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A new species of *Gudeodiscus* Páll-Gergely, 2013 from China, with extraordinary conchological and anatomical features (Gastropoda, Pulmonata, Plectopylidae)

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Abstract

A new species of the Plectopylidae, *Gudeodiscus longiplica* is described from northern Guangxi Province, southern China. The shell, anatomical and radular characters are figured and described. This new species is characterized by long plicae on its parietal shell wall, which have not been observed in any other *Gudeodiscus* species. In contrast, the long parietal plicae are characteristic for the genera *Plectopylis* and *Chersaecia*, which mainly inhabit Thailand and Myanmar. These two genera are, however, only distantly related to the new species, as other characters (anatomy, protoconch sculpture, parietal plicae) suggest. The male portion of the genital structure of the new species is characterized by two separate penial caeca with different lengths, but similar in outer and inner structure. The relevance of this anatomical character is discussed. *Gudeodiscus longiplica* **sp. n.** occurs sympatrically with *Gudeodiscus soosi* Páll-Gergely, 2013. The anatomy and radula characters of the latter species are also described and figured.

Keywords

Taxonomy, systematics, anatomy, sympatric species, duplicated organ, plesiomorphic character

Introduction

The Plectopylidae are composed of flat-shelled terrestrial snail species which are characterized by a complex, internal armature structure. The armature is composed of plicae (horizontal structures) and lamellae (vertical structures) on both the palatal and parietal sides of the body whorl. These barriers are situated ¼ – ½ whorl behind the aperture, thus, usually not visible from the aperture. Instead, the palatal plicae can be seen through the semi-transparent shell wall, whereas small holes must be made in the shell at appropriate sites to examine the parietal plication. The morphology of these plicae and lamellae serve as primary diagnostic characters for species recognition and identification. In addition to these peculiar conchological features, unique traits in the anatomy of Plectopylidae have been reported, i.e. "disposable" calcareous, hook-like granules inside the penis lumen which are probably spent during mating (Páll-Gergely and Hunyadi 2013, Páll-Gergely et al. 2015a).

The most speciose genus in the Plectopylidae is *Gudeodiscus* Páll-Gergely, 2013, mainly distributed in the Chinese Guangxi Province and northern Vietnam, but some species have been reported from eastern Yunnan, southern Hunan and southern Guangdong provinces. More than half of the recorded 24 species of *Gudeodiscus* are known from only empty shells at this time (Páll-Gergely and Hunyadi 2013, Páll-Gergely and Asami 2014, Páll-Gergely et al. 2015a), including *Gudeodiscus soosi* Páll-Gergely, 2013 reported from three nearby localities in northern Guangxi. After *G. soosi* was described, we had the opportunity to examine four additional specimens (shells and ethanol-preserved bodies), from the original sample. Examination revealed that two specimens were *Gudeodiscus soosi*, but the other two were an undescribed species, which is described herein.

Material and methods

Determination of number of shell whorls (precision to 0.25 whorl) follows Kerney and Cameron (1979: 13). Shells and radulae were directly observed without coating under a low vacuum SEM (Miniscope TM-1000, Hitachi High-Technologies, Tokyo). Individual buccal masses were removed and soaked in 2 M KOH solution for 5 h before extracting the radula, which was preserved in 70% ethanol. We use the terms "proximal" and "distal" in relation to the central of the body.

Abbreviations

JUO	Collection Jamen Uiriamu Otani (Koka, Japan)
NHMUK	The Natural History Museum (London, UK)
OK	Collection Kenji Ohara, Nishinomiya Shell Museum (Nishinomiya, Japan)
PGB	Collection Barna Páll-Gergely (Mosonmagyaróvár, Hungary)

Taxonomic descriptions

Family Plectopylidae Möllendorff, 1898

Genus *Gudeodiscus* Páll-Gergely, 2013

2013 *Gudeodiscus* Páll-Gergely, In: Páll-Gergely & Hunyadi, Archiv für Molluskenkunde 142(1): 4, 8.

Type species. *Plectopylis phlyaria* Mabille, 1887, by original designation.

Subgenus *Gudeodiscus*

2015 *Gudeodiscus* (*Gudeodiscus*), — Páll-Gergely, et al., ZooKeys 473: 13.

***Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n.**
http://zoobank.org/3917B197-23F5-4274-B5AA-3B232691848A
Figures 2A–E, 3A–C, 4A–C, 5, 7A–B, 8A, 9A–C, 10B

Material examined. Guangxi (广西), Tiane Xian (天峨县), Liupai Zhen (六排鎮), Shuiliandong (水帘洞), 354 m, 25°00.623'N, 107°09.994'E, leg. Ishibe, T., Ohara, K., Okubo, K. & Otani, J. U., 21.10.2011, NHMUK 20150375 (holotype = shell + body in ethanol + radula on double-faced adhesive tape), NHMUK 20150376/1 paratype (= shell + body in ethanol), JUO/2 paratypes (= shells), PGB/1 paratype (= shell, ex coll. J.U. Otani); Same locality and collection data, OK/4 (= corroded paratypes; these four shells are paratypes of *G. soosi* as well).

Description of the shell (Figs 2–4). The description is based on the holotype and a paratype (NHMUK 20150376) which was opened in order to examine the parietal plicae.

Shell small, dextral, corneous-light brown, translucent, nearly flat, only the protoconch is elevated; whorls 6.75; suture shallow at the protoconch but very deep, even groove-like, near the aperture; protoconch lighter in colour than the rest of the shell, 2 whorls; its surface very finely granulated, matt, and rather regularly ribbed near the suture, ribs becoming weak anteriorly; radial sculpture weakest on the protoconch and becoming stronger towards the end of the protoconch; the umbilical side of the protoconch is not ribbed, but finely granulated, matt; from dorsal view the first three whorls of the teleoconch are irregularly wrinkled, glossy, and lighter coloured than the rest of the shell; this sculpture changes gradually (after approx. 2.5 whorls) to a more strongly ribbed, somewhat darker, less glossy surface which possesses fine periostracal filaments on the ribs; these radial periostracal filaments are most prominent near the suture; the ribbed dorsal surface gradually changes to a smooth, glossy surface at the edge of the body whorl; umbilicus wide, funnel-shaped, shows all whorls; aperture slightly oblique

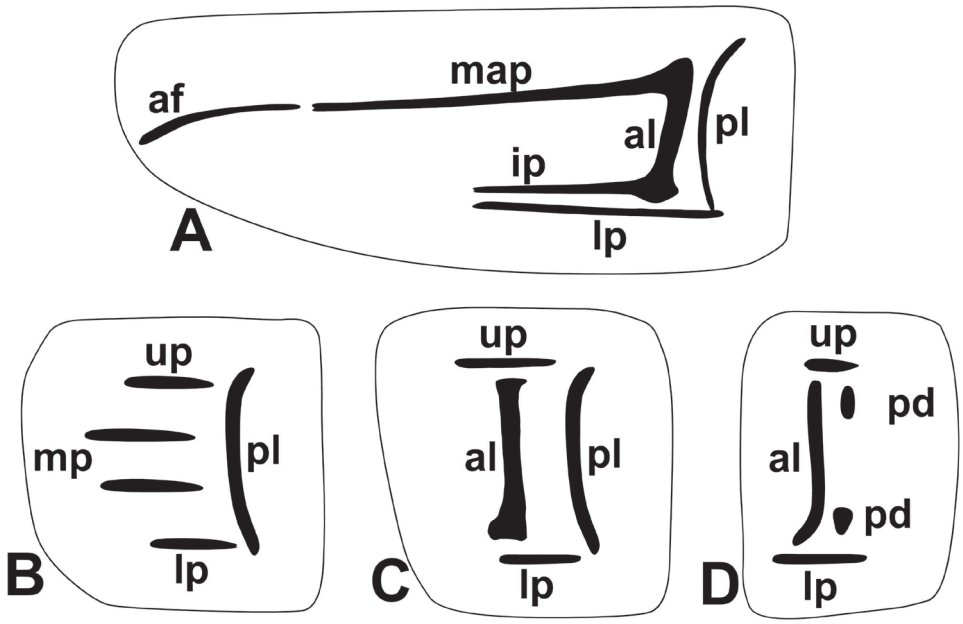

Figure 1. Nomenclature of the parietal plicae and lamellae of *Gudeodiscus* Páll-Gergely, 2013 (**A–C**) and *Endothyrella* Zilch, 1960 (**D**). **A** *Gudeodiscus longiplica* sp. n. **B** *Gudeodiscus emigrans quadrilamellatus* Páll-Gergely, 2013 **C** *Gudeodiscus phlyarius* (Mabille, 1887) **D** *Endothyrella* sp. Abbreviations: af: apertural fold; al: anterior lamella; ip: intermediate plica; lp: lower plica; map: main plica; mp: middle plicae; pd: posterior denticle; pl: posterior lamella; up: upper plica. In order to make the comparison easier, all figures show a dextral specimen (**D** is reversed). After Páll-Gergely and Hunyadi (2013) and Páll-Gergely et al. (2015b).

to the shell axis, peristome white, moderately thickened and very much reflexed; parietal callus strong, elevated but rather blunt; it is angled when it joins the apertural fold; apertural fold long, but free from the main plica.

