species are presently known to science:

Caledonica acentra Chaudoir, 1869
C. affinis (Montrouzier, 1860)
C. bavayi Fauvel, 1882
C. fleutiauxi Deuve, 1981
C. longicollis Fauvel, 1903
C. luiggiorum Kudrna, 2016
C. lunigera Chaudoir, 1860
C. mediolineata (Lucas, 1862)
C. mniszechii (Thomson, 1856)
C. myrmidon Fauvel, 1882
C. pulchella (Montrouzier, 1860)
C. rivalieri Deuve, 1981
C. rivalieriana Kudrna, 2016
C. rubicondosa Deuve, 2006
C. tuberculata Fauvel, 1882
C. viridicollis Deuve, 1987

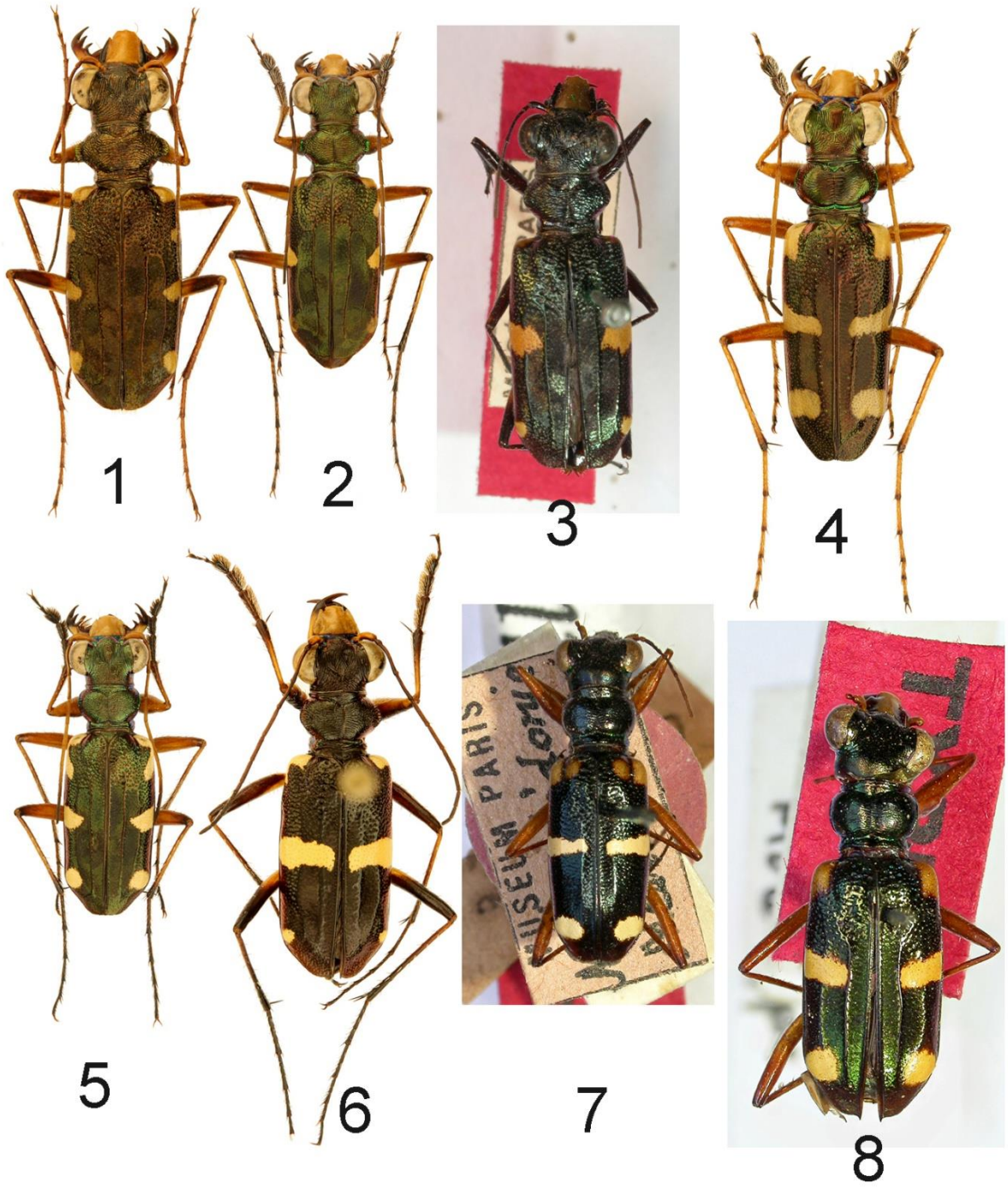

Plate I. Members of the genus *Caledonica*: 1. *C. acentra*, female, 3 km NW Sarramea, 15 mm. 2. *C. bavayi*, male, 15 km NW Koumac, Foret et riviere de Nehoue, 12.5 mm. 3. *C. fleutiauxi*, female, no locality data, 14 mm. 4. *C. affinis*, male, 10 km SE Koumac, 14 mm. 5. *C. lunigera*, male, Paita, Mt.Mou, near Noumea, 11.2 mm. 6. *C. mediolineata*, male, Port Boisé, 13.1 mm. 7. *C. pulchella*, female, no locality data, 11.2 mm. 8. *C. viridicollis*, female, 12.5 mm.

