Department of Zoology
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PhD Thesis

Phylogenetic relationships of loach fishes of the superfamily Cobitoidea (Teleostei: Cypriniformes) based on molecular data analyses

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České Budějovice 2008

Declaration

I hereby declare that I have elaborated the present thesis on my own and that I have listed the used resources of scientific information in the list of literature and acknowledgments. The manuscripts have been completed together with the named co-authors.

Prohlášení

Prohlašuji, že jsem předloženou disertační práci vypracovala samostatně a že jsem uvedla všechny použité zdroje vědeckých informací v seznamu literatury či poděkování. Rukopisy byly zkompletovány společně s jmenovanými spoluautory.

České Budějovice, 28.4.2008 Vendula Šlechtová

ANNOTATION

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The superfamily Cobitoidea belongs to the largest clade of primary freshwater fishes - the order Cypriniformes and its distribution ranges across most of Eurasia and northernmost parts of Africa. Up to know, there have been very few studies that attempted to solve the systematics and taxonomy of this morphologically highly diverse group. The present theses aimed to solve the phylogenetic relationships within the loach fishes of the superfamily Cobitoidea with means of molecular genetic methods. Altogether, about 50 genera and 150 species were analysed within the frame of this study for one or two out of the three following DNA fragments: nuclear gene RAG-1, mitochondrial cyrochrome *b*, mitochondrial 12S rRNA. The present study provides an outline of the main lineages of the loach fishes and hypothesis about their phylogeny, including the mysterious, up to now controversially discussed genera (*Barbucca*, *Ellopostoma*, *Psilorhynchus*, *Serpenticobitis* and *Vaillantella*). The study supports the existence of six families (Balitoridae, Botiidae, Catostomidae, Cobitidae, Gyrinocheilidae and Nemacheilidae), and establishes two further families for the enigmatic genera *Ellopostoma* and *Vaillantella.* From the remaining enigmatic genera, *Barbucca* and *Serpenticobitis* turned out to belong to Balitoridae and *Psilorhynchus* is a member of Cyprinidae. In the frame of this study, the number, outline and relationships of the genera of two families (Cobitidae and Botiidae) were studied further more in detail and one paper is focused on the intrageneric phylogeny of the genus *Pangio*, revealing a large undetected diversity, three main lineages within the genus and confirmed the synonymy of the proposed genera *Eucirrichthys* and *Cobitophris* with *Pangio.* A phylogeographic study on the European loach *Misgurnus fossilis* detected the lowest genetic diversity of any European freshwater fish studied so far; giving rise to the idea that genetic diversity is correlated to autecology.

CONTENTS

PROLOGUE

Even the longest and most exciting journeys have to start with the first small step at home. In our case, the journey to the diversity of loaches across Eurasia started several years ago with a yellow bucket full of small fishes carried from the nearby creek Pšovka to the Laboratory of Fish Genetics in IAPG (Liběchov, Czech Republic). The one to launch the story was a single female of respectable size. These fishes were expected to be *Cobitis taenia*, a species that was until that times believed to be widespread from most of central and northern Europe as far as to Japan. To the big surprise of the researchers (and maybe also of the little fish), this fish showed a strange number of black-violet little dots called chromosomes and a strange number of bands on a gel called isozymes. It became obvious that these fishes are not *Cobitis taenia*. The journey had started….

Since these innocent times, there have been many studies done, plenty of surprising, unexpected and interesting cases found and a lot of scientific discussion held about the diversity of the genus *Cobitis* and the phenomenon of loach hybrid complexes. The fame of these fishes reached so high that scientists even established a loach “fan club of cobitophils” and organized regular meetings called conferences about “The genus *Cobitis* and related genera”. Here everyone could present his or her fantastic findings about these peculiar fishes. Even ‘intergeneric’ hybrid complexes have been found and excitedly discussed. The topic was so complex with several ‘dimensions’ that each scientist had a smaller or bigger piece to add into the puzzle (as Petr Ráb described the situation). But adding one piece often resulted in discovering a new dimension, and a hybridisation between genera was difficult to incorporate into the existing pieces of knowledge. Until once, during the Xth European Ichthyological Congress, one unnamed scientist shouted into the discussion “But what is *Cobitis*?!!! How can you define the genus?!!!” But to the surprise of the researchers, there was no answer to this “simple” question. And so it happened that I was sent out on my part of the journey to find more pieces of the puzzle and to fill them into the picture.

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Most of the work was conducted in the Laboratory of Fish Genetics in Institute of Animal Physiology and Genetics (Liběchov, Czech Republic) and I would like to thank to the whole team for helpful discussions, help in laboratory or just easy chatting when I needed it.

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DISCLAIMER ACCORDING TO ARTICLE 8.2 ICZN

This thesis, although dealing with topics that may affect the taxonomy of certain taxa involved, is explicitly not issued for the purpose of zoological nomenclature and does not intend to make new names available or to contain any other nomenclatural act.

INTRODUCTION

Loach fishes of the superfamily Cobitoidea

The Earth's largest and most diverse clade of primary freshwater fishes, the order Cypriniformes, is divided into two superfamilies: Cobitoidea (loach-like fishes) and Cyprinoidea (carp-like fishes) (Nelson, 1994, 2006). Due to the enormous diversity of cypriniform fishes, the internal phylogenetic relationships are poorly understood. At the time when this PhD project started, there were many studies that attempted to reveal the phylogenetic relationships of families, genera and species of the superfamily Cyprinoidea (e.g. Briolay et al., 1998; Zardoya and Doadrio, 1999; Durand et al., 2002; Wang et al., 2007), but very few was known about the classification and phylogeny of cobitoid fishes and this fact was reflected in the controversial discussions about the main taxonomic units within this group.

Loach fishes inhabit bentic or more rarely semibentic freshwater habitats in Eurasia and northernmost parts of Africa and managed to occupy an incredible variety of ecological niches. Some species inhabit mountainous or piedmont rapids, which in some cases led to strong morphological adaptations allowing the fish to attach to stones in very strong currents (sucker-belly loaches of the family Balitoridae), others occur in black standing water habitats of peat swamps (e.g. genus *Kottelatlimia*, Cobitidae), others are restricted to leaf litter patches in pristine rain forest creeks (e.g. *Pangio*, Cobitidae), while some species can be found mainly in big rivers (Botiidae) (Kottelat, 1998, 2001; Kottelat and Lim, 1993; Tan, 2006). Other taxa (several genera of the family Cobitidae) developed adaptations that allow them to dig, hide and feed in fine sand using the gill apparatus for filtration of food particles out of the sand (Robotham, 1982). In some extreme cases, the strong adaptations for a life in the sand allow the fish to move fast (in a kind of swimming way) through the sand (genus *Acantopsis*, Cobitidae). Except Botiidae, most loach fishes are stationary, which is probably due to their strong morphological and ecological speciation. There is very few known about the ecology of Botiidae but the empirical observation of their seasonal occurrence in certain localities lead to an idea that they undergo spawning migrations (Roberts, 1993; Sokheng et al., 1999; Udomritthiruj, pers. com.).

Taxonomy of loaches: state-of-the-art in the beginning of the project (2004)

The systematics and taxonomic classification of the superfamily Cobitoidea is in a rather poor stage, and shows serious flaws like the lack of a proper definition of the group itself, an uncertain number of major lineages (families) inside and their phylogenetic relationships to each other, doubts on the monophyly of the currently recognised families, a basically unknown number of genera and their phylogenetic position within the group, up to an unclear definition of many species (which is nevertheless a matter of the species concept). In September 2004, one of the world's most experienced specialists for the taxonomy of loach-like fishes, Maurice Kottelat, gave a congress talk about the diversity of Cobitoidea, in which he emphasised the poor state of their taxonomic and phylogenetic understanding using the following formulations (Kottelat, 2004b):

- 'The infrafamilial relationships are only superficially known.'
- 'Some of the usually recognised subfamilies are poorly supported and probably paraphyletic'
- 'Many (if not most) genera are artificial assemblages …'
- '… 'intuitive' taxonomy suggests that at least 30 more lineages should be recognised and named.'
- 'The systematic position of several genera is still unsettled.'

The biggest and often discussed problem is the definition of the superfamily Cobitoidea itself. The first to mention this group was Regan (1911) who, basing on the scull morphology, collected the loach fishes under a family Cobitidae, within which he further recognised two subfamilies, Cobitinae and Noemacheilinae. The superfamily Cobitoidea thus originally consisted of a single family. Later, on the basis of more extensive osteological analyses, Berg (1940) divided the subfamily Cobitinae into Cobitinae and Botiinae. Since, the family Cobitidae was considered to consist of three subfamilies: Cobitinae, Botiinae and Noemacheilinae. Many authors later accepted this classification. Several minor studies on the osteological characters of Cobitidae (e.g. Kobayasi, 1954, 1956; Alexander, 1964b) followed, but since most of them have been based on too few species or on just few characters, they did not contribute much to the understanding of the relationship within loach fishes.

The topic of phylogenetic relationships between the family Cobitidae *sensu* Berg and the other lineages of cypriniform fishes have attracted the attention of several researchers beginning with the study of Hora (1932) who created the family Homalopteridae and divided it into the subfamilies Homalopterinae and Gastromyzoninae. Whereas the former subfamily he considered as related to Berg's family Cobitidae, the latter one he assigned to close relationship of Cyprinidae. Later, Hora (1950) suggested to treat these two groups as two independent families (Homalopteridae and Gastromyzonidae) of the superfamily Cyprinoidea. Nevertheless, this opinion was not always accepted.

The most extensive study concerning the topic of questionable internal relationships within the family Cobitidae *sensu* Berg and its relation to the two other potentially closely related families of Homalopteridae and Gastromyzonidae *sensu* Hora was carried out by Sawada (1982). Due to its broad focus and methodological and taxonomic accuracy this study can be considered as the basic literature about loach phylogeny, hence I would like to devote to this study a little bit more lines in the following text. The attempt of Sawada was to clarify the phylogenetic relationships within Cobitoidea and to improve the knowledge about their position within the order Cypriniformes with use of osteological characters. He was the first author who adopted the Hennigian cladistic approach and the principles of parsimony suggested by Nelson (1970) to elucidate the phylogeny and zoogeographical history of the superfamily Cobitoidea. He attempted to reconstruct the branching pattern of the superfamily and tried to deduce the evolutionary history of the group. The phylogeography was accomplished by estimating the centre of origin and geographical dispersal route of Cobitoidea.

On the basis of several synapomorphies, Sawada suggested a new system of the superfamily Cobitoidea consisting of two families: Cobitidae (spined loaches) and Homalopteridae (hill-stream loaches). In the former one he recognised two subfamilies: Cobitinae and Botiinae. As synapomorphy of this group he considered the movable suborbital spine derived from the prefrontale. The subfamily Noemacheilidae, which was formerly regarded as a member of Cobitidae (Berg, 1940; Nalbant, 1963) he transferred to the family Homalopteridae, which hence consisted of two subfamilies Noemacheilinae and Homalopterinae. The subfamilies Homalopterinae and Gastromyzoninae *sensu* Hora (1950) he fused into a single subfamily. The study of Sawada also deals in detail with the lineages and genera within the single subfamilies, but to this point we will return later in the text. Now let us focus on the main arrangement of the superfamily Cobitoidea.

Although there has not been such a detailed and extend study concerning this topic either on morphological or on the molecular characters since the comprehensive publication of Sawada, several classifications were suggested by different authors. Kottelat (1988a) stated that the family name Homalopteridae is a junior synonym to Balitoridae, therefore this finding changed the name of the subfamily Homalopterinae to Balitorinae. Siebert (1987) studied the relationships of Cypriniformes and suggested to include also the families Catostomidae (suckers) and Gyrinocheilidae (algae eaters) into Cobitoidea. However, his opinion appeared only in form of his PhD thesis but has never been published, therefore I cannot refer to any details. Nelson (1994) was the first one to apply Siebert's (1987) system of “enlarged” Cobitoidea in his famous book “Fishes of the World”. However, this suggestion still appears puzzling, since as loaches most ichthyologists usually imagine elongated fishes with several pair of barbells. A 15-year jump from Siebert’s study brings us to the classification of loaches *sensu stricto* (without the families Catostomidae and Gyrinocheilidae) elaborated by Nalbant (2002). In his study, he suggested to separate the subfamily Botiinae from the family Cobitidae and elevated this group to family Botiidae. This suggestion was followed by Kottelat (2004a) and Nalbant (2004) and was later supported by molecular studies included in this thesis (Šlechtová et al., 2007) as well as by some other authors (Saitoh et al., 2006; Tang et al., 2005).