Parietal wall with two lamellae; the anterior is very much elevated, rather straight but oblique to the shell axis; its lower end is situated more anteriorly than the upper end; the posterior lamella is much weaker (lower) than the anterior, it is C-shaped; the two separate lamellae are well distinguishable, but they are connected to each other by a white calcareous layer; main horizontal plica long, it is connected to the upper end of the anterior lamella; the main plica almost reaches the apertural fold, but in both examined specimens the two structures are free from each other; lower plica long, starts from the lower end of the posterior lamella and ends before the ending point of the main plica; lower plica free from the anterior lamella in case of the holotype, but in the paratype there is a weak connection between them; middle plica strong, starts from the lowermost point of the anterior lamella, and ends in the same position as the lower plica. In case of the paratype there are some additional short plicae in contact with the other, above mentioned plicae, namely: one above the anterior end of the main plica,

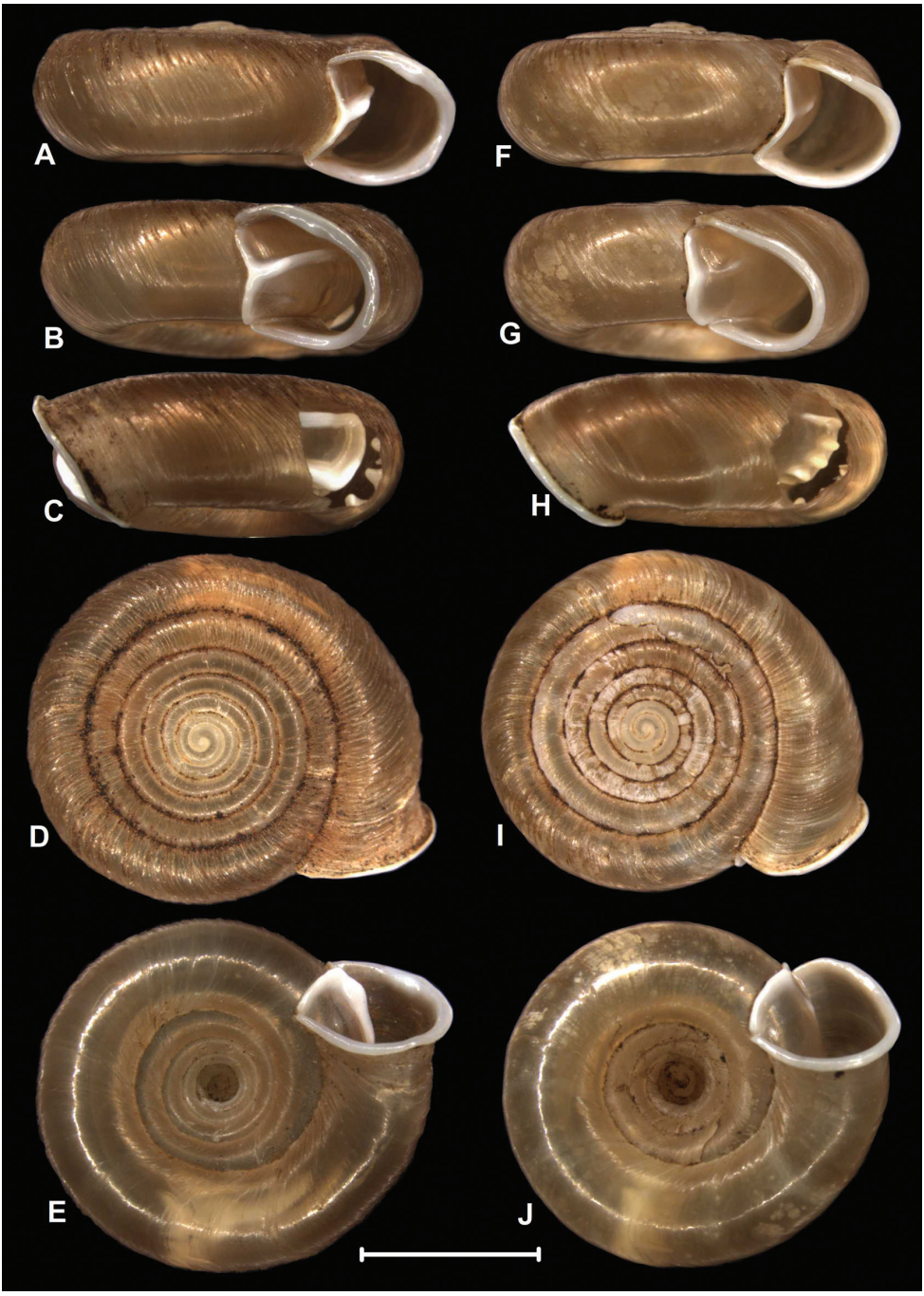

Figure 2. Shells of sympatric Chinese *Gudeodiscus* Páll-Gergely, 2013 species. **A–E** holotype of *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. **F–J** *Gudeodiscus* (*Gudeodiscus*) *soosi* Páll-Gergely, 2013, shell from the type locality (coll. JUO). Figures **C** and **H** were taken after they have been opened in order to observe the inner plicae. Scale bar: 5 mm.

one above the middle plica, and one above the anterior end of the lower plica. Palatal wall with six plicae; the first is long, slender, it is situated near the suture; the second is situated in comparatively large distance from the first; the second plica is even longer than the first, its posterior part is curved downwards; the last (6th) plica is relatively short, with pointed anterior and blunt posterior ends; the middle plicae (3rd to 5th) are complicated, with a shape similar to curly brackets when looking through the semi-transparent shell wall; the anterior leg of the "curly brackets" are longer than the posterior ones; when observing from inside, the middle plicae have a triangular, pointed tip.

Measurements (in mm). D = 11.7, H = 4.6 (holotype); D = 10.3–12.2, H = 4.3–5.0 (paratypes, n = 5).

Characters of the genital structure (Figs 5, 7A–B, 8A). Two specimens were anatomically examined. The right ommatophoral retractor passes between the penis and the vagina. Atrium slender, long; penis moderately long, with slimmer distal part; there are two penial caeca, both of them with their own retractor muscle which merge to a single fascicle after some distance; retractor muscle very long, branched off from the columellar muscle; one of the penial caeca is larger and slimmer than the other; the larger one is approximately half of the length of the penis; epiphallus enters penis laterally (at the meeting point of the penis and the larger penial caecum); penis internally with approximately 16 low, longitudinal folds; just distally from the joint of the epiphallus there is a single, straight, transversal row of small "pockets" which are formed by the longitudinal folds; we have not found calcareous objects in these pockets; larger penial caecum internally with a longitudinal, main row of rounded papillae; this main row consists of 8–9 papillae; there are other, smaller papillae arranged in 2–3 longitudinal rows on the inner wall; there are a few additional papillae adjacent to the ones of the main row; we have found a single calcareous granule with pointed tip and rounded, widened base in one of the papillae; the smaller penial caecum has a single, longitudinal row of papillae with approx. five papillae; the papillae are very well visible throughout the semi-transparent wall of the caeca; epiphallus as long as the longer penial caecum, internally with three longitudinal folds; vas deferens enters epiphallus apically, it is very slender, but gets thicker near the proximal portion of the vagina; vagina extremely long, approximately two times longer than the penis and the penial caecum combined; the distal part is very slender; the proximal portion is slightly thicker than the penis; the inner wall of the vagina is with irregular, low, longitudinal folds, which are the strongest at the proximal end of the vagina (closer to the joining point with the vas deferens); the bursa copulatrix starts a bit distally than the middle point of the proximal portion of the vagina; its base is thickened, but gets slimmer after a short distance; the stalk is slender and very long, the bursa is gradually thickened at the end; diverticulum starts at the end of the vagina, therefore the base of the diverticulum and the base of the bursa copulatrix are situated very far from each other; diverticulum very slender, without thickening at its end; it is approximately as long as the bursa copulatrix; a long, slender, glass-like, fragile spermatophore have been found in the diverticulum; spermoviduct very slender, long.