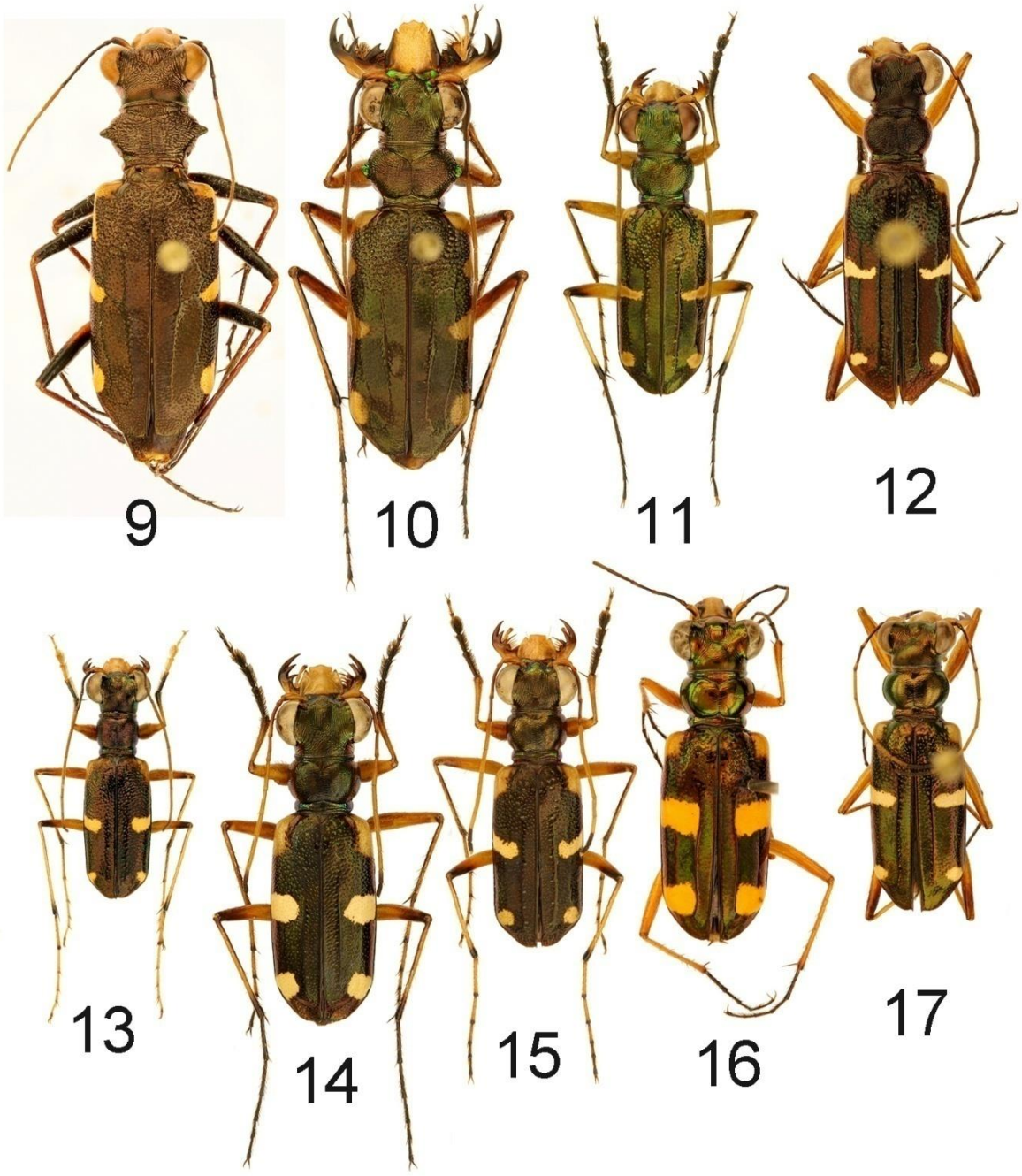

Plate II. Members of the genus *Caledonica*: 9. *C. tuberculata*, female, Ile des Pins, 20.5 mm. 10. *C. mniszechii*, male, 15 km NW Koumac, foret et riviere de Nehoué, 18 mm. 11. *C. rivalieriana*, male, 3 km NW Sarramea, 11.5 mm. 12. *C. rivalieri*, male, Plaine des Lacs, 11.2 mm. 13. *C. longicollis*, male, antenna forest, 2 km S Touho, 9 mm. 14. *C. luiggiorum*, male, 10 km SE Koumac, 9.8 mm. 15. *C. myrmidon*, male, Col de la Pirogue, Mt. Mou, 9.6 mm. 16. *C. rubicondosa*, female, Touho, 11.4 mm. 17. *C. rubicondosa*, male, Mandjelia, 11.5 mm (Holotype of *C. rivalieri laevioricollis*).

Aims of this master thesis

All previous taxonomic revisions of the genus *Caledonica* (Deuve 1981, Kudrna 2016) were based only on morphological characters. This is the first study to use molecular data, using one mitochondrial and one nuclear gene, offering clarification of intrageneric phylogenetic relationships and therefore confirming or disproving monophyly and thus validity of individual species. Moreover, relationships of populations of one of the most widespread species *Caledonica mniszechii* are interpreted using haplotype analysis.

MATERIAL AND METHODS

All *Caledonica* species determinations were made by the author and were based on morphology as described in the last revision of the genus (Kudrna 2016) and on the comparison of the specimens with the type material and the author´s reference collection.

Field work

The fundamental prerequisite for this phylogenetic analysis was availability of a significant number of samples belonging to a maximum number of species, with high quality preservation suitable for molecular analysis. All specimens collected during the author´s three previous expeditions were killed in ethyl acetate and deposited in a fridge or dried in a reference collection over a long period. Therefore their use for molecular analyses was doubtful. The only option to get fresh samples in 96% alcohol was new collecting in situ. For this reason, another expedition to New Caledonia main island was organized from December 2015 to March 2016.

To collect fresh samples, localities discovered during the three previous expeditions were mainly visited. All localities where samples were collected are summarised in Fig. 4. A map with individual species occurrence is given in Fig. 5. If available, several specimens of every species were collected from the maximum number of available localities, to obtain samples with the highest intraspecific variability (Tab.1).

Each individual specimen was deposited in a separate sealable Eppendorf tube with 96% alcohol and with all relevant information, including the date, height above sea level and GPS position.

Fig. 4. A map of New Caledonia with marked localities of the samples used in this study (Map downloaded from www.d-maps.com).