As pointed out before, there were some voices suggesting the families Catostomidae (suckers) and Gyrinocheilidae (algae eaters) to be included into the superfamily Cobitoidea. Wu et al. (1981) have concluded from osteological characters that the cobitoid loaches form a monophyletic lineage with the gyrinocheilids and catostominds, whereas cyprinids and homalopterids (i.e. balitorids) form another big group, a sister lineage to the former one. Most taxonomists did not follow this hypothesis. The classification of Siebert (1987) as mentioned above, considers also the families Catostomidae and Gyrinocheilidae to belong to Cobitoidea together with formerly proposed Cobitidae and Balitoridae.

Harris and Mayden (2001) published a study on the molecular phylogeny of Catostomidae where, besides the internal relationships within the family, they have attempted to show the position of catostomids within Cypriniformes. For this purpose they have chosen some representatives of the family Cyprinidae and ‘other additional taxa from the superfamily Cobitoidea’: *Formosania lacustre* (as *Crossostoma lacustre*), *Chromobotia macracanthus* (as *Botia macracanthus*,), *Misgurnus anguillicaudatus* and *Gyrinocheilus aymonieri*. They did not specify the families of these taxa, but we can easily deduce that they belonged to the families Balitoridae, Botiidae, Cobitidae and Gyrinocheilidae, respectively. From the article it becomes clear that the authors consider Catostomidae and Gyrinocheilidae as members of the superfamily Cobitoidea. Nevertheless, in the results they mention that ‘all analyses yielded a monophyletic Cobitoidea and within Cobitoidea, Cobitidae were sister to a clade of Gyrinocheilidae plus Catostomidae’. They do not discuss the results in detail, only present a simplified tree showing the position of the three mentioned families without the family Cyprinidae. The relationship to the other lineages was not mentioned.

Gilles et al. (2001) used mitochondrial DNA sequences to infer the phylogeny of European cyprinids. Their outgroup taxa came out of the families Catostomidae, Cobitidae, Balitoridae and Gyrinocheilidae from the order Cypriniformes but they have chosen also taxa from other closely related fish orders (Siluriformes and Characiformes). The results of their study show that Cypriniformes form a monophyletic lineage with a clear split into two main lineages: one including all cyprinid fishes and the other the members of the families Botiidae, Cobitidae, Balitoridae and Gyrinocheilidae. The position of Catostomidae is neither shown nor discussed in the article. However, the study was focused on the internal relationships within Cyprinidae, hence we cannot criticise the lack of interest in branching pattern within the whole order Cypriniformes.

Later, based on molecular phylogenetic analyses of Cypriniformes, Liu et al. (2002) proposed that relationships within the Cobitoidea are: (Catostomidae + (Gyrinocheilidae + (Botiinae + (Homalopteridae + (Cobitinae + Noemacheilinae)))). Clements et al. (2004) provided the most recent hypothesis regarding cypriniform relationships. Based on sequences of the growth hormone gene, their analyses resolved a well-supported clade of Cobitidae as sister lineage to Catostomidae plus Cyprinidae. The study of a completely different character, the karyotypes, with an attempt to trace the chromosomal rearrangements to infer the phylogeny within Cypriniformes (Suzuki, 1996) brought again a different hypothesis suggesting the following phylogenetic relationships: (Cyprinidae + (Catostomidae + (Cobitidae + (Gyrinocheilidae + (Homalopteridae))))).

Kottelat (2004b) considered intrafamilial relationships as too superficially known to use formal rank for the five main lineages he recognised in his system of Cobitoidea: cobitines, botiines, balitorines, nemacheilines and newly also the family Psilorhynchidae, which was never before considered as a member of this group. Again he refers only to loaches *sensu stricto* and does not discuss the position of Gyrinocheilidae and Catostomidae.

An overview about the proposed taxonomical hypotheses of Cobitoidea is given in Table 1 (taken from Šlechtová et al., 2007). Taking into account just these few examples, we can see that there have been several, often controversial opinions concerning the taxonomy of Cobitoidea, especially regarding the inclusion of the families Catostomidae and Gyrinocheilidae, which might be a result of lack of focus on the proper choice of representative ingroup taxa as well as appropriate outgroup. One of the biggest problems rises up hand in hand with the poor understanding of the outlines of the major cobioid lineages.

Table 1

Overview about the various grouping systems of Cobitoidea. F = family, Subf. = subfamily.

Regan 1911	Hora 1932	Berg 1940	Hora 1950
F. **Cobitidae**	F. **Cobitidae**	F. **Cobitidae**	F. **Cobitidae**
Subf. Cobitinae	Subf. Cobitinae	Subf. Cobitinae	Subf. Cobitinae
Subf. Noemacheilinae	Subf. Noemacheilinae	Subf. Botiinae	Subf. Noemacheilinae
	F. **Homalopteridae**	Subf. Noemacheilinae	F. **Homalopteridae**
	Subf. Homalopterinae		F. **Gastromyzonidae**
	Subf. Gastromyzoninae		

Roberts 1972	Sawada 1982	Siebert 1987[a]	Nalbant 2002
F. **Cobitidae**	F. **Cobitidae**	F. **Cobitidae**	F. **Cobitidae**
Subf. Cobitinae	Subf. Cobitinae	Subf. Cobitinae	F. **Botiidae**
Subf. Botiinae	Subf. Botiinae	Subf. Botiinae	Subf. Botiinae
Subf. Noemacheilinae	F. **Homalopteridae**	F. **Balitoridae**	Subf. Vaillantellinae
Subf. Vaillantellinae	Subf. Homalopterinae	Subf. Ellopostominae	F. **Noemacheilidae**
F. **Homalopteridae**	Subf. Noemacheilinae	Subf. Noemacheilinae	no mentioning of
Subf. Homalopterinae		F. **Gyrinocheilidae**	Gyrinocheilinae and
Subf. Gastromyzoninae		F. **Catostomidae**	Catostomidae

Kottelat 2004[b]	Tang et al. 2006
Cobitines	F. **Cobitidae**
Botiines	F. **Botiidae**
Balitorines	F. **Nemacheilidae**
Nemacheilinae	F. **Balitoridae**
Psilorhynchidae	Subf. Balitorinae
no mentioning of	Subf. Gastromyzoninae
Gyrinocheilinae and	F. **Gyrinocheilidae**
Catostomidae	F. **Catostomidae**

[a] unpublished PhD thesis, but system adopted by Nelson (1994)
[b] Kottelat intentionally does not use formal rank names due to the superficial knowledge about the phylogenetic relationships of the lineage

Kottelat (2004b) considered intrafamilial relationships as too superficially known to use formal rank for the five main lineages he recognised in his system of Cobitoidea: cobitines, botiines, balitorines, nemacheilines and newly also the family Psilorhynchidae, which was never before considered as a member of this group. Again he refers only to loaches *sensu stricto* and does not discuss the position of Gyrinocheilidae and Catostomidae. Table 2 lists the number of valid genera and nominal and valid species of each of the recognized group. It shows that nowadays there are about 775 valid species of loaches and about half of this diversity occurs in Southeast Asia, where discovery of many new species is still expected (the gross estimate is about 200 – 300 species).

Table 2

Overview about the proposed lineages of Cobitoidea with the numbers of species and genera according to Kottelat (2004b).

Lineage	No. genera	No. nominal species	No. valid species
Psilorhynchidae	1	11	6
Cobitines	19	270	127
Botiines	7	87	50
Balitorines	27	208	157
Nemacheilines	33	627	435
Total	88	1203	775

Enigmatic cobitoid genera

Besides the discussion of the large groups within Cobitoidea, there are several genera with more or less unknown phylogenetic relationships, which most likely belong to Cobitoidea (e.g. *Ellopostoma*, *Barbucca*, *Psilorhynchus, Tuberoschistura*, *Yunnanilus*, *Vaillantela*) (Kottelat, 2004a, 2004b; Nalbant, 2002). The resolution of their systematic position is hampered mainly by their odd morphological characters, which do not allow us to settle these questionable genera unambiguously into one or the other existing family.

The genus *Vaillantella* Fowler, 1905 includes only three taxonomically recognised species (type species *V. euepiptera*), which are distributed in the southern Malayan peninsula, Sumatra and Borneo (Roberts, 1989; Kottelat et al., 1993; Lim, 1993). It is unique among loach fishes due to its unusually long dorsal fin (52-73 branched fin rays). Former investigations of various morphologic characters led Nalbant & Bănărescu (1977) to establish the subfamily Vaillantellinae within the Cobitidae, but other authors considered *Vaillantella* as member of Nemacheilinae (Sawada 1982, Roberts 1989, Kottelat 1990a, 1994). Recently, Nalbant (2002) suggested to include *Vaillantella* into the family Botiidae, an opinion strongly refuted by Kottelat (2004a). The controversial opinions about the phylogenetic position of *Vaillantella* points on the need to include also other than morphologic characters; especially molecular markers could help to resolve its identity.

Another questionable genus I would like to focus on is *Ellopostoma* Vaillant, 1902, currently with two described species (type species *E. megalomycter*). Due to some osteological characters similar to loach fishes, this otherwise highly distinctive fish was originally assigned to the family Cobitidae (Vaillant, 1902). *Ellopostoma* is a moderately elongated, small-scaled fish with very large nostrils and eyes, and very small inferior mouth with a single pair of barbels (Roberts, 1972). Weber and de Beaufort (1916) doubted that this fish should belong to Cobitoidea and regard it as a cyprinid. Later Roberts (1972) examined and redescribed the type specimens, pointed on its similarity to Kneriidae and in his publication discusses in detail the osteological characters of *Ellopostoma* in respect to cobitoids and knerinids but without a final decision on its taxonomical position. Nevertheless, nowadays most taxonomists agree that *Ellopostoma* is a loach fish but its phylogenetic position within Cobitoidea is still under discussion: Basing on absence of a suborbital spine, *Ellopostoma* has been tentatively placed in the family Balitoridae (Kottelat, 1989; Kottelat et al., 1993). Later Bănărescu and Nalbant (1995) in their review of the Nemacheilinae point on the uncertain position of this genus within Balitoridae. Most likely this peculiar fish represent an outstanding lineage within cobitoid loaches but to resolve its systematic position requires comprehensive morphological study with appropriate comparative material. The confusion about the phylogenetic position of *Ellopostoma* can best be illustrated by a citation from Roberts (1972): ‘No modern ichthyologist has found it possible to assign a firm systematic position to this strange fish.’ Until now this fish has never been included into the molecular analyses, probably due to its rarity (during 110 years, only three publications mention catches of this genus) and neglectable knowledge of its habitat preferences.

Another genus with unsolved relationships is *Serpenticobitis* Roberts 1997, which is distributed with three species (type species *S. octazona*) in the middle and lower Mekong basin (Roberts, 1997). Representatives of this genus resemble Cobitidae due to the presence of a movable suborbital spine, but the structure of the swimbladder capsula is rather like in nemacheiline loaches (Nalbant, 2002; Roberts, 1997). This confusing combination of characters gives *Serpenticobitis* a high impact to understand the outlines of the families Cobitidae and Balitoridae: if *Serpenticobitis* belongs to Balitoridae, the suborbital spine looses power as identification character for the families Cobitidae and Botiidae. In fact, Nalbant (2002) considers the moveable suborbital spine to be a synapomorphy of the Cobitoidea, which was secondarily reduced in ‘in vaillantellin botiids, most of nemacheilids and in misgurnoin cobitids’ (meaning in sense of the classification followed here: Balitoridae without *Serpenticobitis* and the genera *Misgurnus* and *Paramisgurnus* of the Cobitidae). Nalbant (2002) states that an independent parallel evolution of the character in *Serpenticobitis* is unlikely due to the complexness of the structure that includes several scull bones and muscles. However, *Serpenticobitis* needs to be studied thoroughly by molecular genetic methods.

The genus *Barbucca* Roberts 1989 includes a single species described from western Borneo, *B. diabolica* (Roberts, 1989), but was recently found also in eastern Thailand (Udomritthiruj, pers. com.). It is a dwarf loach, reaching only up to 24 mm SL and lives in swift waters between stones (Roberts, 1989). Roberts (1989) provided the original description of the genus and its only species, which in fact is the only publication dealing with this genus in detail. According to Roberts (1989) it seems to be closely related to some species of *Nemacheilus* from the Sunda Islands, but differs in the small size, the presence of breeding tubercles on the caudal peduncle in females (versus only in males in *Nemacheilus*) and particularly the presence of a patch of hook-shaped tubercles on the cheek of males. Some of the characters described by Roberts (1989) may be simply the consequence of dwarfism, but the number of unique features could also indicate that *Barbucca* represents a distinct lineage within the Nemacheilinae.