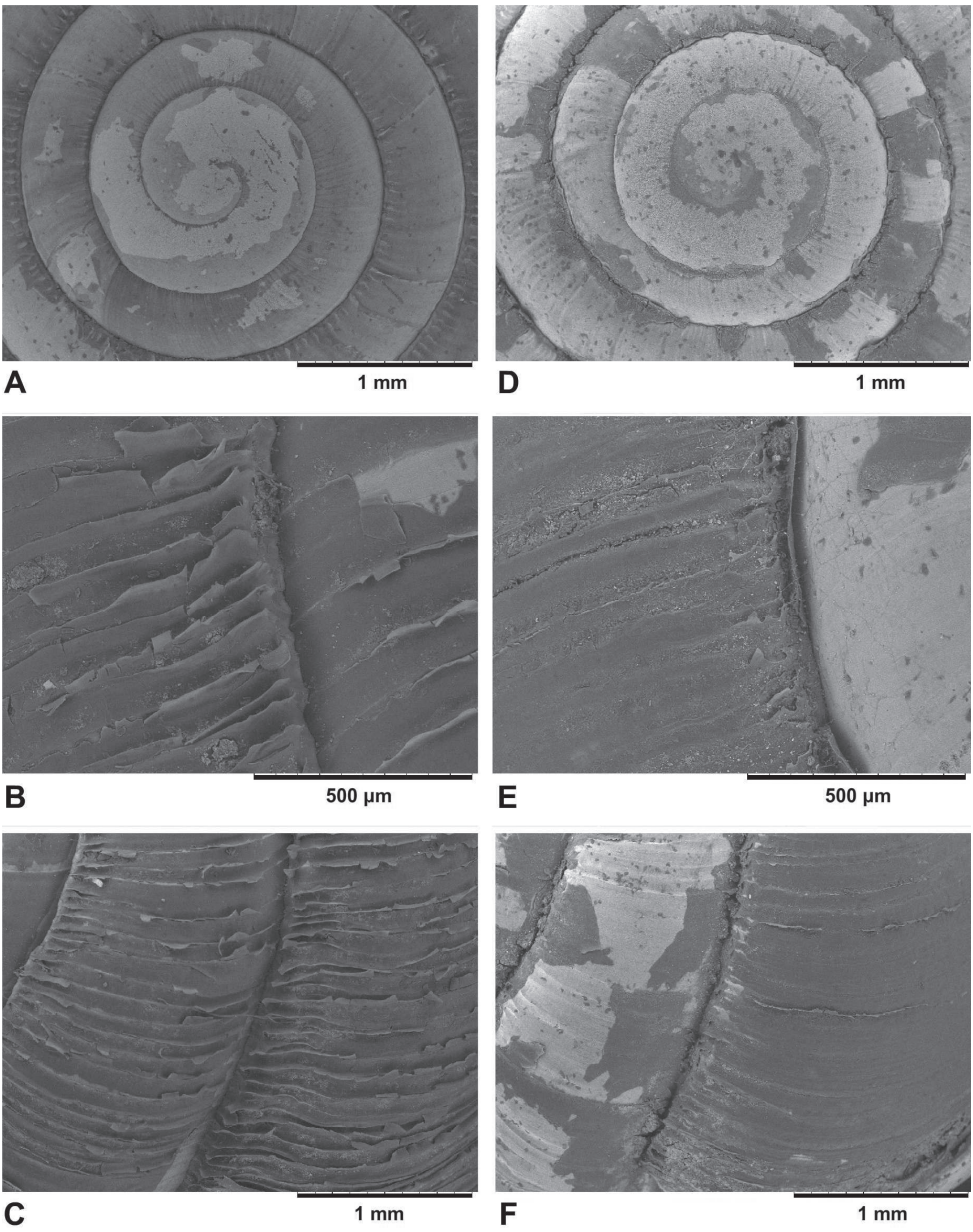

Figure 3. Protoconch (**A, D**) and teleoconch sculpture (**B–C, E–F**) of sympatric Chinese *Gudeodiscus* Páll-Gergely, 2013 species. **A–C** holotype of *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. **D–F** *Gudeodiscus* (*Gudeodiscus*) *soosi* Páll-Gergely, 2013, shell from the type locality (coll. JUO) **B** and **E** shows the last and penultimate whorls opposite of the aperture **C** and **F** shows the last and penultimate whorls near the aperture.

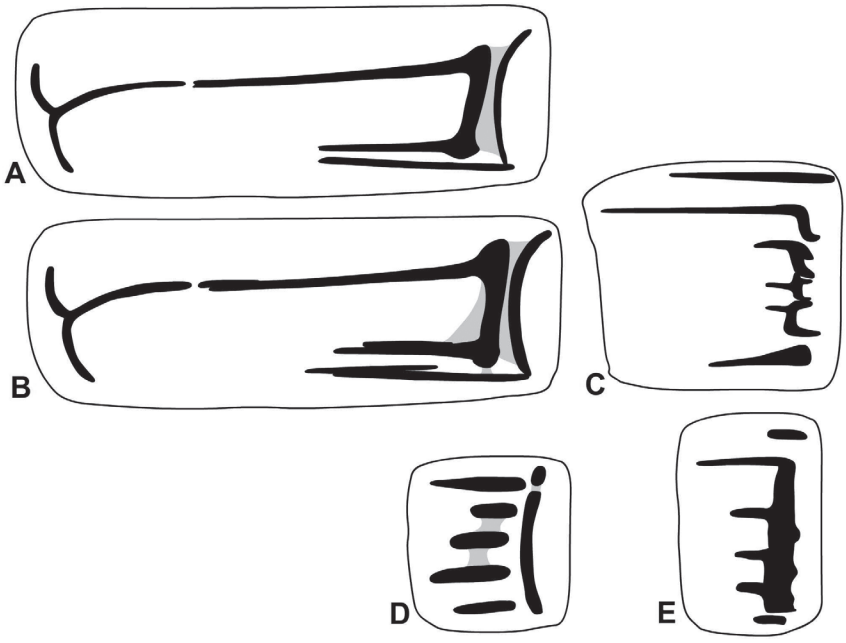

Figure 4. Parietal (**A, B, D**) and palatal plication of *Gudeodiscus* Páll-Gergely, 2013 species. **A** holotype of *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. **B–C** paratype of *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. (NHMUK 20150376) **D–E** *Gudeodiscus* (*Gudeodiscus*) *soosi* Páll-Gergely, 2013 (coll. JUO).

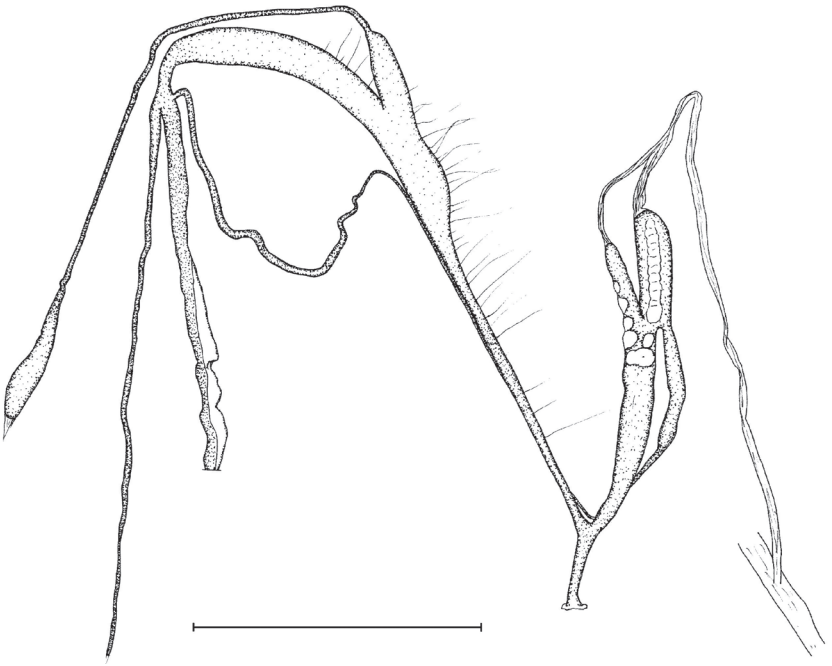

Figure 5. Genital anatomy of *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. Scale bar: 5 mm.