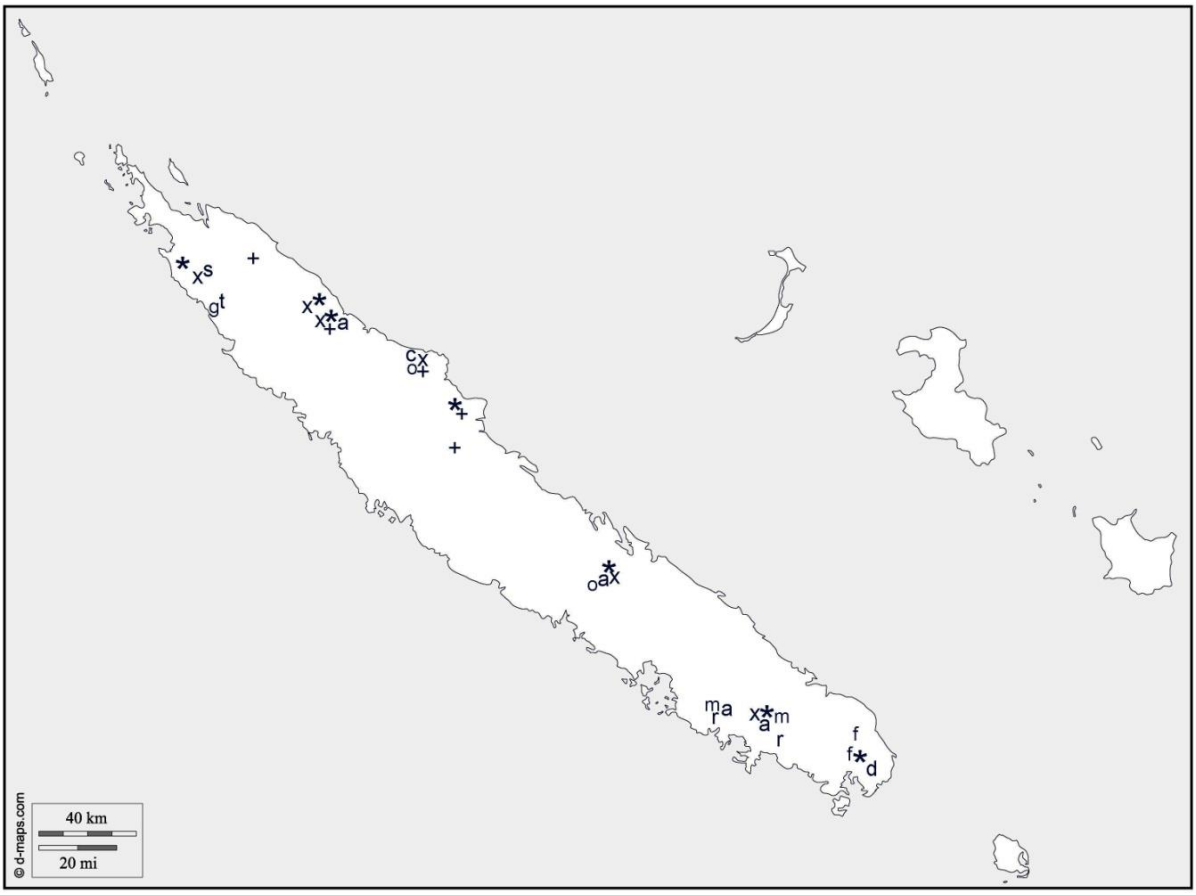

Fig. 5. A map of New Caledonia with marked occurrence of individual species of the genus *Caledonica* used in this study. *C. acentra* a, *C. affinis* s, *C. bavayi* x, *C. fleutiauxi* f, *C. longicollis* c, *C.luiggiorum* g, *C. lunigera* r, *C. mediolineata* d, *C. mniszechii* *, *C. myrmidon* m, *C. rivalieriana* o, *C. rubicondosa* +, *C. tuberculata* t. Map downloaded from www.d-maps.com).

Table 1. Localities visited with collected species of *Caledonica* used in this study. *C. acentra*- ***ace***, *C. affinis*- ***aff***, *C. bavayi*- ***bav***, *C. fleutiauxi*- ***fle***, *C. longicollis*- ***lon***, *C.luiggiorum*- ***lui***, *C. lunigera*- ***lun***, *C. mediolineata*- ***med***, *C. mniszechii*- ***mni***, *C. myrmidon*- ***myr***, *C. rivalieriana*- ***riv***, *C. rubicondosa*- ***rub***, *C. tuberculata*- ***tub***.

	ace	*aff*	*bav*	*fle*	*lon*	*lui*	*lun*	*med*	*mni*	*myr*	*riv*	*rub*	*tub*
Aoupinié												X	
Grand Kaori/Nord				X					X				
Haut Coulna	X		X						X			X	
Karembe						X							X
Koumac		X	X										
La Coulée							X						
Mandjelia												X	
Mont Koghis	X		X						X	X			
Mont Mou	X									X			
Netcha				X									
Ouane Batch			X						X				
Paita, Val Boisé							X						
Plateau de Dogny	X		X						X				
Poindimié									X			X	
Port Boisé								X					
Riviere Nehoué									X				
Sarramea											X		
Touho			X		X						X	X	

Laboratory work

DNA extraction

For easy orientation during all the procedures, every species received a unique two letter abbreviation and each specimen within the species a unique number.

All extractions were made from samples deposited in 96% alcohol one month after return from the expedition. We extracted flight muscle from each specimen, which provides a sufficient amount of tissue with minimum risk of contamination. Flight muscle was obtained by lifting the beetle's elytron and extracting the majority of the flight muscles from one side with sharp forceps. Then, the beetle was returned to the tube with 96% alcohol and deposited in a freezer for potential subsequent use.

The extracted tissue was placed into a 1.5 ml centrifuge tube and DNA extracted using a commercial Genomic DNA Mini kit (Genomed). Using a pipette, 200 µl of GT Buffer and 20 µl of proteinase K was added to the tissue, shaken vigorously and incubated at 60°C for about 12 hours (overnight).

On the second day, lysis was continued by adding 200 µl of GBT Buffer and shaking for several seconds. The mixture was incubated for at least 20 minutes. During this time, the tube was inverted every 5 minutes.

We added 200 µl of 96% alcohol and immediately shook the lysate vigorously for about 10 seconds. The GS Column was placed into a 2 ml collection tube, all the mixture transferred into this GS Column and centrifuged at 14–16000 g for 2 minutes. After this procedure, the mixture was washed: the GS Column was placed into a new Collection tube, 400 µl of W1 Buffer was added to the GS Column and the mixture centrifuged at 14–16000 g for 30 seconds. Then, the GS Column was placed into a new empty Collection tube, 600 µl of Wash Buffer was added and centrifuged at 14–16000 g for 30 seconds. Another three minutes of centrifugation at 14–16000 g were used to dry the column matrix.

For DNA elution, about 25–50 µl of pre-heated (60°C) Elution Buffer was added to the centre of the column matrix. After about 5–10 minutes, when the Elution Buffer was completely absorbed, the purified DNA was transferred into a 1.5 ml Eppendorf tube by centrifugation at 14–16000 g for 30 seconds. Then, the extracted DNA was deposited in a freezer at –20°C.

PCR

For polymerase chain reaction (PCR) amplification, one mitochondrial and one nuclear gene was selected. Cytochrome C oxidase subunit I (COI) was used as the mitochondrial gene, because this is one of the most popular and frequently used markers for genetic studies. The fragment of COI was amplified using primers HybLCO (forward) and HybHCO (reverse) (Tab. 2).

The nuclear gene is CAD–a carbamoylphosphate synthetase. PCR amplifications of CAD followed Maddison (2012) (Tab. 3), but only with the first reaction protocol using CD806F and CD1231R primers. The second reaction with CD1098R2 as the reverse primer was not used.

Table 2. Primers with their sequences used in this study.

GEN	ORIENTACE	NÁZEV	SEKVENCE
COI	reverse	HybHCO	5′-ATTAACCCTCACTAAAGTAAACTTCAGGGTGACCAAAAAATCA-3′
COI	forward	HybLCO	5′-TAATACGACTCACTATAGGGGGTCAACAAATCATAAAGATATTGG-3′
CAD	forward	CD806F	5′-GTNGTNAARATGCCNMGNTGGGA-3′
CAD	reverse	CD1231R	5′-TCCACGTGTTCNGANACNGCCATRCA-3′

Every PCR reaction contained 2 µl of DNA, 12.5 µl of PPP Combi Mastermix Top Bio, 8.5 µl of PCRH_2O, 1 µl of forward primer and 1 µl of reverse primer, making 25 µl in total. Two thermocyclers were used for amplifications: Biometra, type TProfessional standard 96 Gradient and TC-X-D produced by BIOER.

Table 3. PCR thermal cycling conditions for primers used in this study.