Close-up look on families

COBITIDAE (Spined Loaches)

Although the distribution areas of several cobitid genera (*Cobitis*, *Sabanejewia*, *Misgurnus*) reach up to Western Europe and in case of *Cobitis* also to the northernmost Africa (Morocco) (Kottelat and Freyhof, 2007), the greatest diversity is located in Southeast Asia (Nalbant, 1963, 1994). Like most of the other cobitoid families, they include small bottom-dwelling fishes, but members of Cobitidae have the most elongated, sometimes vermiform body shape. The most distinctive character of cobitids is an erectable suborbital spine, the character which that was formerly considered as synapomorphy of Cobitidae and Botiidae (Sawada, 1982). However, Cobitidae differ from Botiidae in the arrangement of barbels and the ossified swim bladder (Nalbant, 2002). Overviews about the genera included into

Cobitidae were given by Nalbant (1963) and Nalbant (1994). In these papers Nalbant considers 12 and 16 genera as valid, respectively. Kottelat (2004a) considered even 19 genera, but did not provide a list of these genera. However, no study has been carried out to clarify the phylogenetic relationships between these genera. The spelling of the family name is grammatically incorrect, the grammatically correct spelling would be 'Cobititidae', but since 'Cobitidae' was used such often, the International Commission on Zoological Nomenclature fixed the family spelling Cobitidae by plenary power (Kottelat, 1986; Opinion 1500).

BOTIIDAE

The freshwater fish family Botiidae is one of the best-known groups of the highly diversified cypriniform fishes and is distributed on the Indian subcontinent and in East and Southeast Asia. These semibenthic fishes reach from small to medium body size (40-300 mm SL) and are generally found in slow to moderately running waters. For a long time, they were considered a subfamily of the family Cobitidae and believed to include only two genera (Fang, 1936; Nalbant, 1963; Sawada, 1982; Taki, 1972). Recently, basing on morphological characters Nalbant (2002) and Kottelat (2004a) established a family rank for botiid loaches and by that they separated them from the family Cobitidae. Nalbant (2002) also included the above-mentioned *Vaillantella* into the family Botiidae as an independent subfamily Vaillantelinae. This step was in disagreement with the opinions of Kottelat (1994), Roberts (1989) and Sawada (1982) that *Vaillantella* is a nemacheilid loach. Consequently the new taxonomic position of this genus was later again refused by Kottelat (2004a).

The important character, which led some authors to the conclusions that botiid and cobitid loaches are closely related, is the presence of a movable suborbital spine (Sawada, 1982) Originally, this character was considered as synapomorphy of both groups although in both groups they differ in structure. While in cobitids the spine is always bifurcated and arranged in horizontal plan (Nalbant, 1963), in botiid loaches the spine can be either simple or bifid, but in latter case the both thorns are always situated vertically to each other. At the beginning of this study, botiid loaches were still considered within Cobitidae.

Formerly, only two genera, *Botia* and *Leptobotia*, the former one with three subgenera *Botia, Sinibotia* and *Hymenophysa* (Fang, 1936; Nalbant, 1963; Sawada, 1982; Taki, 1972) were considered as valid but later, but Nalbant (2002) suggested to recognize six genera within Botiidae and Kottelat (2004) described yet another genus *Chromobotia*, so that at present, Botiidae contain seven genera.

BALITORIDAE (River Loaches)

This family is by far the most species-rich and diverse group of loach-like fishes and collects fishes of many different body plans inhabiting moderately to very fast flowing waters of Eurasia. As mentioned above, the most important characters that were used to define this group are the absence of a moveable suborbital spine and the double-chamber structure of the swimmbladder capsula. Within the Balitoridae (at that times called Homalopteridae), Sawada (1982) recognised two groups, Nemacheilinae and Balitorinae, as comprising a monophyletic lineage. His opinion was followed later by Menon (1987), Kottelat (1990a) and others, but the tremendous differences between the two subfamilies led many authors to the opinion that they represent two independent lineages; therefore I will describe the two subfamilies separately.

Subfamily Nemacheilinae (River loaches)

Nemacheilinae represent the largest group within loaches (Bănărescu & Nalbant, 1995; Kottelat, 2004a). These loaches range throughout Eurasia with most species in the Indian subcontinent, Indochina and China (Nelson, 1994). To Europe reach only the distribution areas of the genera *Barbatula* and *Oxynemacheilus* (Kottelat and Freyhof, 2007). Nemacheilinae occur typically in moderately to fast flowing water between the stones and gravel on the bottom of various waters from small creeks to large rivers. However, there are several genera known from standing, muddy water (e.g. *Yunnanilus, Lefua*; Kim and Park, 2002; Serov et al., 2006) as well as from very fast torrents (e.g. *Turcinemacheilus*; Breil and Bohlen, 2001). Several cave species are known from India, Iran, China, Thailand and Malaysia (Kottelat, 1990a, 1990b, 2004c). The number of genera is particularly difficult to estimate, since many species and genera are poorly known and have no clear definition. Therefore, much confusion exists about the correct genus for many species. For example, at least six different genera have been used for two of the European stone loaches (species names *barbatula* and *bureschi*): *Barbatula, Cobitis*, *Nemacheilus, Nemachilus, Noemacheilus, Orthrias, Oxynoemacheilus* (Kottelat, 1997; Eschmeyer, 2005). The poor definition of many genera leads to artificial assemblages that are constantly topic of changes. The biggest artificial assemblage is the genus *Schistura*, which contains at present at least 160 species over most of Asia, but according to Kottelat (1990a) is a “catch-all” genus. Regularly, species or species-groups are separated from *Schistura* and new generic names are established, e.g. *Sectoria*, *Physoschistura, Tuberoschistura* and *Neonoemacheilus* (Kottelat, 1990a). In such unstable taxonomic environment it is no surprise that only very few attempts have been done to provide overviews about the diversity of nemacheiline loaches and that the few existing ones do not agree with each other. At present, Bănărescu & Nalbant (1995) and Kottelat (1990a) give the most complete lists, mentioning 23 and 31 genera, respectively. The Nemacheilinae certainly are in urgent need of a proper phylogenetic investigation, but in such a big and diverse group this is a very ambitious task.

Subfamily Balitorinae (Hillstream or Sucker-belly loaches)

Balitorinae (formerly Homalopterinae) inhabit typically very fast flowing waters of the Oriental area from India through Southeast Asia including Sumatra, Java and Borneo, to China and Taiwan (Nelson, 1994). Their flattened head and body, horizontally oriented enlarged pelvic and pectoral fins with rays that bear adhesive pads (Roberts, 1982) on the ventral surface enable them to live in mountain stream and rivulets. Also in this group, cave species were recorded (Kottelat, 1988a, 1988b). Hora (1932) divided Balitorinae (as family Homalopteridae in this work) into two subfamilies: Homalopterinae and Gastromyzoninae, but later he considered these two subfamilies as families. Subsequently some authors treated Balitorinae as a single family (e.g. Nelson, 2006; Wu et al., 1981; Roberts, 1989) and Sawada (1982), basing on osteological characters, confirmed that homalopterines and gastromyzonines form a monophyletic lineage. Kottelat (1988a) considered the formerly established name Homalopteridae as a junior synonym of Balitoridae. The International Commission of Zoological Nomenclature (Opinion, 1998) confirmed this opinion. The two former subfamilies Balitorinae (Homalopterinae) and Gastromyzoninae are listed in Nelson (1994) as tribes Balitorini and Gastromyzontini. The first group includes 13 genera and about 68 at present described species and the latter one comprises about 15 genera and 52 species (Nelson, 1994).

Aims of the study and their development throughout the time

The present taxonomic overview reveals that Cobitoidea are large and highly diverse group of freshwater fishes. The comparably low number of phylogenetic studies and studies aiming to overview this group or single lineages reflect that not too many attempts have been done to bring this diversity into a reliable and stable taxonomic system. Consequently, a lot of uncertainty, conflicting opinions and artificial groupings are reported, which shows that the group is in urgent need of revision.

The original aim of my PhD study was to reconstruct a phylogeny of the family Cobitidae with the use of molecular markers and to provide a stable basement for their systematics. Since spined loaches are rather numerous and widespread group with many taxa restricted to remote areas, the collection of samples required several field trips, patience, resistance against leeches as well as tolerance to failure to find the missing species, I started my analyses on a smaller group of fishes, which was during the preparation of this study still believed to belong among Cobitidae – the botiid loaches. Many botiid species are available from the ornamental fish trade, which helped me to build up the starting collection for the study. Moreover, this group is well defined and there have never been doubts about its monophyly. However, many things have changed during the progress of the study. When enlarging the dataset with more taxa, it became evident that botiid and cobitid loaches even do not represent sister lineages and the idea to enlarge the focus of the study arose and, besides resolving the phylogeny of Cobitidae, to bring light into the dark corners of the systematics of the superfamily Cobitoidea at the higher taxonomic level. Thus, the general tasks of the present project after these adaptations were 1) to resolve the outline of the major groups of “the loach bush” and their basal cladogenesis and to compare the results with the formerly proposed systematic hypotheses in order to define outline and number of the families within, 2) to construct phylogenies of the families Cobitidae and Botiidae and to identify, evaluate and define the genera in these families, 3) to clarify the phylogenetic position of as many enigmatic taxa as possible in order to incorporate them into the final ‘puzzle of the loach-fishes’, 4) to elucidate the phylogenetic relationships of species within selected genera of Cobitidae and to reconstruct their evolution from the biogeographic point of view as well as from the development of important morphologic and cytogenetic characters.

Unfortunatelly, the river and sucker-belly loaches are such huge and complex groups with complicated and unsettled systematics that their detailed revision reaches far behind the time frame of this study.

Short elucidation to the papers

In the few following paragraphs I would like to provide short explanations to some potential questions that might rise up in readers mind but cannot be understood from the single papers.

As mentioned above, the botiid loaches still belonged to the Cobitidae when the design of this study was fixed and with the designation of a new family Botiidae another new hypothesis arose regarding the composition of Cobitidae. This hypothesis of course needed evaluation like the former hypothesis about the composition of Cobitidae; therefore we did not exclude the phylogenetic investigations of botiid loaches after their separation as distinct family. Moreover, the botiid loaches provide a perfect example to study the impact of polyploidisation on the evolution of loaches.

The article entitled “Families of Cobitoidea …” focuses on the relationships of the main lineages (proposed families) within the superfamily Cobitoidea and their relationship to Cyprinoidea as well as on the phylogenetic position of genera with uncertain systematic affinities. Another paper, entitled ‘*Ellopostoma* …’ deals also with the phylogenetic position of an enigmatic taxon, therefore a reader may come to the conclusion that the two papers should have been joined into a single manuscript. However, the reason why I could not combine the results into a single article came right from the rarity of the enigmatic taxa, first of all *Ellopostoma* going hand in hand with the problems to obtain any fresh material of this genus or other matherial suited for DNA or karyotype analyses. During the 106 years since its original description by Vaillant (1902), only two publications mention freshly caught material, and our first trials to catch these fishes remained unsuccessful. Therefore, we decided to go on with the enigmatic taxa in hand and to leave *Ellopostoma* for later. However, the article was just released when fortune smiled on us and we caught *Ellopostoma* on the second expedition to the Tapi River.

Once having fresh and even life specimens of this extraordinarily rare and enigmatic fish in hands, we took the chance to combine my DNA sequence data with observations on its habitat obtained during catching, with morphologic data elaborated by Mgr. Radovan Harant (University of South Bohemia) and with cytogenetic data prepared by Doc. Ing. Petr Ráb, DrSC. and Ing. Marie Rábová, PhD (IAPG). Altogether, the combination of data led to a paper that aims to give a deeper understanding of the formerly basically unknown *Ellopostoma*, hereby newly proposed family Ellopostomatidae.

Further, I took the chance of the *Ellopostoma* paper to revise the position of the genus *Psilorhynchus*, which was identified as related to Cyprinidae in the first paper, but without showing if it is embedded into Cyprinidae or a second family of Cyprinoidea. With the improved taxon sampling it turned out that *Psilorhynchus* indeed is a genus of Cyprinidae, not a distinct family. Moreover, the improved sampling of outgroup taxa including ostaryophysan as well as one non-ostariophysan taxa should ensure the internal sorting of the cypriniform lineages and indeed, their arrangement did not change in comparison to the former tree that contained only one ostaryophysan outgroup taxon.