Figure 6. Genital anatomy of *Gudeodiscus* (*Gudeodiscus*) *soosi* Páll-Gergely, 2013. Scale bar: 5 mm.

Characters of the radula (Fig. 9A–C). Radula elongated, but not very slender, central tooth present, laterals 7 or 8 (it is difficult to decide whether the 8th row belong to the laterals or the marginals), standing in straight lines (perpendicular to the central column); marginals approximately 11–12; marginals are placed in slightly oblique rows; central tooth wide-based triangular, smaller than the endocone of the first lateral, but approximately as large as the ectocone of the first laterals; laterals bicuspid, ecto-

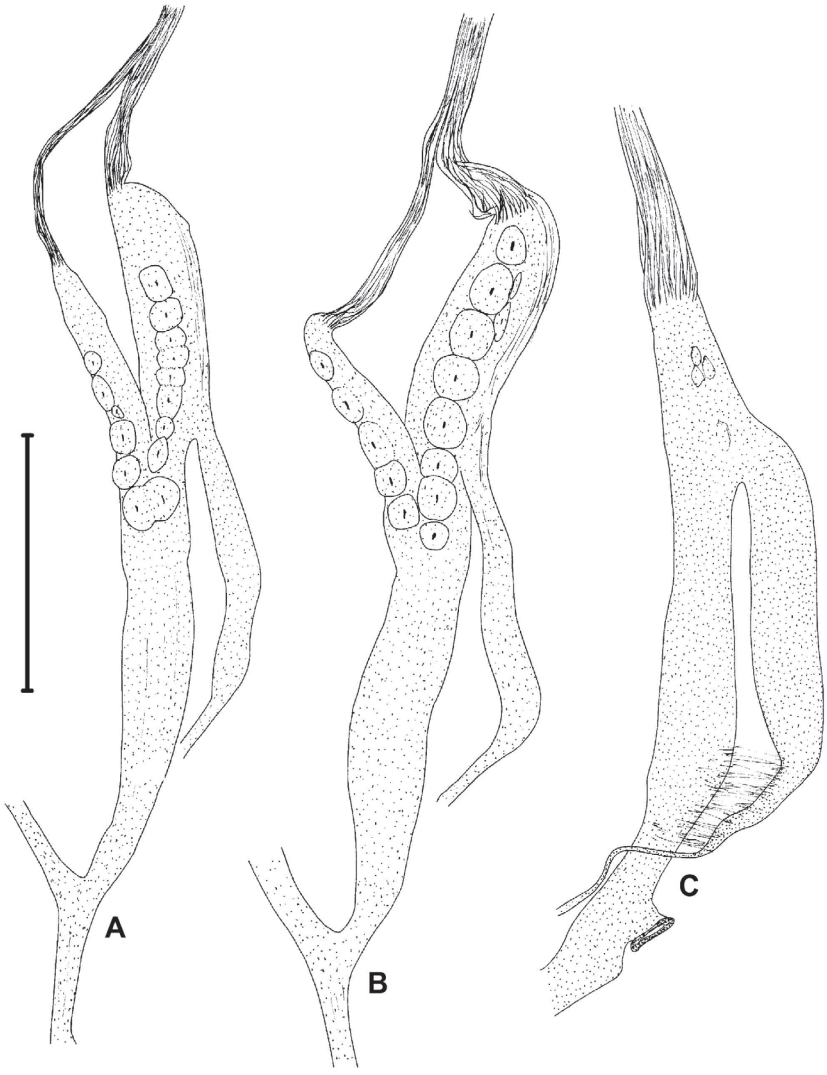

Figure 7. Male genitalia of *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. (**A, B**) and *Gudeodiscus* (*Gudeodiscus*) *soosi* Páll-Gergely, 2013 (**C**). Scale bar: 2 mm.

cones triangular, endocones have rather parallel margins with triangular, pointed tip; marginals usually tricuspid (= the endocone has two cusps); occasionally the innermost cusp is also divided into two cusps resulting in three cusps of the structure equivalent to the endocone of the laterals; some of the external marginals have both the endocone and the ectocone divided into two cusps; all cusps pointed, the incision between the innermost two cusps (= two cusps of the endocone) is deep.

Differential diagnosis. *Gudeodiscus longiplica* sp. n. differs from all other *Gudeodiscus* species by the morphology of the parietal plicae and lamellae, and the presence of

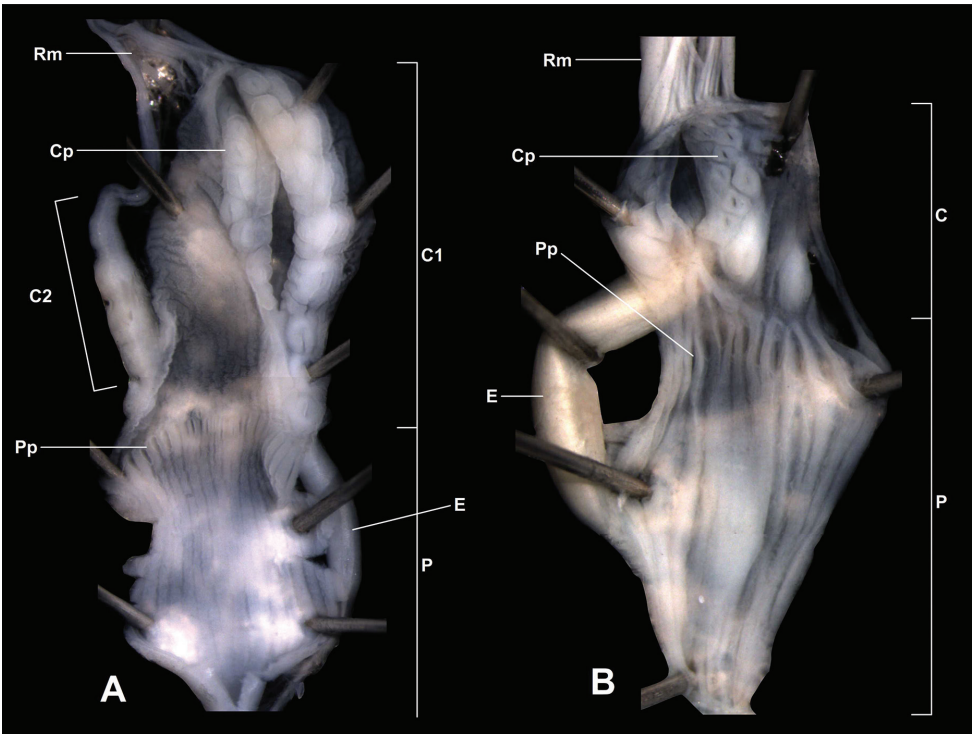

Figure 8. Opened penis and larger caecum of *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. (**A**) and *Gudeodiscus* (*Gudeodiscus*) *soosi* Páll-Gergely, 2013 (**B**). Abbreviations: C: penial caecum; C1: larger penial caecum; C2: smaller penial caecum; Cp: papilla on the inner wall of the larger penial caecum; E: epiphallus; P: penis; Pp: pockets on the penis wall; Rm: retractor muscle. Note that the most proximal portion of the penis is not shown on the left figure.

two penial caeca. It differs from the sympatric *G. soosi* by the presence of two well-developed parietal lamellae and three horizontal plicae (main, lower, and middle), as well as the apertural fold (*longiplica* sp. n.: long; *soosi*: short), the palatal plicae (*longiplica* sp. n.: first two long; *soosi*: first very short, second moderately long), the shell shape (*longiplica* sp. n.: dorsal side flat; *soosi*: dorsal side slightly domed) and the fine sculpture of the dorsal side (*longiplica* sp. n.: several radial periostracal folds; *soosi*: nearly smooth).

The long parietal plicae of *Gudeodiscus longiplica* sp. n. is similar to those of some, mostly sinistral species of *Chersaecia* and *Plectopylis*, which inhabit north-eastern India, Myanmar, northern Thailand, and northern Malaysia. The anatomy of *Plectopylis* and *Chersaecia* is insufficiently known, therefore we cannot use the anatomical characters of *Gudeodiscus longiplica* sp. n. to reject a close relationship with *Plectopylis* and *Chersaecia*. *Gudeodiscus longiplica* sp. n. has a regularly ribbed protoconch, whereas *Plectopylis* and *Chersaecia* species have finely tuberculated or smooth embryonic whorls (Schileyko 1999, Páll-Gergely et al. 2015b). Moreover, the palatal plicae of *Chersaecia* and *Plectopylis* are different (see Discussion).