COI			
Step	Temperature	Time	Cycles
Initial denaturation	94°C	3 minutes	1
Denaturation	94°C	15 seconds	35
Annealing	50°C	1 minute	
Extension	72°C	1 minute	
Final extension	72°C	2 minutes	1
Hold	10°C	∞	
CAD			
Initial denaturation	94°C	2 minutes 30 seconds	1
Denaturation	94°C	30 seconds	9
Annealing	65°C with touchdown (-0.5°C in every step)	30 seconds	
Extension	72°C	2 minutes	
Denaturation	94°C	30 seconds	35
Annealing	55°C	30 seconds	
Extension	72°C	2 minutes	
Hold	10°C	∞	

Electrophoresis

Agarose gel (1%) electrophoresis was used for verifying the success rate of DNA extraction, PCR reaction and purifications. This agarose gel was prepared by mixing an appropriate amount of agarose powder with 1x TAE in a laboratory boiling flask and microwaving until the agarose powder was completely dissolved. After the solution was cooled to about 45–50°C, SYBR Safe DNA gel stain was added at a volume of 1 µl of SYBR to every 10 millilitres of the gel. This solution was carefully stirred and subsequently poured into a prepared gel tray. All bubbles were pushed away and a well comb was inserted.

After the gel had set, it was immersed into the electrophoresis unit and completely covered with 1x TAE. DNA samples mixed with loading buffer were carefully loaded into the wells of the gel. For identifying the size of the products, a molecular-weight size marker was also loaded into one of the wells in the gel. Typically, the gel was run at 70 volts for 30 minutes. After this procedure, the gel was carefully removed from the electrophoresis unit and DNA fragments (bands) visualised using UV light. If needed, mostly for indicative purposes, the concentration of some of the samples was measured. For this purpose a Qubit 2.0 fluorometer from Thermo Fisher Scientific was used.

Purifying DNA

All samples were purified with a GenepHlow PCR Cleanup Kit (Geneaid) before sending for sequencing, following the original protocol.

The whole reaction product was transferred to a 1.5 ml microcentrifuge tube, 5 volumes of PB Buffer were added to the sample and vortexed. The sample mixture was transferred to a DHF Column placed in a Collection tube and centrifuged at 15000 g for 30 seconds. The DHF Column was then placed in a new Collection tube and the sample was washed by adding 600 µl of Wash Buffer and letting it stand for 1 minute. After this, the sample was centrifuged at 15000 g for 30 seconds, the DHF Column was placed in an empty Collection tube and centrifuged again, this time for 3 minutes to dry the column matrix.

The dried Column matrix was transferred to a new 1.5 ml microcentrifuge tube and 20–30 µl of Elution Buffer or water was added to the centre of the column matrix. After ca. 5 minutes, when the Elution Buffer or water was completely absorbed, the DHF Column with purified DNA was centrifuged at 16000 g for 20 minutes at room temperature. Before adding, for better absorption the used Elution Buffer or water was pre-heated to 70°C.

Sequencing

Products with successful amplifications were sent for sequencing. Amplicons of COI gene were sent to Macrogen laboratory (http://www.macrogen.com/en/main/index.php). For sequencing of CAD, SEQme company was used (https://www.seqme.eu/cs/). Required DNA sections were only sequenced in one direction. The amplified COI locus has ca 700 bp and CAD locus ca 1120 bp.

Phylogenetic analyses

The quality of resulting sequences was controlled using Geneious Prime, version 2019.0.4 and bad ones with percentage of high quality untrimmed bases lower than 50 were excluded. In Geneious, all sequences were manually checked and bad quality regions in the beginning and the end of each sequence removed. Resulting sequences were aligned separately for each region using Muscle v3.8 (Edgar 2004). If there were enough good sequences of the same species, samples with bad sequences were no longer used. If there were small number of specimens of a certain species, or the locality of a specimen with a bad sequence was important, the entire process from PCR to sequencing was repeated. Some sequences were checked by Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to be sure that there was no contamination by other organisms (e.g. fungi).

Three datasets were constructed- one for each gene (COI and CAD) and one with these two datasets combined together to uncover phylogenetic relationships within the genus *Caledonica*. Maximum likelihood analyses were performed using RaxML-HPC2 on XSEDE (version 8. 2.10) (Stamatakis 2014) implemented on the Cipres Science Gateway (Miller *et al.* 2010), with the GTRGAMMA model selected for bootstrapping phase, and with 1000 bootstrapping iterations.

For the construction of the combined (COI+ CAD) phylogenetic tree the maximum likelihood method was performed using IQ-TREE program (Nguyen *et al.* 2015) with GTR model selected and bootstrap with 1000 replicates. The concatenated dataset contained 91 sequences (including three *Myriochila semicincta* sequences as outgroup).

For all phylogenetic trees used in this study, bootstrap values between 70–90 are understood as moderate support, bootstrap values higher than 90 mean strong support.

Results were visualised using the tree figure drawing tool Fig Tree (version 1.4.3.) and final design editing was made using Adobe Photoshop CS5 Extended, version 12.0.

Maximum parsimony haplotype networks based on mitochondrial (COI) and nuclear (CAD) genes were reconstructed to illustrate relationships among haplotypes of *Caledonica mniszechii* using TCS algorithm (Clement *et al*. 2000) in PopArt (Leigh *et al.* 2015). The final length of sequences for PopArt was 566 bp for COI and 756 for CAD.

RESULTS

Habitat preferences and biology of *Caledonica*

Most of the information about the habitat preference and biology of the species in the genus *Caledonica* provided in this chapter was obtained by the author´s personal observations,

based on the author´s recent study (Kudrna 2016), supplemented by other publications (Deuve 1981, 2015).

Tiger beetles of the genus *Caledonica* are predominantly silvicolous, preferring forest habitats, and have diurnal activity. It seems that *C. acentra*, *C. longicollis*, *C. myrmidon*, *C. rivalieri*, *C. rivalieriana* and *C. rubicondosa* are species with a strict forest specialization. The author never observed these species in habitat other than forest, and always inside the vegetation cover.

On the contrary *C. bavayi* and *C. mniszechii* are found not only inside the forest, but also in open woods, along roads and on solitary trees. This could be one of the reasons why these two species are the most commonly observed and commonest in collections. *C. affinis*, belonging to the same group of not strictly forest dwelling species, is rarer in nature, with only a local and patchy distribution.

C. lunigera most often occurs on the drier, western side of the main island and usually on the trees outside the dense forest (solitary trees in gardens, parks, along the roads, etc.). The author has never observed specimens of *C. lunigera* inside forests.

C. tuberculata is a species rather commonly represented in collections by old specimens, however, fresher specimens are nearly entirely absent. Historical localities include places with dry sclerophyllous vegetation (Païta), but also wet forests (Kanala). The only known recent locality is located in the north-west of Grande Terre, south of Koumac town. The habitat is a small remnant of dense woodland, predominantly with young trees in a heavily eroded hilly area. It is necessary to obtain additional data for a better understanding of the biology of this species.