The study dealing with the family Botiidae included in its original form also an extensive paragraph about biogeographical aspects with the aim to compare the phylogenetic pattern of Botiidae with the published knowledge about the river history of southeast Asia and (Clark et al., 2004; Rüber et al., 2004). Unfortunately, following the suggestions of one anonymous referee, the chapter concerning the phylogeography in its original form had to be removed in the reviewed version. However, since this topic is of broader interest, I still consider it worth publishing and plan to elaborate it into more detailed form in future studies.

The paper concerning the phylogeography of the European *Misgurnus* may appear little bit out of context of the general study. However, *Misgurnus* was the last European cobitid genus whose phylogeography has not been studied. Moreover, some cytogenetic analyses revealed inconsistencies in the observed ploidy levels: usually, all European *Misgurnus* are evolutionary tetraploid and are considered to belong to the same species. However, we had recent records of naturally occurring diploid and triploid specimens, a phenomenon that in other genera indicates the secret existence of more than one species or hybrid complexes. From these facts rose a suspicion that there is an overlooked hidden diversity among the European *Misgurnus* and the present phylogeographic overview was supposed to be a pilot study with the aim to reveal potential existence of hidden mitochondrial diversity.

The Appendix 2 containing photographs of fishes is supposed to provide a visual picture of the fishes under consideration.

All the included studies have been presented on international conferences in forms of oral presentations.

NOTE: The manuscripts are not sorted in chronological order, but in a descending hierarchical taxonomic order. This means first I present the overview about the families of Cobitoidea, then the phylogenetic studies on the families Botiidae and Cobitidae, and then the manuscripts dealing with single genera.

Paper I

Families of Cobitoidea (Teleostei; Cypriniformes) as revealed from nuclear genetic data and the position of the mysterious genera *Barbucca*, *Psilorhynchus*, *Serpenticobitis* and *Vaillantella*

Vendula Šlechtová, Jörg Bohlen and Heok Hui Tan

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Short communication

Families of Cobitoidea (Teleostei; Cypriniformes) as revealed from nuclear genetic data and the position of the mysterious genera *Barbucca*, *Psilorhynchus*, *Serpenticobitis* and *Vaillantella*

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1. Introduction

The order Cypriniformes represents the world's largest clade of primary freshwater fishes (Nelson, 2006), and is divided into two monophyletic superfamilies: Cyprinoidea (carp like fishes) and Cobitoidea (loach fishes). It is generally accepted that Cyprinoidea contain a single family, Cyprinidae, although a few authors consider also the family Psilorhynchidae within Cyprinoidea (e.g. Nelson, 2006). The subfamily Cobitoidea includes several families, but their number varies between authors. Based on morphological and mitochondrial DNA data, a number of different classifications have been proposed, the most important ones are listed in Table 1. In general, the large number and diversity of the various classification concepts reflect the poor stage of knowledge about the major lineages of Cobitoidea. One of the major changes in recent times was the separation of Botiidae from Cobitidae; these two families have been treated for a long time as very closely related since both share the morphologic character of a moveable suborbital spine (Nelson, 1994). Kottelat (2004b) explicitly points on the lack of understanding of the natural lineages within Cobitoidea; he further suggests to include the genus *Psilorhynchus* into Cobitoidea. Siebert (1987) proposed that the Gyrinocheilidae and Catostomidae be included into Cobitoidea. Tang et al. (2006) presented the first classification hypothesis that was based on DNA data. Basing on sequences of mitochondrial cytochrome *b* and control region, they suggested that Gyrinocheilidae and Catostomidae indeed represent the sister lineage to the loaches sensu stricto and that Balitoridae and Nemacheilidae represent two separate families. According to their results, balitorid and nemacheilid fishes do not represent sister lineages, although most former classifications have assumed a sister-relation between these two loach groups, usually as subfamilies of a single family Balitoridae. The classification hypothesis of Tang et al. (2006) surely deserves attention, but since it is based on the rather fast evolving mitochondrial cytochrome *b* it has limited ability to resolve the relationships among the families of Cobitoidea and has to be tested with other, better suited markers with lower mutation rate Fig. 1.

The recently developed hypotheses deal only with the major lineages within Cobitoidea, while there still remain a number of taxa with uncertain phylogenetic position (Kottelat, 2004b), among them the genera *Vaillantella*, *Serpenticobitis*, *Barbucca* and *Psilorhynchus*, which have not been included into any genetic study. The uncertainties about their phylogenetic relationships document the lack of suited characters in the definition of major lineages in loach fishes Fig. 2.

Fishes of the genus *Vaillantella* are morphologically the most unusual among loaches. They differ from all other loach fishes by their unusually long dorsal fin base with a large number of branched rays (52–73); in comparison, the largest number of branched dorsal fin rays in other loach fishes is 28 in *Enobarbus maculatus* (Cobitidae). Former investigations of various morphological characters led Nalbant and Bănărescu (1977) to establish the subfamily Vaillantellinae within Cobitidae, while other authors considered *Vaillantella* as a member of Nemacheilinae (Sawada, 1982; Roberts, 1989; Kottelat, 1990, 1994). Recently, Nalbant (2002) included *Vaillantella* as subfamily Vaillan-

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Paper II

Molecular phylogeny of the freshwater fish family Cobitidae (Cypriniformes: Teleostei): delimitation of genera, mitochondrial introgression and evolution of sexual dimorphism

Vendula Šlechtová, Jörg Bohlen and Anebel Perdices

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Molecular phylogeny of the freshwater fish family Cobitidae (Cypriniformes: Teleostei): Delimitation of genera, mitochondrial introgression and evolution of sexual dimorphism

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Abstract

The family Cobitidae represents a characteristic element of the Eurasian ichthyofauna. Despite diverse features of sexual dimorphism, comparably few morphological characters have been utilized for taxonomic studies resulting in many unresolved puzzles. Here we present the phylogenetic relationships of Cobitidae as inferred from the mitochondrial cytochrome *b* gene and the nuclear gene RAG-1. Analyses of both markers show a group of eight nominal genera, which all occur in Europe and eastern, northern and western Asia, forming a monophyletic lineage (northern clade) while all other clades inhabit South and Southeast Asia (southern lineages). While all eight southern lineages correspond to genera as defined by morphological studies, only four lineages were reliably recovered within the northern clade, and of these only one (*Sabanejewia*) corresponds to a formerly considered genus. The genera *Cobitis*, *Iksookimia* and *Niwaëlla* were polyphyletic. A comparison of the two markers shows several incongruities within the northern clade and mitochondrial introgression at least in the genus *Misgurnus*. Mapping the characters of sexual dimorphism on our cladogram, we identified five character states that are diagnostic for certain lineages. Estimations of the divergence times dated the separation of the northern clade from the southern lineages to the middle Eocene (46 MYA) and the origin of "*Cobitis*" *misgurnoides*, the basal taxon of the northern clade, during early Oligocene (30–35 MYA). The geographic distribution of the major clades supports recently developed hypotheses about the river history of East Asia and further suggests that a range expansion of the northern clade in late Miocene (15 MYA) led to the colonisation of Europe by three already distinct genera.

Keywords: Molecular phylogeny; Spined loaches; Eurasia; Cytochrome *b*; RAG-1; Sexual dimorphism; Mitochondrial introgression; Hybridisation

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1. Introduction

The freshwater fish family Cobitidae represents a characteristic element of the ichthyofauna of Eurasia, where most freshwater systems host one or more members of this family. Although the family has a Palaearctic distribution, its greatest diversity is found in Southeast Asia (Nalbant, 1963, 1994). Species of Cobitidae rarely reach 15 cm in total length and, as a result of their strong adaptation to benthic habitats, have an elongated or very elongated body covered with thick skin, strongly reduced scales and small, sometimes reduced eyes (Roberts, 1989; Sterba, 1957). Cobitids were recognised as a natural assemblage based on the presence of a movable bifurcated suborbital spine,

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Paper III

Molecular phylogeny of the Southeast Asian freshwater fish family Botiidae (Teleostei: Cobitoidea) and the origin of polyploidy in their evolution

Vendula Šlechtová, Jörg Bohlen, Jörg Freyhof and Petr Ráb

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Molecular phylogeny of the Southeast Asian freshwater fish family Botiidae (Teleostei: Cobitoidea) and the origin of polyploidy in their evolution

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Abstract

The freshwater fish family Botiidae is represented by seven genera on the Indian subcontinent and in East and Southeast Asia and includes diploid as well as evolutionary tetraploid species. We present a phylogeny of Botiidae including 33 species representing all described genera using the mitochondrial cytochrome *b* and 12s rRNA genes to reconstruct the phylogenetic relationships among the genera and to estimate the number of polyploidisation events during their evolution. Our results show two major lineages, the subfamilies Leptobotiinae with the genera *Leptobotia* and *Parabotia* and Botiinae with the genera *Botia*, *Chromobotia*, *Sinibotia*, *Syncrossus*, and *Yasuhikotakia*. Our results suggest that two species that were traditionally placed into the genus *Yasuhikotakia* form a monophyletic lineage with the species of *Sinibotia*. A review of the data on the ploidy level of the included species shows all diploid species to belong to Leptobotiinae and all tetraploid species to Botiinae. A single polyploidisation event can therefore be hypothesised to have occurred in the ancestral lineage leading to the Botiinae.

Keywords: Loach fishes; Tetraploidy; Polyploidisation; Cytochrome *b*; 12s rRNA

1. Introduction

Botiid loaches represent an interesting model to study the role of polyploidisation in vertebrate evolution since they include diploid (with $2n = 50$ chromosomes) as well as evolutionary tetraploid species ($2n = 98$–100 chromosomes). From the 26 species surveyed by Suzuki and Taki (1996) in a review of ploidy level in botiid fishes, 11 were diploid and 15 tetraploid. Polyploidisation is well known as an important evolutionary force in plants and indications for its importance in the evolution of animals are constantly accumulating (Le Comber and Smith, 2004; Soltis and Soltis, 1995, 1999). Polyploidisation events seem to be more common than they had been until recently believed (Leggatt and Iwama, 2003; Soltis and Soltis, 1999) and recurrent formations of polyploid taxa were already called the norm rather than the exception (Soltis and Soltis, 1999). Examples of fish groups, in which changes in ploidy level have been already identified as key events in their evolution include Acipenseridae (Ludwig et al., 2001), Cyprinidae (Alves et al., 2001; David et al., 2003), Catostomidae (Ueno et al., 1988), and Salmonidae (Crespi and Fulton, 2004; Phillips and Ráb, 2001). Multiple origins of polyploidy were demonstrated in African barbs (Tsigenopoulous et al., 2002) and Cobitidae (Janko et al., 2003). However, to evaluate the origin of polyploidisation within Botiidae and its contribution to its evolution, it is necessary to understand the phylogenetic relationships between the diploid and tetraploid species.

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Paper IV

Low mitochondrial divergence indicates a rapid expansion across Europe in the weather loach, *Misgurnus fossilis* (L.)

Jörg Bohlen, Vendula Šlechtová, Ignacio Doadrio and Petr Ráb

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Low mitochondrial divergence indicates a rapid expansion across Europe in the weather loach, *Misgurnus fossilis* (L.)

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Several phylogeographic studies using mtDNA sequence data have revealed an expressed geographic structure in nearly every European freshwater fish species studied. The authors present a phylogeographic study of *Misgurnus fossilis* on the base of 43 specimens from 17 localities across a major part of the known distribution area of *M. fossilis*. Despite the large geographic distance between the sampling points and their origin from different major European river systems, only eight closely related haplotypes in the sequences of the whole mitochondrial cytochrome *b* were detected. The most common haplotype I included more than 60% of specimens and occurred in the North Sea basin in northern Germany, in the Danube and Elbe basins in the Czech Republic, in the Nieman basin in Poland and in the Dniester and Vistula basins in the Ukraine. Since the highest number of haplotypes (six out of eight) and the most divergent haplotypes were found in the Danube, the authors tentatively consider the Danube to have acted as a refuge area for *Misgurnus* during the glaciation maxima in the Pleistocene. From this refuge, the species presumably recolonized Central and Eastern Europe but failed to stretch to Western Europe.

Key words: colonization; fresh water; glaciations; phylogeography; Pleistocene; refuge.