Etymology. This new species is named for its long plicae on the parietal wall.

Type locality. Guangxi (广西), Tiane Xian (天峨县), Liupai Zhen (六排鎮), Shuiliandong (水帘洞), 354 m, 25°00.623'N, 107°09.994'E.

Distribution. *Gudeodiscus longiplica* sp. n. is known only from the type locality.

Gudeodiscus (*Gudeodiscus*) *soosi* Páll-Gergely, 2013

Figures 2F–J, 3D–F, 4D–E, 6, 7C, 8B, 9D–F

2013 *Gudeodiscus soosi* Páll-Gergely, In: Páll-Gergely & Hunyadi, Archiv für Molluskenkunde 142(1): 31–32, figs 42a–b, 66.

Characters of the genital structure (Figs 6, 7c, 8B). Two specimens were anatomically examined. Shells, ethanol-preserved bodies and radulae on double-faced adhesive tape are deposited in coll. JUO.

One of the specimens was aphallic, i.e. the male part of the genitalia was entirely missing. The right ommatophoral retractor passes between the penis and the vagina of the second specimen. Atrium extremely short, slender, long; penis moderately long, spindle shaped; inner wall of the penis with approx. 14 low longitudinal folds which join each other in the direction of the atrium resulting in fewer number of folds posteriorly; on the penial wall of the apical part of the penis there are slit-like "pockets" arranged in a transversal row; no calcareous granules have been found inside these pockets; the penial caecum is situated on the apical portion of the penis, it is approx. one third of the length of the penis; its inner wall is ornamented with several rhomboid papillae with holes in the middle of each papillae; no calcareous granules were found in them; retractor muscle inserts on the apical part of the penial caecum; retractor muscle very long, branched off from the columellar muscle; epiphallus enters penis laterally, at the joint of the penis and the larger penial caecum; its inner wall with three strong longitudinal folds; vagina slightly longer and thicker than the penis; the inner wall of the vagina is with irregular, low, longitudinal folds; the bursa copulatrix starts on the proximal part of the vagina; its base is not thickened; the stalk is slender and very long, the bursa is oval, more thickened than in the other species; diverticulum starts at the end of the vagina, therefore the base of the diverticulum and the base of the bursa copulatrix are very far from each other; diverticulum very slender, without thickening at it end; it is approximately as long as the bursa copulatrix; spermoviduct contained several developing eggs.

Characters of the radula (Fig. 9D–E). Radula elongated, but not very slender, central tooth present, laterals 7 or 8 (it is difficult to decide whether the 8th row belong to the laterals or the marginals), standing in straight lines (perpendicular to the central column); marginals approximately 12–13; marginals are placed in slightly oblique rows; central tooth wide-based triangular, smaller than the endocone of the first lateral, but approximately as large as the ectocone; laterals bicuspid, ectocones triangular, endocones have rather parallel margins with triangular tip; marginals usually tricuspid

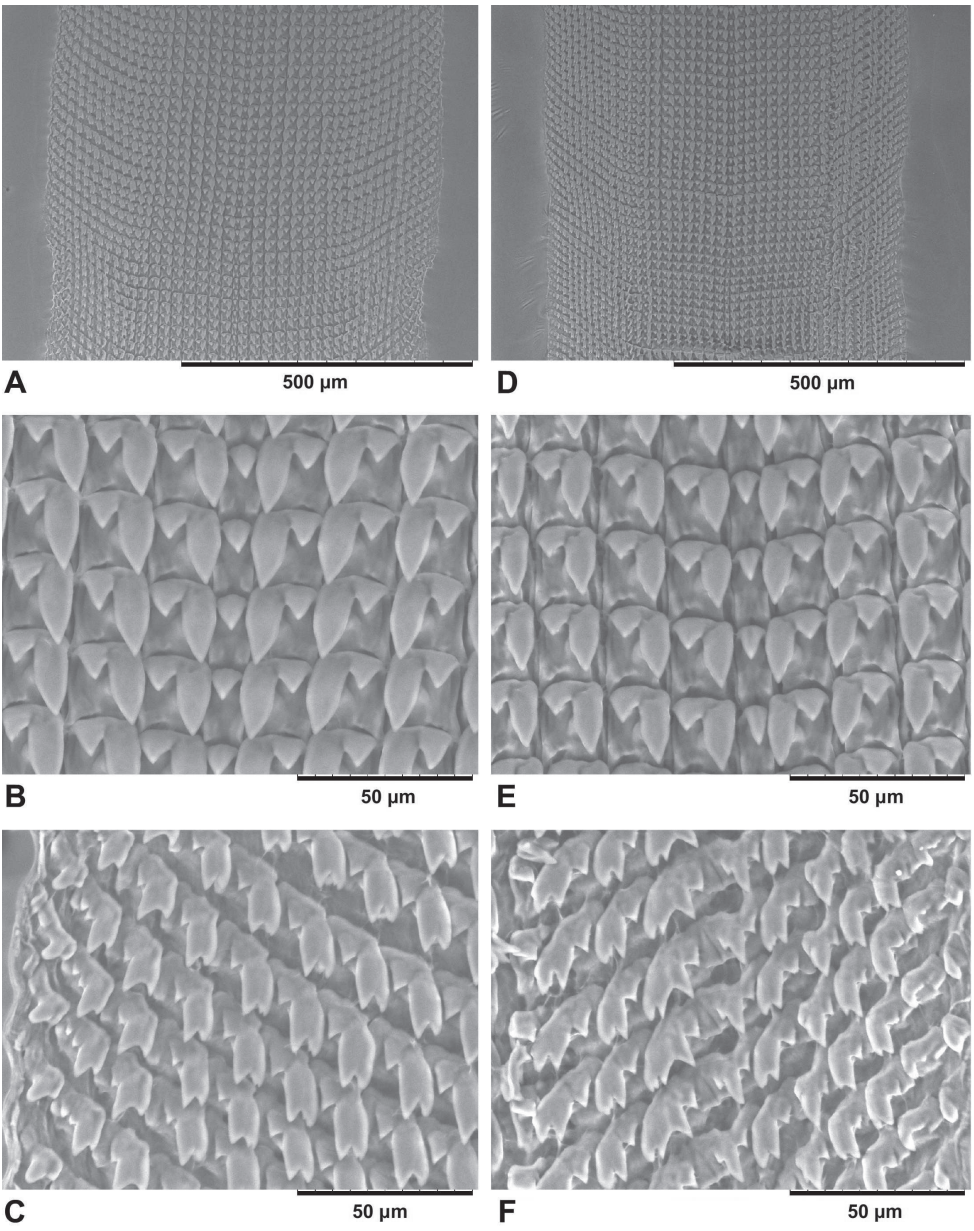

Figure 9. Radula of *Gudeodiscus* species. **A–C** *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. **D–F** *Gudeodiscus* (*Gudeodiscus*) *soosi* Páll-Gergely, 2013. **A, D** middle section of the radula plate **B, E** central tooth and first 3–4 lateral teeth **C, F** marginals.

(= the endocone has two cusps); occasionally the innermost cusp is also divided into two cusps resulting in three cusps for the structure equivalent to the endocone of the laterals; some of the external marginals have both the endocone and the ectocone di-

vided into two cusps; all cusps pointed, the incision between the innermost two cusps (= two cusps of the endocone) is deep.

Differential diagnosis. See under *Gudeodiscus longiplica* sp. n.

Remarks. We cannot rule out the possibility that the aphallic individual was a hybrid (see Schilthuizen et al. 2011).

Discussion

Gudeodiscus soosi and *Gudeodiscus longiplica* sp. n. share an anatomical character that differentiate them from all other anatomically-known species of the Plectopylidae, including *Gudeodiscus*. The origination sites of the bursa copulatrix and the diverticulum are distantly situated from each other because the bursa copulatrix of both species branches off the vagina at approximately the middle vaginal section. In contrast, in all other members of Plectopylidae the bursa copulatrix and the diverticulum originate very near each other with both attaching at the proximal end of the vagina. This, however, does not warrant a genus-group-level distinction of *Gudeodiscus longiplica* sp. n. and *G. soosi* from other plectopylids. The general shell characters and the inner morphology of the penis (presence of a transversal row of slit-like "pockets") places these two species in the genus *Gudeodiscus*. Furthermore, the retractor muscle inserts at the end of the penial caecum without additional curtain-like muscle fibres (characteristic for the subgenus *Veludiscus* Páll-Gergely, 2015) on the apical part of the penis. This trait places *Gudeodiscus longiplica* sp. n. and *G. soosi* in the subgenus *Gudeodiscus* (*Gudeodiscus*). The morphology of radular teeth also agrees with the other members of the subgenus *Gudeodiscus*. Namely, the central tooth is as large as the ectocone of the first laterals, the marginals are tricuspid or even quadricuspid with rather pointed inner cusp, and there is a deep incision between the two inner cusps. In contrast, *Gudeodiscus* (*Veludiscus*) species are characterized by central teeth smaller than the ectocone of the first laterals, and the inner cusps of the marginals are rather blunt with shallow incision between the two innermost cusps.