Very little is known about the biology of *C. fleutiauxi*. The species is only known from three type specimens and an additional five recent specimens. The author found a single male just outside the "Foret Nord" forest reserve, and one female in secondary vegetation along a small brook in the vicinity of a little forest remnant and a plantation of Caribbean pines (*Pinus caribaea*) near to Netcha in southern province. Localities of all specimens known to science (Foret Nord, Netcha, Baie de Prony, Pic du Grand Kaori) indicate that this species could be strictly localised in the very southern tip of Grande Terre only, probably on the edges of or inside forest remnants in prevailing macchia vegetation. As in the previous species, additional data are required.

For *C. luiggiorum*, the type locality is the only locality known to science. The habitat is a big, open natural garden with young trees and grassy places situated in the north-western drier part of the island. *C. pulchella* and *C. viridicollis* are both known from single historic

specimens without exact locality and completely unknown biology. *C. mediolineata* is found only rarely. The author observed adults of this species only once, on the trunks of larger trees and shrubs in macchia habitat in the far south of Grande Terre (Port Boisé). The area of their occurrence was limited to a few hundred meters and adults were entirely absent in the neighbouring small forest. According to personal communication with entomologists from IRD (L´Institut de recherche pour le développement) in Noumea, another population of this species was observed in secondary vegetation, mostly of Caribbean pines in the vicinity of forest of Mt. Koghis.

Most of the adult life of specimens of *Caledonica* is confined to tree trunks, usually at a height of one to three meters. They search for straight trunks with smooth bark, and although good fliers, when disturbed, they prefer to repeatedly hide on the opposite side of the trunk. Only when disturbed continuously do they fly to another tree and immediately turn to a head-down position after landing. This is their usual position on the trunks.

Females oviposit into soil, often by the bases of trees or the edges of forest trails. Nothing is known about their larval biology, however, most probably, like most Cicindelidae larvae, they inhabit along narrow burrow and rapidly attack other insects in the close vicinity.

Phylogeny

Genomic DNA was obtained from 99 specimens comprising all 13 species, and also for three specimens of *Myriochila (Myriochila) semicincta*, a tiger beetle used as an outgroup for all trees. Sequences of COI of suitable quality were received for 48 specimens and of CAD for 79 specimens.

Phylogenetic trees

The phylogenetic tree based on COI for 12 *Caledonica* species (data unavailable for *C. luiggiorum*) (Fig. 6) shows strong support for all species except one branch of *C. rubicondosa* without support. One sample of *C. rivalieriana* (PS2) is located on its own branch outside the *C. rivalieriana* group. *Caledonica mniszechii* is divided into two clades, each with strong support (100), but lacking support as sister groups.

The phylogeny of *Caledonica* species based on CAD dataset (Fig. 7) does not have enough support for species delimitation. Out of 13 species only 5 (*C. acentra, C. fleutiauxi, C. longicollis, C. luiggiorum* and *C. mediolineata*) have strong support and one species– *C. rubicondosa* has moderate support. Moreover, *C. myrmidon* is located inside

C. rubicondosa, but without any supported association of *C. myrmidon* with any specific subset of *C. rubicondosa*. In addition, *C. mniszechii* is divided into two clusters, where only one cluster is supported. Sample PS3 is separated from other samples of *C. rivalieriana.*

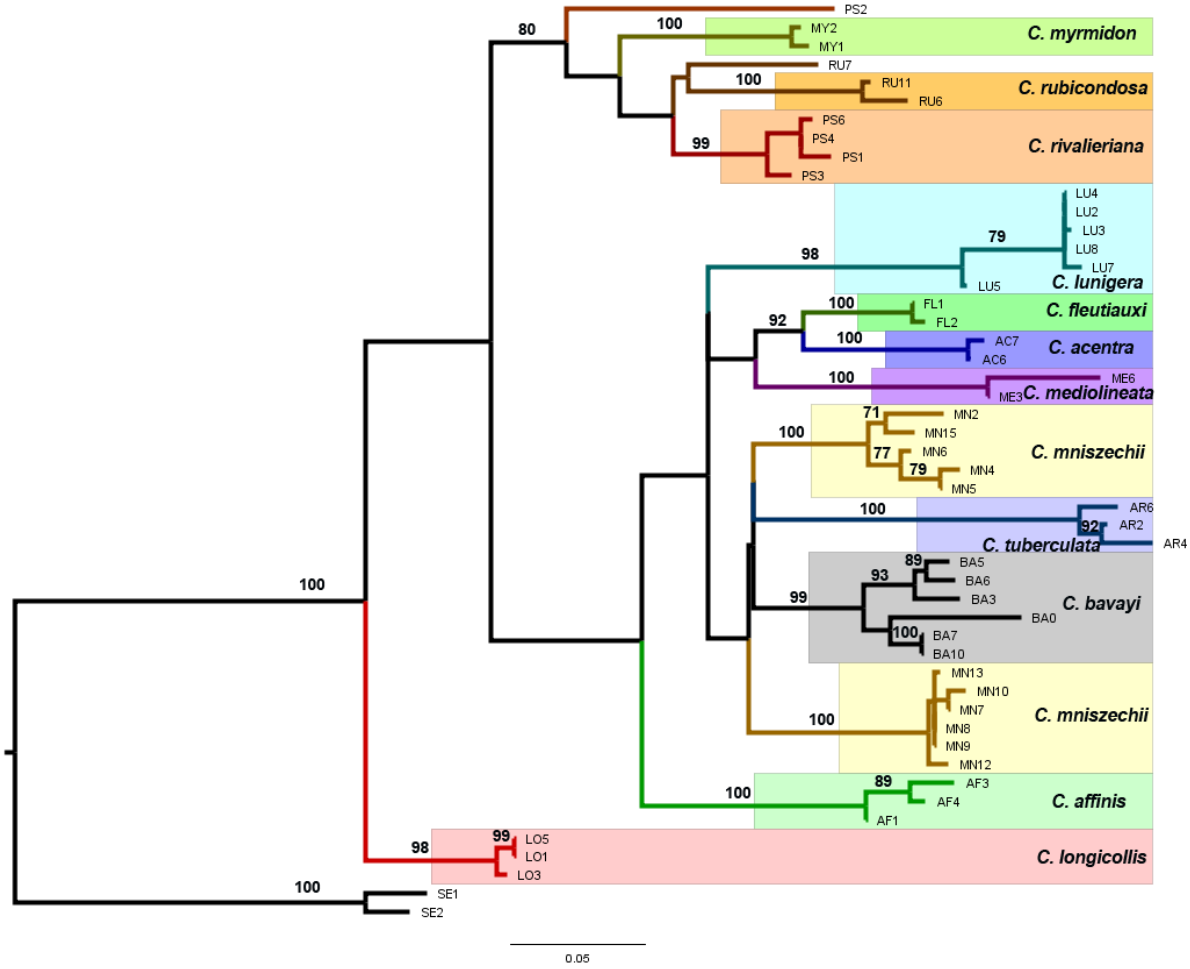

Fig. 6. Phylogeny of *Caledonica* species, reconstructed from the COI dataset as a result of maximum likelihood inference performed with RAxML. For each node, the ML bootstraps (if equal or higher than 70) are presented above the branch leading to that node. All nodes with support lower than 70 are considered as collapsed and therefore the support number is not given. For increased clarity, branches of individual species are coloured, with their names added, and in addition, supported species have a coloured background. 'SE' is the outgroup, *Myriochila semicincta*.