INTRODUCTION

Most phylogeographic studies on European freshwater fishes using molecular genetics as a tool have revealed that the species are separated into several allopatric major lineages, often with high numbers of haplotypes (Durand *et al.*, 1999; Nesbo *et al.*, 1999; Bernatchez, 2001; Perdices *et al.*, 2003; Šlechtová

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Paper V

Phylogenetic position, karyotype and anatomy of the enigmatic loach genus *Ellopostoma* (Teleostei: Cobitoidea) with description of a new family of freshwater fish

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Manuscript

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Running title: The enigmatic loach *Ellopostoma*

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Abstract

The superfamily Cobitoidea is an important element of the Eurasian ichthyofauna, containing seven families and about 100 genera. However, the genus *Ellopostoma* cannot be assigned to any of the existing families, but has been considered as member of the families Nemacheilidae, Balitoridae and Cobitidae. It is morphologically characterised by a very small protrudable mouth, a single pair of barbels and huge eyes; a unique combination of character states among Cobitoidea. Due to the very rare occasions of collection, *Ellopostoma* has never been included into a genetic study. Recently, we obtained life specimens of *E. mystax* and present here its phylogenetic position according to nuclear sequence data (RAG-1 gene), and report on its karyotype and morphology. According to the molecular genetic data, *Ellopostoma* is a member of the superfamily Cobitoidea. It does not belong to any of the described families, but represents an independent lineage. Cytogenetically, *Ellopostoma* is characterised by a diploid chromosome number 2n = 48, a karyotype that is dominated by bi-armed chromosomes and a simple NOR phenotype. This karyotype parallels those described from other evolutionarily diploid cobitoid loaches. In 13 morphologic characters *Ellopostoma* expressed character states that do not occur in other members of Cobitoidea. These unique characters can be grouped according to their function as related to a) feeding, b) senses and c) maneuvering and reflect specific adaptations to the ecological niche inhabited by *Ellopostoma*. Our results demonstrate that *Ellopostoma* is a loach fish, but morphologically and genetically distinct from other loaches and should be considered as an own family.

Key words: molecular genetics, RAG-1, cytogenetics, chromosomes, morphology, systematics

Paper VI

Phylogeny of the Southeast Asian freshwater fish genus *Pangio* (Cypriniformes: Cobitidae) and its use as model in biogeography.

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Manuscript

Phylogeny of the Southeast Asian freshwater fish genus *Pangio* (Cypriniformes; Cobitidae) and its use as a biogeographic model

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Abstract

The genus Pangio is one of the most species-rich of the loach family Cobitidae and widespread across South and Southeast Asia. Its internal diversity has never been studied in detail under a clear phylogenetic approach, but three generic names have been synonymised with Pangio and four 'species-groups' were erected according to the most obvious morphologic characters. We present here phylogenetic analyses of the genus Pangio basing on sequence data of the mitochondrial cytochrome b gene, the nuclear recombination-activating gene 1 (RAG 1) and a combined dataset of 109 specimens from 18 morphologically identified species. Our data reveal the existence of three major lineages inside the dataset. The type species of the two synonymised genera included into the analyses were nested inside Pangio; therefore our data support their status as synonyms of Pangio. Two of our major lineages were congruent with formerly proposed species-groups, the remaining two species-groups were joined in the third major lineage; therefore we can refer to the lineages as anguillaris-group, kuhlii-group and shelfordii-group. The application of a molecular clock approach dated the age of the lineages between 33 and 29 million years. On the species level, our data suggest about 30 monophyletic lineages, indicating that there is a number of undescribed species within Pangio. In at least one case, horizontal gene flow between two co-occurring species was detected. The usability of Pangio as biogeographic model was tested on the shelfordii-group that is distributed across Sundaland and two cases of faunal exchange between Borneo and the Malay Peninsular, two cases of vicariance and two cases of speciation, all of these during Miocene, could be detected.

Key words: eel loaches, phylogeography, species group, Sundaland, *Eucirrichthys, Cobitophis*

Introduction

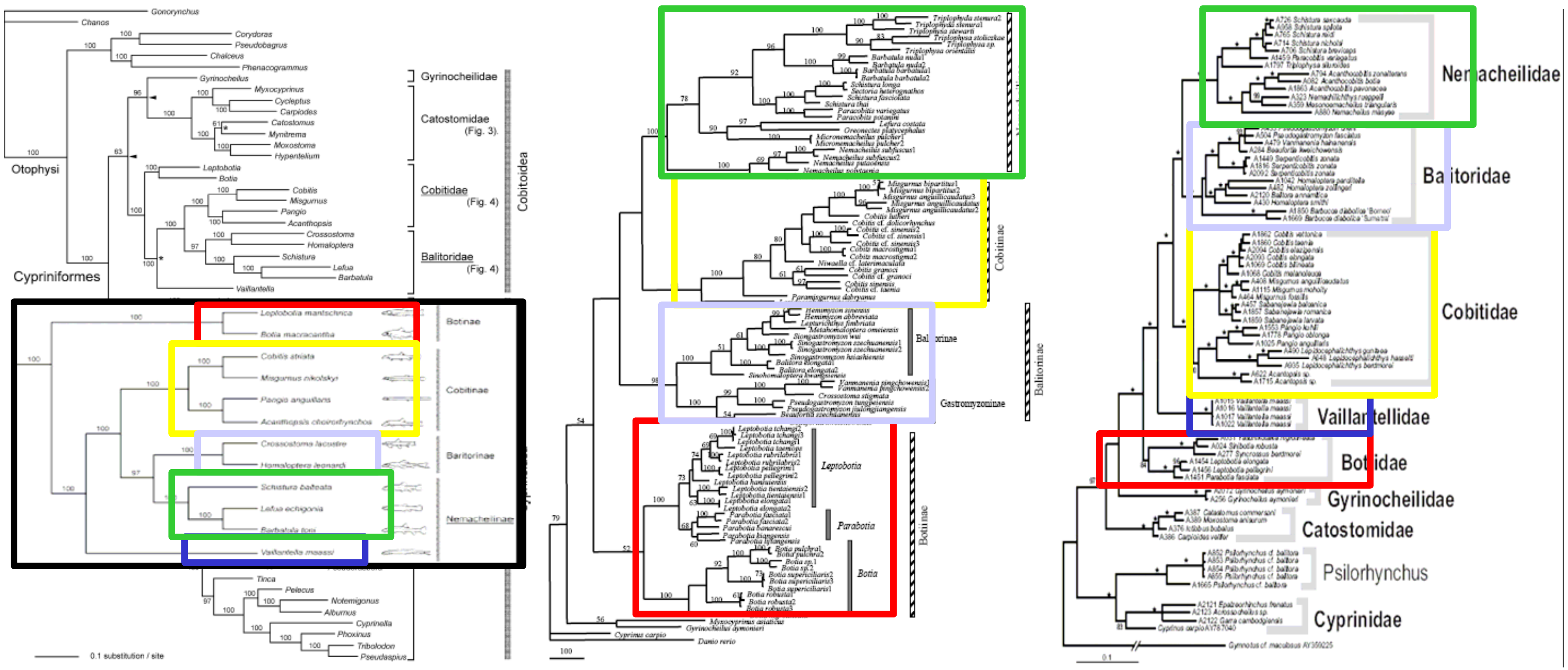
The freshwater fish family Cobitidae represents a characteristic element of Eurasian ichthyofauna that occurs with about 19 genera and 130 species in nearly all water systems from Portugal to Japan (Bănărescu, 1990; Nelson, 2006). As recently shown, this family includes a distinct, monophyletic group of taxa that are distributed in Europe, northern and East Asia ('northern lineage') that stems out of a paraphyletic assemblage of well-differentiated genera that are distributed in South and Southeast Asia ('southern lineages') (Šlechtová et al., 2008). While several genera of the northern lineage have been used as a

SUMMARY OF RESULTS AND CONTRIBUTION OF THE STUDY TO CURRENT RESEARCH

The present thesis intended to study the phylogenetic structures and interrelations among fishes of the superfamily Cobitoidea at the level of superfamily, family and, in case of two families, genera. In order to reach this aim, the number of lineages and their outlines were estimated using modern methods of molecular genetics. With these methods, we could confirm the validity of five families and establish two new families. In the frame of this project, the number, outline and relationships of the genera of two families (Cobitidae and Botiidae) were studied.

The general impact of the present study has to be seen in the frame of the high interest in biodiversity research during the last decade. Since molecular genetics nowadays offers a set of new and convincing tools to study biodiversity into formerly unreachable details and resolution, the reconstruction of the phylogeny and evolutionary history of animals has become a major topic in biological science. The background for this activity is the growing understanding that a high biodiversity is one of the most important control mechanisms to stabilise ecosystems locally as well as globally. In this context, my study is a contribution to understand the biodiversity of one of the most typical and widespread freshwater fish groups of Eurasia. A proper definition of the investigated subject is the crucial base for any kind of biological research. In the case of natural lineages, a proper definition is the requirement for all kinds of studies of evolution, biogeography and comparative morphology of the group in question.

On the level of the Cobitoidea, the present study clarified the number of major lineages and their phylogenetic relationships to each other. Four formerly recognised families were supported including Gyrinocheilidae and Catostomidae (which were not always considered as Cobitoidea), Cobitidae and the very recently erected family Botiidae. An indication for a split between Nemacheilidae and Balitoridae was presented and two new families were identified. The new results allowed further to give a clear definition of the termini ‘Cobitoidea’ and ‘loaches’, which were formerly used in inconsistent way. To bring the results of the present study into a broader frame, we can compare them with those of other studies, which were published during the work on this thesis. Although other studies usually did not aim to study the diversity of Cobitoidea in a detailed way and have rather poor coverage of the relevant taxa. In Fig. 1, the results of such studies are illustrated next to my results



Saitoh et al. 2006
Whole mitochondrium

Tang et al. 2006
Cytochrome *b* + D-Loop

Šlechtová et al. 2007
RAG-1

Fig. 1. Comparison of the results of the recent studies which included cobitoid taxa.

and the identified families are marked with different colours. As can be seen, all three studies identified the same families, although they differed in the marker (whole mitochondrial genome in study of Saitoh et al., 2006; cytochrome *b* and D-loop in study of Tang et al., 2006; and nuclear gene RAG-1 in the study of Šlechtová et al., 2007) and the composition of taxa and were carried out independently in different institutions and by differed researchers. This broad agreement between such different studies can be taken as signal that the outline of the families as proposed in these studies comes close to the natural situation and gives rise to the hope that we have reached a stable taxonomic and phylogenetic platform for further scientific investigations of various kinds.

The phylogenetic relationships between the identified families revealed a number of surprises. First, the Botiidae and Cobitidae did not turn out as sister lineages as it was proposed for long time. In fact, these two groups were placed in the same family since the first time they have been classified by Berg (1940). The interesting contribution was the finding of clear split between Nemacheilidae and Balitoridae, two groups that experienced rich and conflictful taxonomical history and went through many changes. Following Sawada (1982) they have been recently classified as a single family Balitoridae (Nelson, 1994; Nelson, 2006). Tang et al. (2006) considered Nemacheilidae to be closer to Cobitidae than to Balitoridae, although the used mitochondrial markers were not able to resolve this relationship and Balitoridae and Nemacheilidae formed trichotomy with Cobitidae. In the studies of Saitoh et al. (2006) and Šlechtová et al. (2007) Nemacheildae and Balitoridae still formed sister lineages, In both cases (Balitoridae/Nemacheilidae and Botiidae/Cobitidae), there have been morphologic characters that had appeared to be so convincing that they had been assumed as synapomorphies without testing of these dogmas. In the case of Botiidae and Cobitidae, this morphologic character was the presence of an erectable suborbital spine formed by lateral ethmoid bone (Berg, 1940; Sawada, 1982; Nalbant, 2002); while in the case of Nemacheilidae and Balitoridae the characters were the arrangement of barbels and the shape of the swim bladder (Bănărescu and Nalbant, 1995; Nalbant, 2002). My analyses show these characters to be not as reliable as formerly proposed for the definition of the families. In general, my phylogenetic analyses can help to re-evaluate the suitability of morphologic characters that were formerly used to define the lineages within Cobitoidea and that were also used to reconstruct the evolutionary history of the group. In the next step, new characters may be chosen as most useful for this purpose and will provide future ichthyologists with an improved set of characters.

Another important improvement in the composition of Cobitoidea that comes from my studies is the finding of two new families of vertebrates. The enigmatic genera *Vaillantella* and *Ellopostoma* turned out to form independent lineages that reach the rank of families.

This new insight may end the long-term controversial discussion about their phylogenetic relationships. As explained in more detail in the introduction, the genus *Vaillantella* was successively considered a member of Nemacheilidae, of Cobitidae and of Botiidae; while for the genus *Ellopostoma*, all trials to associate it with other families have resulted in opinions of little conviction.