Gudeodiscus longiplica sp. n. has two surprising characters that need further discussion. Firstly, the two long, anteriorly-elongated parietal plicae that are in contact with the anterior lamellae, and secondly, its two separate penial caeca, which are similar in inner and outer morphology, but differ in size.

Long parietal plicae

Gudeodiscus, *Halongella* Páll-Gergely, 2015, *Sicradiscus* Páll-Gergely, 2013 and *Sinicola* Gude, 1899 are known as genera lacking long horizontal parietal plicae on the parietal wall. Species belonging to these four genera possess two vertical lamellae (Fig. 10J), a single lamella (Fig. 10E–F), or a single lamella with denticles anteriorly, which are situated in the position of the anterior lamella (Fig. 10G). In some *Sicradiscus* and

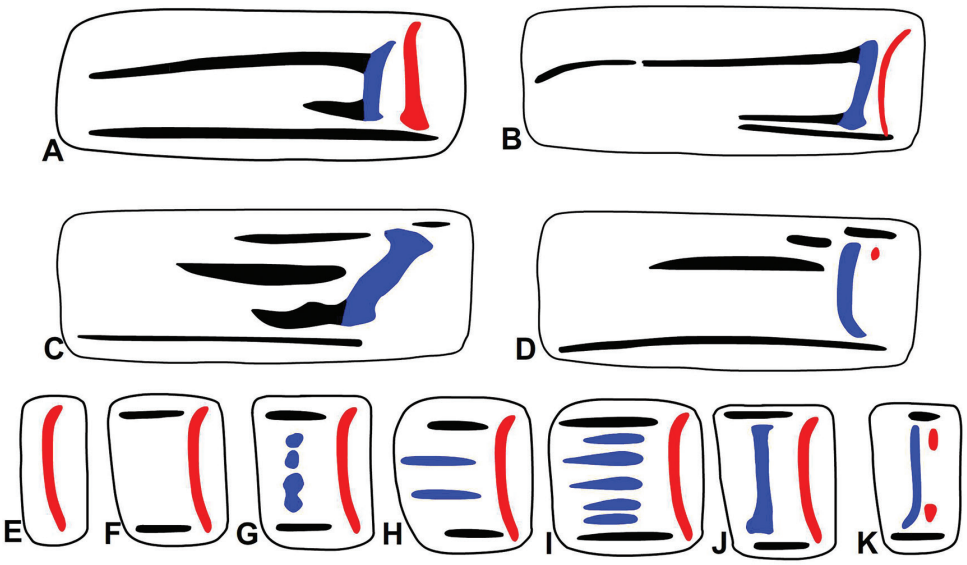

Figure 10. Parietal plication of Plectopylidae species (diagrammatic figures). **A** *Plectopylis leucochila* Gude, 1898 (after Gude 1898) **B** *Gudeodiscus* (*Gudeodiscus*) *longiplica* sp. n. **C** *Endothyrella brahma* (Godwin-Austen, 1879) **D** *Endothyrella williamsoni* (Gude, 1915) **E–J** character states of *Gudeodiscus*, *Halongella*, *Sicradiscus* and *Sinicola*; **K** *Endothyrella* sp. (some *Sicradiscus* has also similar parietal lamellation) (mainly after Páll-Gergely & Hunyadi 2013 and Páll-Gergely et al. 2015b). To allow better comparison all figures show dextral specimens (**A, C, D**, **J** are reversed), thus, the aperture is situated left from the armature. Red colour indicates the posterior, blue colour indicates the anterior lamella (and their respective homologous structures).

in most *Endothyrella* Zilch, 1960 species there is a single lamella and one or two denticles on its posterior side (Fig. 10K). These posterior denticles are probably homologous with the posterior lamella. Only some taxa, namely two subspecies of *Gudeodiscus emigrans* (Möllendorff, 1901) and *Sinicola reserata hensanensis* (Yen, 1939) are reported to have four relatively short, anteriorly elongated plicae (Fig. 10H). *Gudeodiscus ursula* Páll-Gergely, 2013 has seven parallel plicae, the uppermost and the lowermost being conspicuously longer and slimmer than the middle ones (Fig. 10I) (Páll-Gergely and Hunyadi 2013). The single, vertical, curved lamella of these three species is probably homologous with the posterior lamella of other *Gudeodiscus* species which possess two lamellae. The middle horizontal plicae anterior to the single lamella, however, are situated at the position of the anterior lamella. Thus, the two middle plicae are probably homologous with the anterior lamella. There are even transitional character states between the two lamella-type (Fig. 10J) and the single lamella plus four parallel plica-type (Fig. 10H) (see Páll-Gergely and Asami 2014: figures 5F–H). In *Gudeodiscus longiplica* sp. n., the anteriorly elongated plicae are connected to the well-developed anterior lamella (Fig. 10B). Therefore, the long horizontal plicae of *G. longiplica* sp. n., and those of the above-mentioned two *Gudeodiscus* and one *Sinicola* species cannot be

homologous. Instead, the horizontal parietal plicae of *Gudeodiscus longiplica* sp. n. are probably homologous with those of the genera *Plectopylis* and *Chersaecia*, where long plicae commonly occur. Among the plectopylid genera possessing ribbed embryonic whorls (*Gudeodiscus*, *Sicradiscus*, *Sinicola*, *Halongella*, *Endothyrella*) only some *Endothyrella* species possess long horizontal lower and/or main parietal plicae (Fig. 10C–D) (Páll-Gergely et al. 2015b). The long plicae are probably plesiomorphic characters in the Plectopylidae.

Double penial caecum

Both anatomically examined specimens of *Gudeodiscus longiplica* sp. n. had two separate penial caeca, both having their own fascicle of retractor muscle. The two caeca were different in length but the outer appearance and the inner structure were similar. The same anatomical trait in both specimens suggests that the two caeca are characteristic for the species and do not represent rare teratological event. No other members of the Plectopylidae are known to have two separate penial caeca. Moreover, as our non-exhaustive literature survey shows, the duplication of the penial caecum (or any accessory organ in similar relative positions) is a very rare event in stylommatophoran snails.

Two genera of the Cerastidae (= Pachnodidae; superfamily Enoidea), namely *Altenaia* Zilch, 1972 and *Archeorachis* Schileyko, 1998 possess two penial caeca arising from the apical part of the penis. None of these caeca have retractor muscles. One caeca is slender, vermiform, and the other is conic or ovate. Other genera of the Pachnodidae are known to possess only one type of the penial caeca, either vermiform or thick and fleshy (Schileyko 1998). The euconulid genus *Gunongia* Tillier & Bouchet, 1988 and the systrophiid *Tamayoa* Baker, 1925 (see Tillier 1980) also possess two differently looking penial caeca without retractor muscles. Some species of the genus *Deroceras* Rafinesque, 1820 (family Agriolimacidae) have two accessory organs on the apical part of the penis (Wiktor 2000). These organs differ in morphology and function from each other ("penial caecum" and "penial lobe", see Reise et al. 2011, H. Reise & J. Hutchinson, pers. comm., 2015). These accessory organs usually lack retractor muscles, but *Deroceras oertzeni* (Simroth, 1889) has a branched penis retractor running to more or less the ends of two big pockets (bigger one is considered the main penis and the other one an "appendix", see Wiktor 2001). The different morphology of the two caeca of the Pachnodidae, Euconulidae, Systrophiidae and Agriolimacidae are not the result of the duplication of a single organ, as we hypothesise in the case of *Gudeodiscus longiplica* sp. n. Moreover, retractor muscles of the penial appendices are absent in most abovementioned taxa, but present in *Gudeodiscus longiplica* sp. n.