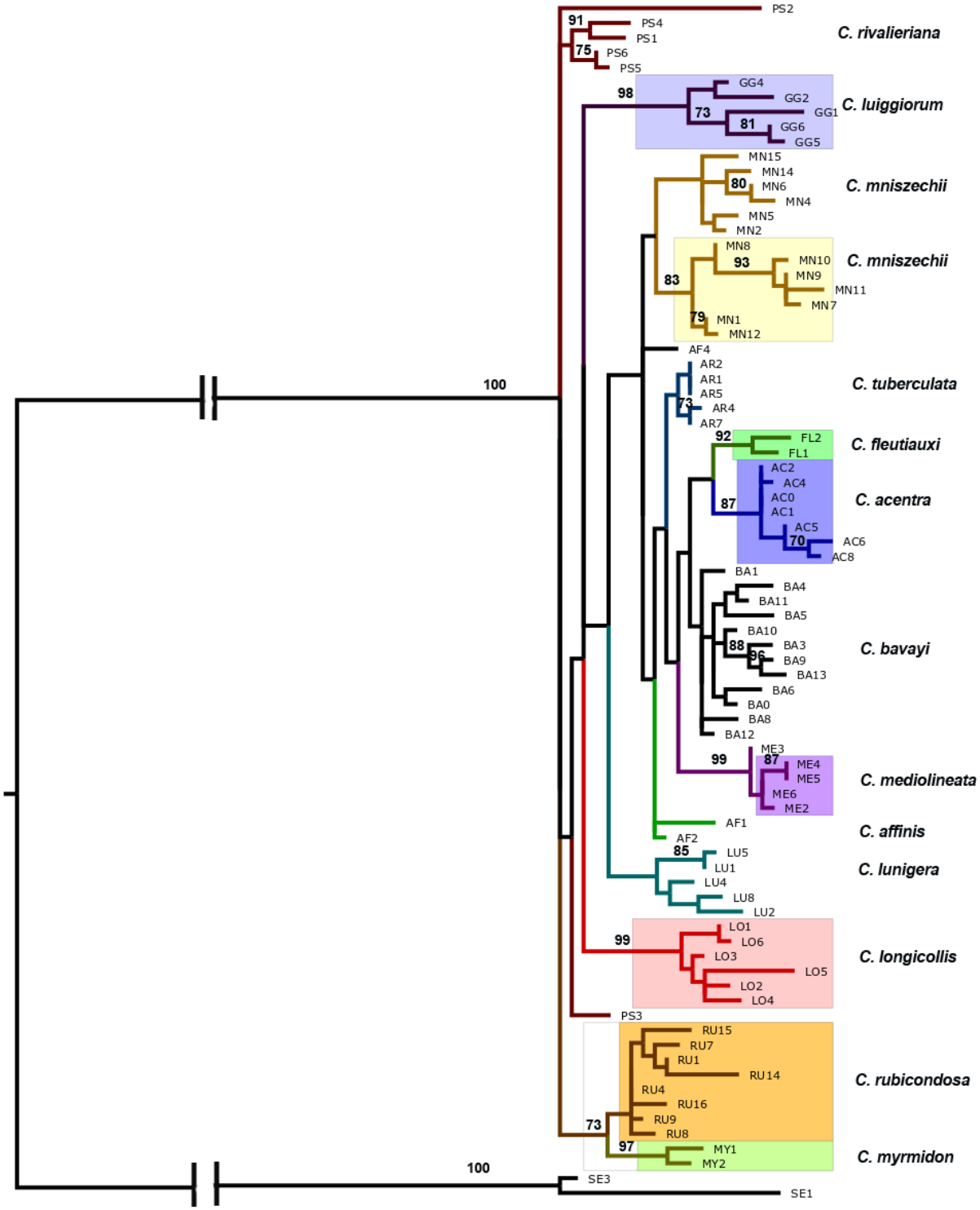

Fig. 7. Phylogeny of *Caledonica* species, reconstructed from the CAD dataset as a result of maximum likelihood inference performed with RAxML. For each node, the ML bootstraps (if equal to or higher than 70) are presented above the branch leading to that node. All nodes with support lower than 70 are considered as collapsed and therefore the support number is not given. For increased clarity, branches of individual species are coloured, with their

names added, and in addition, supported species have a coloured background. 'SE' (*Myriochila semicincta*) and *Cicindela* are used as outgroups.

The combined phylogenetic tree of *Caledonica* (Fig. 8) reveals strong support for *Caledonica acentra, C. affinis, C. fleutiauxi, C. longicollis, C. luiggiorum, C. lunigera, C. mediolineata, C. rivalieriana* and *C. tuberculata* and a moderate support for *C. bavayi*. *C. mniszechii* is divided into two clusters with strong support for each clade and the common clade. *C. rubicondosa* forms two groups with one supported. *C. myrmidon* is located inside *C. rubicondosa*. *C. rivalieriana* sample PS2 has a very isolated position in the tree.