On the level of families, besides the providing definitions of the families, the first reliable placement of most enigmatic taxa of Cobitoidea into families can be considered the biggest contribution of the present study. The enigmatic taxa have disturbed any discussion about the diversity of Cobitoidea since shifts between different lineages always led to major changes in the definition of these families. Now we know that for instance *Psilorhynchus* is not a cobitoid (loach-like) fish but a cyprinoid (carp-like) fish, and therefore no taxonomist has further to wonder which characters are shared with the Cobitoidea and why it has such similarities with Cyprinoidea. Shortly before this article was released, a study based on investigation of the gill-arch osteology of two species of *Psilorhynchus* (*P. sucatio* and *P. balitora*) was published (Conway and Mayden, 2007). In their article they concluded that the genus *Psilorhynchus* is more closely related to non-cyprinid cypriniforms than to members of the Cyprinidae. However, our article initiated a re-evaluation of the case with use of different osteological characters by K. Conway. In his recent revision he found more evidence that *Psilorhynchus* belongs to Cyprinoidea, more precisely to Cyprinidae (Conway, 2007). It was also of particular importance to find the correct phylogenetic position for *Serpenticobitis*, since it shares formerly diagnostic characters with two families, Nemacheilidae and Cobitidae. The fact that *Serpenticobitis* does not belong to either of these two families, but to Balitoridae, has to open our eyes for a fresh evaluation of the ‘diagnostic’ characters. Most interesting, *Serpenticobitis* bears also the suborbital spine mentioned for Botiidae and Cobitidae. Its occurrence in a member of Balitoridae led to the conclusion that this grave character is a symplesiomorphy of ‘loaches’ that was secondarily reduced in several lineages.

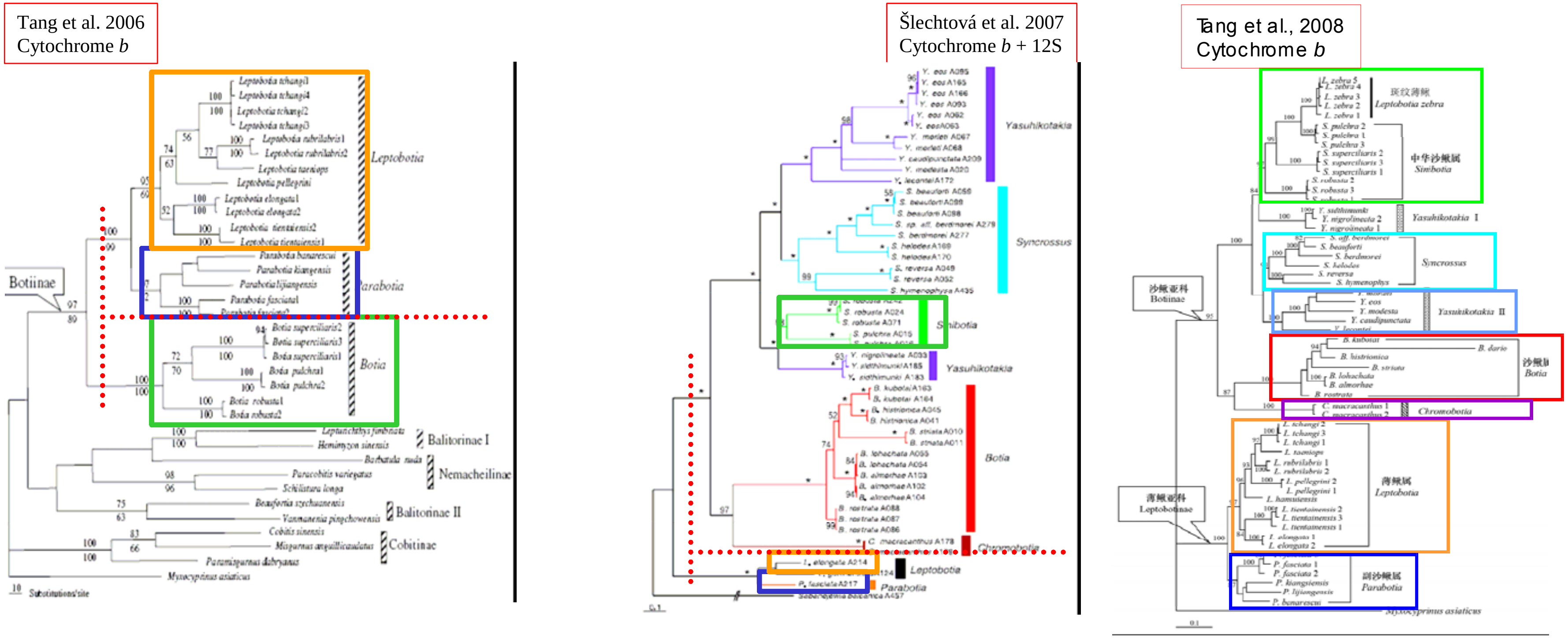
As mentioned above, the precise and reliable definition of the families and the correct classification of the taxa is a basic requirement for all kind of further biological investigations.

Looking on the intrafamily level, I see the contribution of my studies in the identification of the genera within the families Botiidae and Cobitidae and the reconstruction of their phylogeny. As we have done on the level of Cobitoidea, we can compare at least my arrangement of genera of Botiidae with the arrangements from other studies (Fig. 2). As we can see, the studies of Tang et al. (2006, 2008) show the same outline of the genera, although they have been based on a different taxon collection. Additionally, the deep split between the groups of genera that we referred to as Leptobotiinae and Botiinae, respectively, has been found in the comparative studies and Tang et al. (2008) even follow our suggestion to consider them as subfamilies. From the good agreement of the studies it can be concluded that also here we have managed to identify the natural situation. The fast acceptance of our conclusion by other research teams indicates the possibility that this will be the state-of-the-art for the future.

In the case of Cobitidae, my study is the first of its kind and no comparison with other studies is possible. Therefore, my study is the first formulated hypothesis that bases on a complex molecular genetic analyses of Cobitidae and can now undergo testing by other studies using different markers.

A phenomenon that gains increasing attention as general factor for evolution is polyploidisation. It was also repeatedly discussed as a driving force in the evolution of Cobitidae as well as of Botiidae, since in both families diploid as well as polyploid taxa are found. Our analysis of Botiidae has revealed a single evolutionary event to be responsible for the tetraploidy of five of the genera. In opposite, the polyploid taxa within Cobitidae were scattered across the part of the tree we refer to as ‘northern lineage’. It seems to have impact on species or intraspecific level mainly and can be considered to have evolved a number of times independently. Therefore, the comparison of these two loach families can show to other research teams that are

Fig. 2. Comparison of the results of different studies on Botiidae.



Tang et al. 2006
Cytochrome *b*

Šlechtová et al. 2007
Cytochrome *b* + 12S

Tang et al., 2008
Cytochrome *b*

working on the impact of polyploidisation in evolution that polyploidisation has various effects on the evolution of fish groups and has to be studied case by case. The polyploidisation event played undoubtedly a very important role in the diversification of the family Botiidae, although it is unclear at present whether the evolutionarily tetraploidy of one major lineage of botiid fishes is of auto- or allopolyploid origin. Besides some autopolyploid species (e.g. Cobitis biwae 'big race', Kitagawa et al., 2003), the salmonids (family Salmonidae) is the only fish group where an autotetraploid origin after a single polyploidisation event was unambiguously evidenced (Phillips and Ráb, 2001; Crespi and Fulton, 2004), while in several cyprinid (e.g. Chenuil et al., 1999; David et al., 2002) and/or cobitid (Janko et al., 2003) lineages the data-sets indicate allopolyploid, i.e. hybrid, and also polyphyletic (Tsigenopoulous et al., 2002) origins of polyploidy. Alves et al. (2001) also hypothesised that the stage with asexual reproduction after a hybridisation event might be associated with an increase of ploidy level. Then, the two alternative scenarios for the tetraploidy of botiid fishes can be proposed to formulate testable hypotheses 1) single autotetraploid event or 2) hybridisation events probably associated with a stage with asexual type of reproduction shortly after reproductive contacts of distinct taxa and subsequent elevation of ploidy level via interplay of genomes of parental hybridising species.

The analyses of selected genera of Cobitidae under the light of their evolutionary history and biogeography demonstrated that loaches are well-suited model organisms to study such kind of questions. The different results from the analysis of the European *Misgurnus*, which showed the lowest genetic diversity ever reported for a European freshwater fish, and the Southeast Asian *Pangio*, which revealed a great diversity on species as well as intraspecific level, are caused by the different factors influencing their evolution, but reflect very well the geologic history of the inhabited areas. For Europe, a number of freshwater taxa have been studied so far and usually revealed a higher diversity that was formerly considered (e.g. Bohlen et al., 2006; Perdices et al., 2003; Šedivá et al., 2008; Šlechtová et al., 2004; Tsigenopoulos and Berrebi, 2000; Volckaert et al., 2002). However, *Misgurnus* represented the first really lowland species and the differences to the formerly studied riverine and mountainous species indicate that fishes of different autecology underwent different evolutionary histories. This insight is a new contribution to the field of phylogeography of European freshwaters and may provide a promising topic for future research.

In the study of *Pangio*, a big potential for biodiversity studied appeared. My studies demonstrated that a high number of species are still undetected and that a geographically fine-scaled sampling is necessary to cover the existing diversity and to reconstruct properly the biogeographic history of the area under consideration. This conclusion of the study has already motivated the work of ichthyologic taxonomists from the National University of Singapore in the way that some of the taxa detected in the genetic analysis are presently under description as new species (Tan, pers. com.).

I would expect that the biogeographic results from our study on *Pangio* will receive an impact at least as important as the conclusions on biodiversity. Due to its wide occurrence and low migration potential *Pangio* seems to be a well-suited model for biogeographic studies in SE Asia. The reconstruction of several faunal exchange events between Borneo and the Malay Peninsular are up to now the most precise published and clearly show the immense importance of changes of the global sea water level for the biogeography of SE Asia. This is especially important since there is a serious lack of biogeographic studies in the freshwater systems of SE Asia. Up to now, there are only very few studies in this field that can be considered serious: on phylogeny and phylogeography of Badidae carried out by Rüber et al. (2004), of the Chinese catfish family Sisoridae elaborated by Guo et al. (2005) and very recently on the phylogeny and biogeography of the cyprinid genus *Tor* (Nguyen et al., 2008). The study on Badidae deals with the phylogeography of this group in Indochina, an area that was mainly influenced by the Himalayan orogenesis and not by sea level fluctuations. Also, there have been several studies focused on biogeography of Sundaland and the seawater fluctuations connected with this area (Cannon et al., 2003; Bruyn et al., 2005). However, all these studies dealt only with the patterns reflecting the changes during the last glacial maximum. Although there are several papers about historical geography or geomorphology of this area focused mainly on the sea level fluctuations (Bird et al., 2007; Voris, 2000; Rohling et al., 1998; Woodruff, 2003), very few biogeographical studies (Inger et al., 2001) went further in past with the trial to reconstruct the impact of the older geological events on evolution of higher taxonomic units. Therefore, the use of loaches as biogeographic models can be considered a promising approach.

OUTLOOK FOR FUTURE RESEARCH

Looking in February 2008 on the situation of phylogenetic research in Cobitoidea, it appears that the knowledge about the organisation of the Cobitoidea into families has reached a satisfying level that can be used as working platform for the future studies. Also, the generic organisation of the families Botiidae, Cobitidae, Ellopostomatidae and Vaillantellidae among the loaches is resolved in a way that should suit the next investigations. In opposite, the knowledge about the natural groups and their phylogenetic relationships in the families Nemacheilidae and Balitoridae cannot be described other than abysmal. There is no existing idea about the real number of genera in terms of natural evolutionary lineages and most taxonomic works agree than many if not most genera are artificial groupings and that a major part of diversity in these families has not yet been discovered. Therefore, phylogenetic studies of these families on the generic level would be highly needed.

At the same time, the family Nemacheilidae is the most widespread (across all of Eurasia and via the Near East into Ethiopia), the most numerous (at present, some 450 species, but the number increases steadily) and also the ecologically most plastic of all loaches: most Nemacheilidae live in swift creeks and rivers with pebble or stone structure (Kottelat, 1990a), but at least 15 cave species from seven genera are known (Romero and Paulson, 2001), two genera (*Lefua*, *Yunnanilus*) adopted for a pelagic life in swamp habitats and they include also fish inhabiting the highest-altitutes in the world (*Triplophysa* in Himalayan Mountains, up to 5400 m; Kottelat and Chu, 1988). This highly diverse ecology allows Nemacheilidae to inhabit nearly all rivers of Eurasia, and in big parts of the general distribution area, more than one species co-occur. The dense geographical coverage in some areas makes Nemacheilidae a promising target for biogeographic studies. Since their most commonly occupied niches are flowing waters, this group would provide an excellent model to study biogeographic history of piedmont and mountain areas in mainland Eursia. .