In the literature we encountered some reports of retractor muscles with two branches, each of them inserting on the two penial caeca, or the penial caecum and the penis itself. *Furcopenis darioi* Castillejo & Wiktor, 1983, (Agriolimacidae) has a bifurcate "accessory organ" with retractor muscles inserting on both tips of the accessory body (Castillejo and Wiktor 1983, Wiktor 2000). *Testacella scutulum* G.B. Sowerby

I, 1820 and species of the genus *Schistophallus* Wagner, 1915 have the two branches of the retractor muscle inserted on the bifurcated end of the penis (Wagner 1915; *T. scutulum* was mentioned under the name *T. hungarica*). Despite the superficial similarity between the above mentioned pairs of penial accessory structures and the doubled penial caecum of *Gudeodiscus longiplica* sp. n., it is difficult to decide whether these organ pairs represent homologous structures between distantly related taxonomic groups, especially since our knowledge of their function is extremely limited. The very similarly looking pair of penial caeca in *Gudeodiscus longiplica* sp. n. represents a rare and interesting case which requires further investigation.

In most *Gudeodiscus* species the penis has larger pockets for calcareous hook-like granules than the penial caecum. In some examples the caecum was absent (Páll-Gergely and Asami 2014, Páll-Gergely et al. 2015a). Assuming that the penial and caecal calcareous hooks have similar functions, this suggests that whatever function the hooks might have (stimulation, mucus injection, mechanical holdfast, see Páll-Gergely et al. 2015a), the penis makes a larger contribution than the penial caecum. The penial pockets of *Gudeodiscus longiplica* sp. n. and *G. soosi* are smaller and shallower than the caecal ones. We might assume that in the two species in question (but especially in *Gudeodiscus longiplica* sp. n.) the hooks in the penial caecum play a more important role during mating than the hooks in the penis.

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RESEARCH ARTICLE

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Barcoding of Central European *Cryptops* centipedes reveals large interspecific distances with ghost lineages and new species records from Germany and Austria (Chilopoda, Scolopendromorpha)

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Abstract

In order to evaluate the diversity of Central European Myriapoda species in the course of the German Barcode of Life project, 61 cytochrome *c* oxidase I sequences of the genus *Cryptops* Leach, 1815, a centipede genus of the order Scolopendromorpha, were successfully sequenced and analyzed. One sequence of *Scolopendra cingulata* Latreille, 1829 and one of *Theatops erythrocephalus* Koch, 1847 were utilized as outgroups. Instead of the expected three species (*C. parisi* Brolemann, 1920; *C. anomalans* Newport, 1844; *C. hortensis* (Donovan, 1810)), analyzed samples included eight to ten species. Of the eight clearly distinguishable morphospecies of *Cryptops*, five (*C. parisi*; *C. croaticus* Verhoeff, 1931; *C. anomalans*; *C. umbricus* Verhoeff, 1931; *C. hortensis*) could be tentatively determined to species level, while a further three remain undetermined (one each from Germany, Austria and Croatia, and Slovenia). *Cryptops croaticus* is recorded for the first time from Austria. A single specimen (previously suspected as being *C. anomalans*), was redetermined as *C. umbricus* Verhoeff, 1931, a first record for Germany. All analyzed *Cryptops* species are monophyletic and show large genetic distances from one another (p-distances of 13.7–22.2%). Clear barcoding gaps are present in lineages represented by >10 specimens, highlighting the usefulness of the

barcoding method for evaluating species diversity in centipedes. German specimens formally assigned to *C. parisi* are divided into three clades differing by 8.4–11.3% from one another; their intra-lineage genetic distance is much lower at 0–1.1%. The three clades are geographically separate, indicating that they might represent distinct species. Aside from *C. parisi*, intraspecific distances of *Cryptops* spp. in Central Europe are low (<3.3%).

Keywords
Barcode, biodiversity, COI, cryptic diversity, introduced species

Introduction

The German Barcode of Life project – Myriapoda was started in 2012 with the aim to construct a library of reference sequences from the 200 indigenous Diplopoda and Chilopoda species of Germany (Voigtländer et al. 2011). This project, spearheaded by a study of Bavarian myriapods (Spelda et al. 2011), is still in progress. First results of the "German Myriapod Barcoding Group" were presented by Wesener et al. (2015). With the help of a comprehensive gene database, the taxonomical problems and confusion that exists in many myriapod groups on a species and higher level could be solved in combination with morphological character analyses. Additionally, barcoding could make it possible to determine juvenile and female myriapods; such a determination is often impossible with morphological characters only. Furthermore, in combination with other genetic markers, barcoding might allow analyses of the evolutionary history of species or species groups (e.g. Pilz et al. 2007, Oeyen et al. 2014).

Such a problem of taxonomic confusion applies in particular to the family Cryptopidae of the centipede order Scolopendromorpha. The Cryptopidae show an almost worldwide distribution, as they are present on most continents and many islands (Attems 1930). The family shows their highest diversity in the temperate parts of North and South America, Europe and the Mediterranean region, central and southern Africa, Madagascar, and Australia (Bonato and Zapparoli 2011). Many cryptopid taxa are currently difficult to determine and are in need of revisions. While the phylogeny of the family inside the Scolopendromorpha is still not fully resolved (e.g. Murienne et al. 2010; Vahtera et al. 2013), the monophyly of the diverse and cosmopolitan genus *Cryptops* is currently undisputed (Vahtera et al. 2012).

In Germany and most of Central Europe, the only Scolopendromorpha that occur naturally are two widely distributed species of the genus *Cryptops*: *C. parisi* and *C. hortensis* (Voigtländer et al. 2011). Both species are morphologically distinct and relatively easy to identify, at least in the adult stage. However, in the Austrian Inn-valley, unusual specimens previously assigned to *C. hortensis* have been found (Pichler 1987) which might be different from *C. hortensis*, and in later studies were placed in keys (Lewis 2011) under *C. parisi*.

A third species, *C. anomalans*, is a recent addition to the German fauna (Voigtländer 1988; Fründ 1989; Spelda 2006, Decker and Hannig 2011). Although already mentioned as a possible member of the German fauna by Schubart (1964) this species

was most likely introduced from the Mediterranean realm to northern Europe (Eason 1964; Lindner 2005), as it is mainly confined to parks and gardens. Because the species has few records in Germany (Decker et al. 2014), a special effort was undertaken to collect specimens from the limited number of known German populations.

There are only a handful of barcoding and phylogenetic studies applying molecular data of Scolopendromorpha worldwide (Murienne et al. 2010; Simaiakis et al. 2012; Vahtera et al. 2012, 2013; Joshi and Edgecombe 2013; Oeyen et al. 2014; Siriwut et al. 2015). For *Cryptops*, there is only a singular molecular study utilizing barcoding genes and it deals with tropical pacific island species (Murienne et al. 2011). Therefore, this study focusing on Central European/German *Cryptops* is the first of its kind.

Barcoding studies inside the Scolopendromorpha consecutively revealed large interspecific distances (Simaiakis et al. 2012; Joshi and Edgecombe 2013; Oeyen et al. 2014; Siriwut et al. 2015). The only study involving *Cryptops* (Murienne et al. 2011) revealed exceptionally high intra- and interspecific distances, similar to the observations made in other Scolopendromorpha genera (see above), as well as in a recent study on German geophilomorph centipedes (Wesener et al. 2015).

The aim of this study is to see if barcoding of *Cryptops* allows (a) a clear separation of the species found in Germany; (b) enables the detection of potential cryptic lineages in the widespread German species; as well as (c) facilitating the correct identification of morphologically distinct specimens from Central Europe.

Material and methods

Specimen collection and preparation

The focus of the project was *Cryptops* from Germany, which encompass 85% of the here analysed specimens of the genus (Fig. 1). The remaining 15% (11) successfully sequenced specimens of *Cryptops* were collected in adjacent countries. Our sample includes six specimens from Austria, two from Italy, and one each from Croatia, Wales, and Slovenia. One of the Italian specimens is of special importance as it came from the type locality of the subspecies *Cryptops parisi sebini* Verhoeff, 1934. All specimens are stored as vouchers in 95% undenatured ethanol, either at the Museum Koenig, Bonn, Germany (ZFMK), the Senckenberg Museum für Naturkunde, Görlitz (SMNG) or the Bavarian State Collection of Zoology, Munich, ZSM (see Table 1, full specimen information in Suppl. material 1).