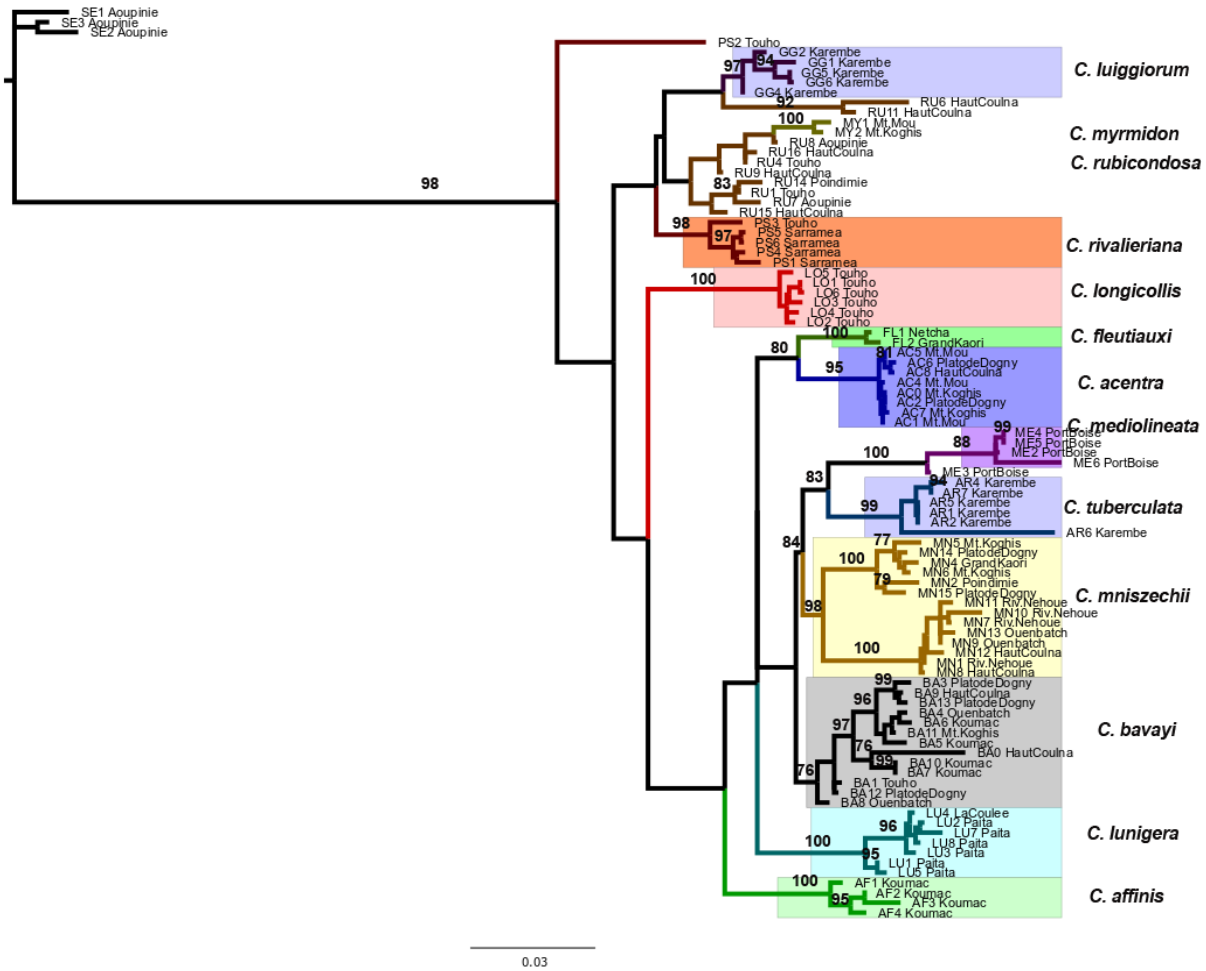

Fig. 8. ML phylogenetic tree resulting from a combined dataset (COI+CAD) analysis. For each node, the ML bootstraps (if equal to or higher than 70) are presented above the branch leading to that node. All nodes with support lower than 70 are considered as collapsed and therefore the support number is not given. For increased clarity, branches of individual

species are coloured, with their names added, and in addition, supported species have a coloured background. ‘SE’ (*Myriochila semicincta*) is used as the outgroup.

Haplotype networks

For a better visualisation of intraspecific variability within *Caledonica mniszechii*, haplotype networks based on mitochondrial (COI) and nuclear (CAD) genes are presented (Fig. 9).

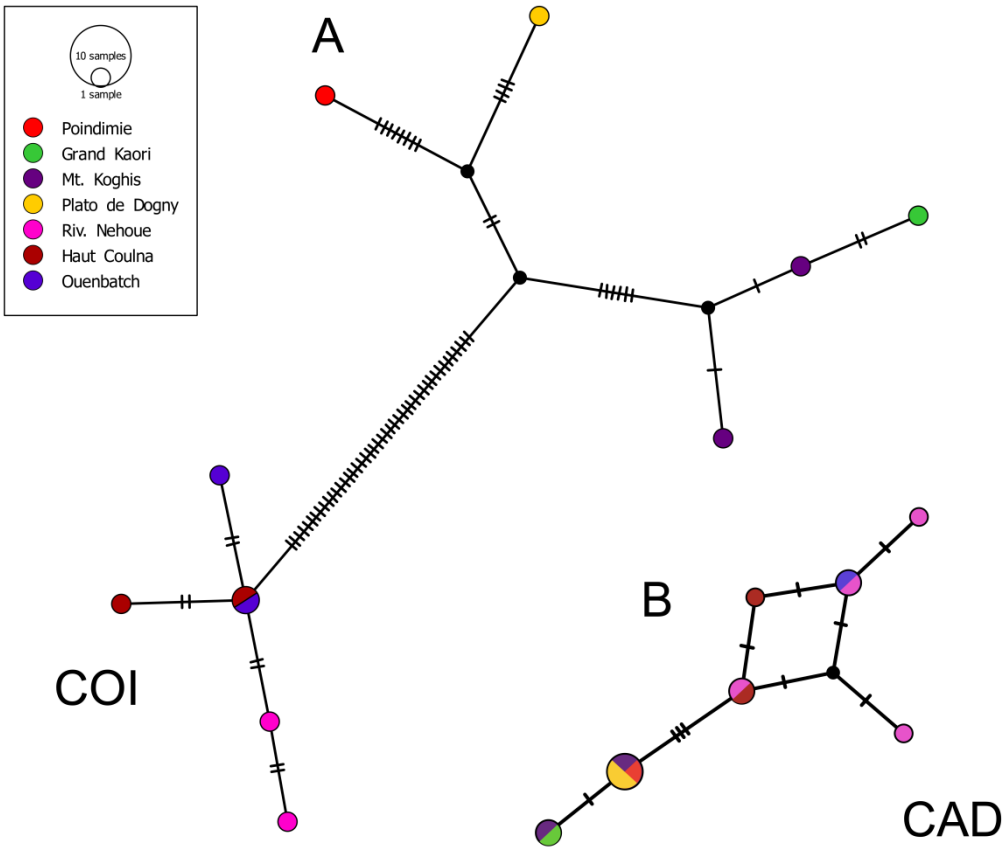

Fig. 9. Maximum parsimony haplotype networks showing the intraspecific genetic diversity for *Caledonica mniszechii* with mutations indicated along branches. (A) mtDNA (COI) and (B) nDNA (CAD).

Discussion

Despite unusually dry weather conditions and near absence of precipitation during the rainy season of 2016, the field sampling was very satisfactory. In total 13 species (of the 16 described species) were collected. *Caledonica fleutiauxi* and *C. mediolineata* were

collected by the author for the first time. Moreover, *C. fleutiauxi* was until then only known from three historic type specimens and three additional, more recently collected specimens.

On the contrary, no specimens of *C. rivalieri* were found, despite two visits to the locality of their former occurrence. The absence of this species is particularly unfortunate, as *C. rivalieri* together with *C. rivalieriana*, *C. rubicondosa* and *C. myrmidon*, form a complex of related species, which is visible both on morphology and DNA, and it would be very beneficial to obtain complete information about their phylogenetic relationships.

At the beginning, we tested two other nuclear genes, Wingless and Elongation Factor α, but despite diverse PCR set ups with two pairs of primers for each of them, we never obtained suitable products. Three other genes, ITS1, GADPH and Arginin K, also did not yield useful results.

We also tested in detail a muscle specific protein MP20 (Aime-Southgate *et al.* 1989). We used primers (MP20-5′, MP20-3′) and protocols for PCR amplification of MP20 according to the study of Pons *et al.* (2004), but the obtained PCR amplified fragments were not cloned. This MP20 gene sequencing also brought only partial success and is therefore not included in this thesis. However, this gene, with additional sequencing and treatment, could be used in a subsequent study.