On the level of families, within the Botiidae there is still a need to confirm, best with the use of other molecular markers, the monophyly of the subfamilies Botiinae and Leptobotiinae, to confirm the genera with a nuclear marker and to solve the phylogenetic position of the species *Yasuhikotakia nigrolineata* and *Y. sidthimunki*. Open topics regarding the Cobitidae are the monophyly of the genus *Acanthopsoides*, description of at least two new genera, the degree of hybridisation in the northern lineage and to study the phylogenetic relationships within *Cobitis s.l.* with a much enlarged sampleset and to try to identify and extract carefully potential genera from this at present unsorted mess.

However, as common in research, opening one topic necessarily leads to many new questions and many more ideas than listed above about what should be solved in future are arising in my mind.

Some of these topics will fill my scientific life for the next years, and I hope to be able to contribute to an increased clarity of our understanding of the diverse world of loaches.

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APPENDIX 1

Following is a list of loach genera including the number of valid species, the name of the type species and comments on their status according to the online Eschmeyer's Catalog of Fishes (ECF) and results of the present study. Since the families Nemacheilidae and Balitoridae were not main aim of the present Thesis (therefore not studied here in detail) and their systematics is in a disastrous stage (in terms of questioner number of species as well as their generic status), I provide here only list of valid genera as reported in ECF.

Balitoridae

Annamia Hora, 1932; type species *A. normani* (Hora, 1931).

Balitora Gray, 1830; type species *B. brucei* Gray, 1830.

Balitoropsis Smith, 1945; type species *B. bartschi* Smith, 1945.

Barbucca Roberts, 1989; type species *B. diabolica* Roberts, 1989.

Beaufortia Hora, 1932; type species *B. leveretti* (Nichols and Pope, 1927).

Bhavania Hora, 1920; type species *B. australis* (Jerdon, 1849).

Cryptotora Kottelat, 1998; type species *C. thamicola* (Kottelat, 1988).

Dienbienia Nguyen and Nguyen, 2002; type species *D. namnuaensis* Nguyen and Nguyen, 2002.

Erromyzon Kottelat, 2004; type species *E. sinensis* (Chen, 1980).

Formosania Oshima, 1919; type species *F. gilberti* Oshima, 1919.

Gastromyzon Günther, 1874; type species *G. borneensis* Günther, 1874.

Glaniopsis Boulenger, 1899; type species *G. hanitschi* Boulenger, 1899.

Hemimyzon Regan, 1911; type species *H. formosana* (Boulenger, 1894).

Homaloptera van Hasselt, 1823; type species *H. ocellata* Van der Hoeven, 1833.

Homalosoma Boulenger, 1901; type species *H. stenosoma* Boulenger, 1901.

Hypergastromyzon Roberts, 1989; type species *H. humilis* Roberts, 1989.

Jinshaia Kottelat and Chu, 1988; type species *J. sinensis* (Sauvage and Dabry de Thiersant, 1874).

Katibasia Kottelat, 2004; type species *K. insidiosa* Kottelat, 2004.

Labigastromyzon Tang and Chen, 1996; type species *L. fangi* (Nichols, 1931).

Lepturichthys Regan, 1911; type species *L. fimbriata* (Günther, 1888).

Liniparhomaloptera Fang, 1935; type species *L. disparis* (Lin, 1934).

Metahomaloptera Chang, 1944; type species *M. omeiensis* Chang, 1944.

Neogastromyzon Popta, 1905; type species *N. nieuwenhuisii* (Popta, 1905).

Neohomaloptera Herre, 1944; type species *N. johorensis* Herre, 1944.

Paraprotomyzon Pellegrin and Fang,, 1935; type species *P. multifasciatus* Pellegrin and Fang, 1935.

Parhomaloptera Vaillant, 1902; type species *P. obscura* Vaillant, 1902.

Plesiomyzon Zheng and Chen, 1980; type species *P. baotingensis* Zheng and Chen, 1980.

Protomyzon Hora, 1932; type species *P. whiteheadi* (Vaillant, 1894).

Pseudogastromyzon Nichols, 1925; type species *P. zebroidus* (Nichols, 1925).

Pseudohomaloptera Silas, 1953; type species *P. tatereganii* (Popta, 1905).

Serpenticobitis Roberts, 1997; type species *S. octozona* Roberts, 1997.

Sewellia Hora, 1932; type species *S. lineolata* (Valenciennes, 1846).

Sinogastromyzon Fang, 1930; type species *S. wui* Fang, 1930.

Sinohomaloptera Fang, 1930; type species *S. kwangsiensis* (Fang, 1930).

Travancoria Hora, 1941; type species *T. jonesi* Hora, 1941.

Vanmanenia Hora, 1932; type species *V. stenosoma* (Boulenger, 1901).

Botiidae

Botia Gray, 1831; 8 species; type species *B. almorhae* Gray, 1831; monophyly supported.

Chromobotia Kottelat, 2004; monotypic genus; type species *C. macracanthus* (Bleeker, 1852); molecular data justify its generic status.

Leptobotia Bleeker, 1870;, 13 species; type species *L. elongata* (Bleeker, 1870); monophyly supported.

Parabotia Dabry de Thiersant, 1872; 7 species; type species *P. fasciatus* Guichenot, 1872; monophyly supported.

Sinibotia Fang, 1936; 6 species; type species *S. superciliaris* (Günther, 1892); mitochondrial data suggest to include also the species *S. nigrolineata* and *S. sidthimunki*, two species that are presently considered to belong to the genus *Yasuhikotakia.*

Yasuhikotakia Nalbant, 2002; 9 species; type species *Y. modesta* (Bleeker, 1865); the inclusion of *Y. nigrolineata* and *Y. sidthimunki* must be justified.

Cobitidae

Acanthopsoides Fowler, 1934; 6 species; type species *A. gracilis* Fowler, 1934; molecular data neither support nor reject the monophyly of this genus.

Acantopsis van Hasselt, 1823; 5 species, type species *A. dialuzona* van Hasselt, 1823; according to morphological and molecular analyses appears as monophyletic lineage, the taxonomy of *Acantopsis* is in very poor stage and there are many undescribed species

Bibarba Chen and Chen,, 2007; monotypic genus; type species *B. bibarba* Chen and Chen, 2007; not included into the study due to very recent designation.

Canthophrys Swainson, 1838; monotypic genus; type species *C. gongota* (Hamilton, 1822). Formerly known as *Somileptes*

Cobitis Linnaeus, 1758; about 40 species; type species *C. taenia* Linnaeus, 1758; molecular data indicate that Cobitis is a polyphyletic genus and is included into *Cobitis* s.l., a large collective group within northern Cobitidae.

'Cobitis' misgurnoides Rendahl, 1944, represents in all molecular analyses a distinct group within the 'northern lineage' and should be described as new genus.

***Cobitis* s.l.**; is hereby newly suggested assemblage within northern Cobitidae collecting all species of the proposed genera *Iksookimia, Kichulchoia, Niwaëlla* and all but one *Cobitis* (exception is *C. misgurnoides*). Specific and detailed studies are necessary to understand the generic classification within *Cobitis s.l*. (a molecular analyses is in progress).

Enobarbus (Day, 1868); monotypic genus; type species *E. maculatus* (Day, 1868); according to Harant and Bohlen (2007 submitted to J. Fish. Biol.) *Enobarbus* is a younger synonym of *Lepidocephalichthys*.

Iksookimia Nalbant, 1993; 6 species; *I. koreensis* (Kim, 1975); monophyly not supported.

Kichulchoia Kim, Park and Nalbant, 1999; monotypic genus; type species *K. brevifasciata* (Kim and Lee, 1995); Status as distinct genus questionable, inside *Cobitis* s.l.

Koreocobitis Kim, Park and Nalbant, 1997;, 2 species; type species *K. rotundicauda* (Wakiya and Mori, 1929); monophyly supported.

Kottelatlimia Nalbant, 1994;, 2 species; type species *K. katik* (Kottelat and Lim, 1992); monophyly supported

Lepidocephalichthys Bleeker, 1863; about, 19 species; type species *L. hasselti* (Valenciennes, 1846); monophyly supported.

Lepidocephalus Bleeker, 1857;, 2 species; type species *L. macrochir* Bleeker, 1854; molecular data justified its generic status (generic status was formerly not always accepted).

Misgurnus Lacepède, 1803; about 6 species; taxonomy in poor stage; type species *M. fossilis* (Linnaeus, 1758); nuclear molecular data do not reject monophyly, mitochondrial data indicate an ancient mitochondrial introgression.

Neoeucirrhichthys Banarescu and Nalbant, 1968; monotypic genus; type species *N. maydelli* Banarescu, 1968; molecular data justify its generic status.

Niwaëlla Nalbant, 1963; 5 species; type species *N. delicata* (Niwa, 1937); monophyly not supported.

Pangio Blyth, 1860; about, 22 species; type species *P. pangia* (Hamilton, 1822); monophyly supported.

Paralepidocephalus Thang, 1935;, 2 species; type species *P. yui* Thang, 1935; not included into the study due to its rarity.

Paramisgurnus Dabry de Thiersant, 1872; monotypic genus; type species *P. dabryanus* (Guichenot, 1872). Molecular data suggest to consider *Paramisgurnus* as synonym of *Misgurnus*.

Protocobitis Yang and Chen, 1993; monotypic genus; type species *P. typhlops* Yang, Chen and Lan in Yang and Chen, 1993; not included into the study due to its rarity.

Sabanejewia Vladykov, 1929;, 10 species; type species *S. balcanica* (Karaman, 1922); monophyly supported.

Ellopostomatidae

Ellopostoma Vaillant, 1902;, 2 species; type species *E. megalomyster* (Vaillant, 1902).

Nemacheilidae

Aborichthys Chaudhuri, 1913; type species *A. kempi* Chaudhuri, 1913.

Barbatula Linck, 1790; type species *B. barbatula* (Linnaeus, 1758).

Dzihunia Prokofiev, 2001; type species *D. amudarjensis* (Rass, 1929).

Heminoemacheilus Zhu and Cao, 1987; type species *H. zhengbaoshani* Zhu and Cao, 1987

Ilamnemacheilus Coad and Nalbant, 2005; type species *I. longipinnis* Coad and Nalbant, 2005.

Indoreonectes Rita and Banarescu in Rita, Banarescu and Nalbant, 1978; type species *I. keralensis* (Rita, Banarescu and Nalbant, 1978); valid or synonym of *Oreonectes*.

Lefua Herzenstein, 1888; type species *L. pleskei* (Herzenstein, 1888).

Mesonoemacheilus Banarescu and Nalbant in Singh et al., 1982; type species M. *triangularis* (Day, 1865).

Micronemacheilus Rendahl, 1944; type species *M. cruciatus* (Rendahl, 1944).

Nemacheilus Bleeker, 1863; type species *N. fasciatus* (Valenciennes, 1846).

Nemachilichthys Day, 1878; type species*N. rueppelli* (Sykes, 1841).

Neonoemacheilus Zhu and Guo, 1985; type species *N. labeosus* (Kottelat, 1982).

Nun Banarescu and Nalbant in Banarescu, Nalbant and Goren, 1982; type species *N. galilaea* (Günther, 1864).

Oreonectes Günther, 1868; type species *O. platycephalus* Günther, 1868.

Oxynoemacheilus Banarescu and Nalbant, 1966; type species *O. persa* (Heckel, 1847).

Paracobitis Bleeker, 1863; type species *P. malapterura* (Valenciennes, 1846).

Paranemachilus Zhu, 1983; type species *P. genilepis* Zhu, 1983

Physoschistura Banarescu and Nalbant in Singh et al., 1982 type species *P brunneanus* Annandale, 1918.

Protonemacheilus Yang and Chu, 1990; type species *P. longipectoralis* Yang and Chu, 1990. .

Schistura McClelland, 1838; type species *S. rupecula* (McClelland, 1838).

Sectoria Kottelat, 1990; type species *S. atriceps* (Smith, 1945).

Seminemacheilus Banarescu and Nalbant, 1995; type species S. *lendlii* (Hankó, 1924).

Sphaerophysa Cao and Zhu, 1988; type species *S. dianchiensis* Cao and Zhu, 1988

Sundoreonectes Kottelat, 1990; type species *S. obesus* (Vaillant, 1902); Valid or synonym of *Oreonectes*.

Traccatichthys Freyhof and Serov, 2001; type species *T. taeniatus* (Pellegrin and Chevey, 1936); Vaĺid or synonym of *Micronemacheilus*.

Triplophysa Rendahl, 1933; type species *T. hutjertjuensis* (Rendahl, 1933).

Troglocobitis Parin, 1983; type species *T. starostini* (Parin, 1983).

Tuberoschistura Kottelat, 1990; type species *T. baenzingeri* (Kottelat, 1983).

Turcinoemacheilus Banarescu and Nalbant, 1964; type species *T. kosswigi* Banarescu and Nalbant, 1964.

Yunnanilus Nichols, 1925; type species *Y. pleurotaenius* (Regan, 1904).

Vaillantellidae

Vaillantella Fowler, 1905; 3 species; type species *V. euepiptera* (Vaillant, 1902)

Appendix 2

Illustrations of representatives of loaches included in the current study either for molecular analyses or for morphological comparisons.

Balitoridae

Barbucca diabolica Roberts, 1989; Balitoridae; Indonesia (Sumatra), Jambi Province; photo: Tan H. H.

Gastromyzon ctenocephalus Roberts, 1982; Balitoridae; ornamental fish trade; A0283; photo: J. Bohlen 2005.

Hemimyzon nanensis Doi and Kottelat, 1998; Balitoridae; Thailand, Chao Phraya R. basin, Mae Nam Yom R.; photo: V. Šlechtová 2005

Homaloptera confuzona Kottelat, 2000; Balitoridae; Thailand, Narathiwat Province; A0557; photo: J. Bohlen 2005.

Homaloptera parclitella Tan and Ng, 2005; Balitoridae; Thailand, Narathiwat Province; A0549; photo: J. Bohlen 2005.

Pseudogastromyzon cheni Liang, 1942; Balitoridae; ornamental fish trade; A0434; photo: J. Bohlen 2005.

Serpenticobitis cingulata Roberts, 1997; Balitoridae; paratype CAS95172, 37 mm SL; photo: J. Bohlen 2007.

Serpenticobitis cingulata Roberts, 1997; Balitoridae; paratype CAS95171, 25 mm SL; photo: J. Bohlen 2007

Serpenticobitis zonata Kottelat, 1998; Balitoridae; Thailand, Chanthaburi province, Mekong R. basin, Mae Nam Pong R., A1449; photo: J. Bohlen 2006.

Vanmanenia hainanensis Chen and Zheng, 1980; Balitoridae; ornamental fish trade; photo: J. Bohlen 2005.

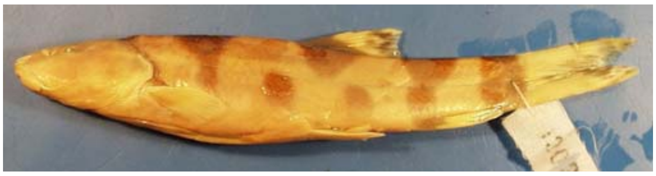
Botiidae

Botia almorhae Gray, 1831; Botiidae; ornamental fish trade; A0102; photo: V. Šlechtová 2004.

Botia striata Narayan Rao, 1920; ornamental fish trade; A0010, photo: V. Šlechtová 2008.

Chromobotia macracanthus (Bleeker, 1852); Botiidae; ornamental fish trade; photo: V. Šlechtová 2008.

Leptobotia elongata (Bleeker, 1870); Botiidae; China China, Upper Chang Jiang R., Chendu, Sichuan Province; A1443; photo: V. Šlechtová 2008.



Leptobotia elongata (Bleeker, 1870); Botiidae; China; A0214/1303180 (Inst. Hydrobiol., Wuhan); photo: J. Bohlen 2004.

Parabotia banarescui (Nalbant, 1965); Botiidae; China; A0216/1303182 (Inst. Hydrobiol., Wuhan); photo: J. Bohlen 2004.

Parabotia fasciata Dabry de Thiersant, 1872; Botiidae; China, Li Jiang R., Guilin, Guangxi Province; A1450; photo: V. Šlechtová 2008.

Sinibotia pulchra (Wu, 1939); Botiidae; ornamental fish trade; A0015; photo: V. Šlechtová 2004.

Syncrossus berdmorei Blyth, 1860; Botiidae; Laos, Mekong R. basin; Xe Bang Fai R.; A0564; photo: V. Šlechtová 2005.

Yasuhikotakia caudipunctata (Taki and Doi, 1985); Botiidae; ornamental fish trade; A2711; photo: V. Šlechtová 2008

Yasuhikotakia morleti (Tirant, 1885); Botiidae; ornamental fish trade; A0282; photo: J. Bohlen 2005.

'Yasihikotakia' sidtimunki (Klausewitz, 1959); Botiidae; ornamental fish trade; photo: V. Šlechtová 2008.

Cobitidae

Acantopsis van Hasselt, 1823 **sp.**; Cobitidae; ornamental fish trade; photo: J. Bohlen 2006.

Cobitis lutheri Rendahl, 1935; Cobitidae; Korea , Jin Am R.; A1930; photo: J. Bohlen 2006

Cobitis pacifica Kim, Park and Nalbant, 1999; Cobitidae; Korea, Cheon Jin R.; A2007 photo: J. Bohlen 2006.

Cobitis pacifica Kim, Park and Nalbant, 1999; Cobitidae; Korea, Cheon Jin R.; A2002 photo: J. Bohlen 2006

Cobitis misgurnoides Rendahl, 1944; Cobitidae; Viet Nam, photo: J. Bohlen 2003

Enobarbus maculatus (Day, 1868); Cobitidae; holotype, India, Madras region; BMNH 1968_10_27_36; photo: V. Šlechtová 2006.

Iksookimia koreensis (Kim, 1975); Cobitidae; Korea, Han Tan R.; A1868; photo: J. Bohlen 2006

Iksookimia longicorpa (Kim, Choi and Nalbant, 1976); Cobitidae; Korea, Seon Jin R., A1918; photo: J. Bohlen 2006.

Iksookimia pumila (Kim and Lee, 1987); Cobitidae; Korea, Baeng R.; A1987; photo: J. Bohlen 2006.

Iksookimia yongdokensis Kim and Park, 1997; Cobitidae; Korea, Dae Seo R.; A1896; photo: J. Bohlen 2006.

Kottelatlimia pristes (Roberts, 1989); Cobitidae; Malaysia, Sarawak, Sarawak R. basin; A1626; photo: V. Šlechtová 2006.

Koreocobitis naktongensis Kim, Park and Nalbant, 2000; Cobitidae; Korea, Geum R., A1970; photo: J. Bohlen 2006.

Koreocobitis rotundicauda (Wakiya and Mori, 1929); Cobitidae; Korea, Jeon R.; A1886; photo: J. Bohlen 2006.

Lepidocephalichthys berdmorei (Blyth, 1860); Cobitidae; Thailand, Mekong R. basin, Mae Nam Fang R.; A0680; photo: V. Šlechtová 2005.

Lepidocephalichthys thermalis (Valenciennes, 1846); Cobitidae; Sri Lanka; photo: J. Bohlen 2006.

Misgurnus fossilis (Linneaeus, 1758); Cobitidae; Czech Republic, Morava R.; photo: V. Šlechtová 2008.
(male with horizontal swelling during spawning season)

Misgurnus Lacepède, 1803, **sp. 2**; Cobitidae; Korea, Chuk San R.; A1958; photo: J. Bohlen 2006.

Neoeucirrhichthys maydelli Banarescu and Nalbant, 1968; Cobitidae; Bangladesh, Brahmaputra R. basin; A0500; photo: J. Bohlen 2006.

Niwäella multifasciata (Wakiya and Mori, 1929); Cobitidae; Korea, Geum R.; A1907; photo: J. Bohlen 2006.

Pangio anguillaris (Vaillant, 1902); Cobitidae; Indonesia; photo: J. Bohlen 2007.

Pangio cuneovirgata (Raut, 1957); Cobitidae; Indonesia, A2637; photo: J. Bohlen 2007.

Pangio doriae (Perugia, 1892); Cobitidae; Malaysia, Sarawak, Sarawak R. basin, Noren R.; A1583; photo: V. Šlechtová 2006.

Pangio cf. oblonga (Valenciennes, 1846); Cobitidae; Indonesia, A2646; photo: J. Bohlen 2007.

Pangio cf. oblonga (Valenciennes, 1846); Cobitidae; ornamental fish trade, A2603; photo: J. Bohlen 2007.

Pangio semicincta (Fraser-Brunner, 1940); Cobitidae; Malaysia, Sarawak, Kumba R. basin; photo: V. Šlechtová 2006.

Pangio cf. semicincta (Fraser-Brunner, 1940); Cobitidae; Indonesia, A2638; photo: J. Bohlen 2007.

Pangio cf. semicincta (Fraser-Brunner, 1940); Cobitidae; Indonesia, A2639; photo: J. Bohlen 2007.

Pangio shelfordii (Popta, 1903); Cobitidae; Malaysia, Sarawak, Sarawak R. basin, Noren R.; A1588; photo: V. Šlechtová 2006.

Pangio superba (Roberts, 1989); Cobitidae; Indonesia; A2636; photo: J. Bohlen 2007.

Sabanejewia balcanica (Karaman, 1922); Cobitidae; Romania, spring ‚Baile 1. Mai‘; photo: V. Šlechtová 2008.

Cyprinidae

Ellopostomatidae

Psilorhynchus balitora (Hamilton, 1822); Cyprinoidea; Myanmar, Ma Gawe R.; A1665/NUS THH99-10; photo: J. Bohlen 2006.

Ellopostoma mystax Tan and Lim, 2002; Thailand, Tapi R. basin, Tapi R.; A2510; photo: V. Šlechtová 2007

Nemacheilidae

Barbatula barbatula (Linnaeus, 1758); Nemacheilidae; Slovakia; Danube R. basin, Ubljanka R.; photo: J. Bohlen 2006.

Mesonoemacheilus triangularis (Day, 1865); Nemacheilidae; Ornamental fish trade; photo: J. Bohlen 2005.

Nemacheilus binotatus Smith, 1933; Thailand; Nemacheilidae; Chao Phraya R. basin; Mae Nan R.; photo: V. Šlechtová 2005.

Nemacheilus pallidus Kottelat, 1990; Nemacheilidae; Thailand, Chao Phraya R. basin; Mae Nan R.; photo: V. Šlechtová, 2005.

Nemacheilus platiceps Kottelat, 1990; Thailand, Chanthaburi province, Mekong R. basin, Mae Nam Pong R; photo: V. Šlechtová 2006.

Nemacheilus selangoricus Duncker, 1904; Nemacheilidae; Malaysia, Johor, Muar R. basin, Segamat R.; photo: V. Šlechtová 2006.

Nemachilichthys rueppelli (Sykes, 1841); Nemacheilidae; ornamental fish trade; A0553; photo: J. Bohlen 2006.

Oreonectes platyceps Günther, 1868; Nemacheilidae; ronamental fish trade; photo: J. Bohlen 2005.

Oxynoemacheilus Banarescu and Nalbant, 1966 ***sp.***; Nemacheilidae; Turkey; Tigris R. basin; photo: J. Bohlen 2007.

Physoschsitura pseudobruneata Kottelat, 1990; Nemacheilidae; Thailand; Chao Phraya R. basin; Mae Nam Yom R.; photo: V. Šlechtová 2005.

Schistura desmotes (Fowler, 1934); Nemacheilidae; Thailand, Chao Phraya R. basin, Mae Teang River; photo: V.Šlechtová 2005.

Schistura kohchangensis (Smith, 1933); Nemacheilidae; Thailand, Chanthaburi province, Mekong R. basin, Mae Nam Pong R; photo: V. Šlechtová 2006.

Schistura maepaiensis Kottelat, 1990; Nemacheilidae; Thailand, Salween R. basin, Mae Pai R.; photo: V. Šlechtová 2005.

Traccatichthys pulcher (Nichols and Pope, 1927); Vietnam; Thua Luu; photo: J. Bohlen 2006.

Yunnanilus brevis (Boulenger, 1893); Nemacheilidae; Myanmar, Lake Inle; photo: J. Bohlen 2006.

Yunnanilus cruciatus (Rendahl, 1944); Nemacheilidae; Vietnam; Thua Luu R.; photo: J. Bohlen 2006.

Vaillantellidae

Vaillantella maassi Weber and de Beaufort, 1912; Vaillantellidae; Thailand, Surat Tani province, Tapi R. basin, Klong Sok R.; A1015; photo: V. Šlechtová 2006.