Moreover, despite repeated attempts, sequencing of some species was unsuccessful with only a few good quality sequences gained (for example sequences of *C. rubicondosa* for COI). Isolating of high quality sequences of *C. mediolineata* for COI was unsuccessful and the species is represented in the dataset only by two lower quality sequences. This could be caused by a mutation in the primer complementary sequence in this species. All six isolates of *Caledonica luiggiorum* for COI contained entirely nonsensical data, as checked by Blast, and therefore this species was completely excluded from COI dataset. Dataset for combined tree contains only CAD sequences of this species.

Both studied genes support a divergence of *C. mniszechii* into two geographically separated populations. One population is represented by samples from following localities: Riviere Nehoué, Haut Coulna and Ouane Batch, all located in northern part of the main island. The other consists of samples from localities Poindimié, Grand Kaori, Mt. Koghis and Plato de Dogny. All these latter localities are in the southern half of the island except for Poindimié that is geographically closer to the northern populations. The distance from the closest locality of the northern population is about 35 kilometres, whereas closest locality of the southern population is ca. 90 kilometres (see Fig. 4., map of New Caledonia with marked localities). For better visualisation, haplotype networks of COI and CAD were also included

in this study. The mitochondrial COI network confirms a close relation between the Mt. Koghis and Grand Kaori populations (both located in southern tip of Grande Terre), between populations from more central areas (Plato de Dogny and Poindimié) and a long genetic distance between both of these populations and the populations in the north.

The explanation could be that *C. mniszechii* is evolving into two separate species with the division line between them lying somewhere between Haut Coulna and Poindimié. The high diversity of haplotypes indicates isolation of populations and low gene flow. This could be explained by geography, as the island´s surface is predominantly mountainous with steep slopes and deep valleys. It would be very beneficial to sample additional specimens in this zone, along with specimens from the central area of Grande Terre between Voh in the north-west, Poindimié in the north-east, Bourail in the south-west and Canala in the south-east. Besides this, it would also be necessary to study the morphology of specimens used in this study and specimens deposited in collections, in detail.

The COI tree divided *C. rubicondosa* into two clusters, where only one cluster has sufficiently high ML bootstrap (100). There is a moderate support (73) for this species in the tree based on nuclear CAD dataset. Both datasets indicate high intraspecific variability. *Caledonica rubicondosa* is a poorly studied species, as populations are only rarely found in the wild. Only five localities for this species, all mountainous, are known (Mandjelia, Touho, Aoupinié, Poindimié and Haut Coulna). They are restricted to a rather small area in the north-east of the island. Molecular divergence lacks any geographical pattern. Moreover, sequencing of this species was repeatedly unsuccessful with only a few sufficient quality isolates gained. Certainly, successful sequencing of more samples from various localities is necessary.

The placement of *C. myrmidon* (represented by only two specimens) inside the clade of *C. rubicondosa* in the CAD and combined trees indicates that *C .rubicondosa* and C. *myrmidon* represent only morphologically different populations of one species. *Caledonica myrmidon* differs from *C. rubicondosa* in the following characters: in body that is distinctly smaller, darker and less shining, in differently shaped median elytral macula that is slightly cranked upwards instead of a straight shape and in a wider pronotum. The reason for such a morphological difference can be explained by the long distance between the populations in the north-east (*C. rubicondosa)* and the south-west (*C. myrmidon)* of the island. However, this is not supported by COI data. Additional sampling of both taxa will probably justify future synonymization of *C. rubicondosa* with *C. myrmidon*.

Probably the most variable species in the genus is *C. rivalieriana*. This species is distinctly morphologically different across its populations. A female specimen from Touho, presented here under the abbreviation PS3 differs in shape of pronotum, elytral pattern and colour from "typical" *C. rivalieriana* and also from another female (PS2) collected at the same place. Other individuals from the recently discovered population in Mont Do Reserve (not used in this study) are more elongate with darker colour and slightly different maculation. Deuve (personal communication) is planning to describe both populations as two new species and he believes that *C. rivalieriana* is in fact a complex of several, closely related undescribed species. In the CAD tree, samples PS1, PS4, PS5 and PS6, originating from Sarramea in the close vicinity of the type locality (Plato de Dogny), have moderate to strong support, but they are divided into two clusters. All phylogenetic trees located the PS2 isolate on its own isolated branch. This branch lacks support, but the separation is not surprising. If PS2 represents one of the separate undescribed species, more specimens are needed to confirm their split. The same kind of pattern is seen in PS3 sample. *C. rivalieriana* could easily represent a complex of species, and discovery of new populations together with additional sampling of specimens from Touho and Mont Do could clarify the situation within this species. Moreover COI and combined tree indicate that PS2 is not necessarily a sister species of *C. rivalieriana.*

The phylogeny confirms the previous impression that *C. myrmidon, C. rivalieriana* and *C. rubicondosa* form a group of closely related species, as already suggested by Kudrna (2016). Based on their morphology, with small body size, oblong median macula (except for some females of *C. rubicondosa)* and reduced or only indistinct pronotal lateral ribs, and also based on their strict forest dwelling behaviour, another species– *C. rivalieri* should be included into this group. However, due to the lack of fresh samples, this species was not included in our molecular phylogeny. If future study, with more included specimens, will confirm samples PS2 or PS3 as a new species, then also these species could belong to this group.

Phylogeny based on CAD tree shows strong support (98) for *C. luiggiorum*. Combined tree based on CAD data only, as COI data are entirely lacking, placed this species within an unsupported clade of *C. rubicondosa* and *C. myrmidon.*

The phylogeny indicates that *C. acentra* and *C. fleutiauxi* are sister species. Both are also close morphologically, with similar medium-sized body, distinct flat lateral ribs on the pronotum, however *C. fleutiauxi* differs in much larger elytral median maculation.

On the other side, it does not show any support for *C. acentra* and *C. bavayi* as sister species, although it is difficult to distinguish both species using morphology, except for the aedeagus and some minor details of colour and shape, as these are two nearly cryptic species.

For future phylogenetic studies on tiger beetles, we recommend to use CAD gene sequences only in combination with other informative genes.

Conclusion

There are 16 species (excluding *C. viridicollis laevioricollis*) recognised in the last revision (Kudrna 2016). This phylogenetic study demonstrated the validity of 12 out of 13 studied species. According to CAD and combined data, *C. rubicondosa* represents only a morphologically different population of *C. myrmidon*. This study also defined *C. acentra* and *C. fleutiauxi* as closely related species. Moreover, it suggests separation of *C. mniszechii* into two species and detected high variability within the populations of *C. rubicondosa*. It also indicates that *C. rivalieriana* could be a complex of two closely related species.

It is evident that the combination of morphological and molecular methods offers much more comprehensive results than either method alone, and the author recommends to use this combination whenever possible.

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