The specimens were collected by hand and transferred to vials containing 95% undenatured ethanol within days of collection. The vials contain an individual GBOL number with which the specimens can be connected to the accompanying data. After conservation the specimens were either sent to the GBOL facility at the ZFMK or to the corresponding laboratory at the ZSM. Upon arrival, all specimens were photographed (images are or will be uploaded to BOLD, http://www.boldsystems.org/), and a tissue sample was removed for DNA extraction. For this specific GBOL subproject,

Table 1. GBOL numbers, GenBank codes, locality data. GBOL number refers to DNA extraction and BOLD registration; L Nr refers to number of Map (Figure 1). SMNG = Senckenberg Museum für Naturkunde, Görlitz, Germany; ZFMK = Zoological Research Museum A. Koenig, Bonn, Germany; ZSM = Zoologische Staatssammlung München, Germany.

L Nr	GBOL	GenBank	Voucher	Species	Locality
	GBOL02755	KU497147	ZSM-ART-JSP130822-001	*Scolopendra cingulata*	Croatia, Istra, Umag
	GBOL02750	KU497149	ZSM-ART-JSP110424-007	*Theatops erythrocephalus*	Croatia, Istra, Brestova
1	ZFMK-TIS-2531556	KM491707	ZFMK-MYR 3450	*Cryptops hortensis*	Germany, Waren (Müritz), Nationalpark Müritz
1	ZFMK-TIS-2531557	KM491678	ZFMK-MYR 3438	*Cryptops hortensis*	Germany, Waren (Müritz), Nationalpark Müritz
2	ZFMK-TIS-1470	KU342047	ZFMK-MYR 3853	*Cryptops hortensis*	Germany, Potsdam, Babelsberg
2	ZFMK-TIS-2507217	KU342045	ZFMK-MYR 3888	*Cryptops hortensis*	Germany, Potsdam, Babelsberg
3	ZFMK-TIS-1543	KM491700	ZFMK-MYR 3684	*Cryptops hortensis*	Germany, Ilsenburg
4	ZFMK-TIS-1528	KM491595	ZFMK-MYR 3679	*Cryptops hortensis*	Germany, Friedeburg (Saale)
4	ZFMK-TIS-2519823	KM491677	ZFMK-MYR 3824	*Cryptops hortensis*	Germany, Friedeburg (Saale)
5	ZFMK-TIS-1289	KU342043	ZFMK-MYR 3551	*Cryptops hortensis*	Germany, Hoyerswerda, Dubringer Moor
6	ZFMK-TIS-15761	KM491615	ZFMK-MYR 1057	*Cryptops hortensis*	Germany, Bonn - Bad Godesberg, Panoramapark
7	ZFMK-TIS-15555	KU342044	ZFMK-MYR 1043	*Cryptops hortensis*	Germany, Niederzissen, Bausenberg
8	GBOL14853	KU497144	ZSM-ART-JSP130930-017	*Cryptops hortensis*	Germany, Enzberg, Kieselbronn
9	GBOL02747	KU497160	ZSM-ART-JSP110312-009	*Cryptops hortensis*	Germany, Zuckerberg SW Stuttgart-Steinhaldenfeld
9	GBOL10885	KU497162	ZSM-ART-JSP110312-009b	*Cryptops hortensis*	Germany, Zuckerberg SW Stuttgart-Steinhaldenfeld
10	GBOL14855	KU497145	ZSM-ART-JSP150118-018	*Cryptops hortensis*	Germany, Kenzingen, Forlenwald
11	GBOL14854	KU497155	ZSM-ART-JSP150117-055	*Cryptops hortensis*	Germany, Badenweiler, Schweighof (Eselsgrabenfelsen),
12	ZFMK-DNA-112780039	KM491565	ZSM-ART-JSP100619-031	*Cryptops hortensis*	Germany, Mainau island, 4 km NNE Konstanz
13	GBOL14858	KU497146	ZSM-ART-JSP150121-039	*Cryptops hortensis*	Germany, Mainau island, 4 km NNE Konstanz
14	ZFMK-DNA-112780041	KU342046	ZSM-ART-JSP110208-005	*Cryptops hortensis*	Italy, Provincia di Sondrio, Chiavenna, Riserva Naturale Marmitte dei Giganti
15	ZFMK-TIS-19439	KM491610	ZFMK-MYR 1948	*Cryptops parisi*	Germany, Bochum, Botanical Garden of the Ruhr-University
16	ZFMK-TIS-1619		ZFMK-TIS-1619	*Cryptops parisi*	Germany, Leipzig-Schönefeld, Partheaue
17	ZFMK-TIS-15786	KM491698	ZFMK-MYR 1082	*Cryptops parisi*	Germany, Schwelm-Erlen, nahe Eingang Erlenhöhle,

L Nr	GBOL	GenBank	Voucher	Species	Locality
18	ZFMK-TIS-15767	KM491624	ZFMK-MYR 1063	*Cryptops parisi*	Germany, Wuppertal, NSG ‚Im Hölken'
19	ZFMK-TIS-6357	KM491666	ZFMK-MYR 3535	*Cryptops parisi*	Germany, Weißenberg, Gröditzer Skala
20	ZFMK-TIS-2517115	KU342051	ZFMK-MYR 2157	*Cryptops parisi*	Germany, Stromberg (Windeck)
21	ZFMK-TIS-19435	KM491556	ZFMK-MYR 2020	*Cryptops parisi*	Germany, Seelbach bei Hamm (Sieg), Marienthal
21	ZFMK-TIS-19436	KM491664	ZFMK-MYR 2019	*Cryptops parisi*	Germany, Seelbach bei Hamm (Sieg), Marienthal
22	ZFMK-TIS-15462	KM491557	ZFMK-MYR 950	*Cryptops parisi*	Germany, Bonn - Oberkassel, unterhalb Steinbruch,
23	ZFMK-TIS-19593	KM491702	ZFMK-MYR 1545	*Cryptops parisi*	Germany, Bonn - Röttgen, Kottenforst, Naturwaldzelle, Oberm Jägerkreuz'
24	ZFMK-TIS-19592	KM491590	ZFMK-MYR 1544	*Cryptops parisi*	Germany, Wachtberg, Kottenforst bei Pech
25	ZFMK-TIS-15753	KM491588	ZFMK-MYR 1045	*Cryptops parisi*	Germany, Niederzissen, Bausenberg
26	ZFMK-TIS-1561	KU342054	ZFMK-MYR 3697	*Cryptops parisi*	Germany, Lichtenberg, NSG Höllental
27	GBOL14862	KU497148	ZSM-ART-JSP150201-159	*Cryptops parisi*	Germany, Lusen, Winterweg
28	ZFMK-TIS-2520349	KM491592	SMNG VNR016538-3	*Cryptops parisi*	Germany, Ludwigsburg, Salonwald
29	GBOL14843	KU497154	ZSM-ART-JSP130903-006	*Cryptops parisi*	Germany, Felswandergebiet (siev.) 4 km E Neuschoenau, 10 km NE Grafenau
30	GBOL14863	KU497157	ZSM-ART-SSP130614-044	*Cryptops parisi*	Germany, 1 km SE Pfuenz, 7 km ESE Eichstaett
31	GBOL11259	KU497163	ZSM-ART-JSP141004-021	*Cryptops parisi*	Germany, W Unterfrohnstetten, 4 km NNW Hengersberg
32	BCZSMMYR00490	JN266284	ZSM-ART-JSP100508-007	*Cryptops parisi*	Germany, Esslingen-St. Bernhard, Laienweg 33
32	GBOL11266	KU497150	ZSM-ART-JSP130530-002	*Cryptops parisi*	Germany, Esslingen-St. Bernhard, Laienweg 33
33	GBOL14856	KU497152	ZSM-ART-JSP150118-024	*Cryptops parisi*	Germany, Esslinger Burg N Esslingen-Stadtmitte
34	GBOL14859	KU497161	ZSM-ART-JSP150124-038	*Cryptops parisi*	Germany, St. Johann-Fohlenhof, 4 km WSW Bad Urach
35	ZFMK-DNA-112780049	KM491560	ZSM-ART-JSP100516-001	*Cryptops parisi*	Germany, Wendelstein, Ueber der Glonn, 1 km WSW Glonnbercha
36	GBOL02712	KU497164	ZSM-ART-JSP130609-018	*Cryptops parisi*	Germany, Schwarzhoelzl, 2 km NE Karlsfeld
37	ZFMK-TIS-9712	KU342050	ZFMK-MYR 1225	*Cryptops parisi*	Austria, Schneeberg unten
38	BCZSMMYR00493	JN266285	ZSM-ART-JSP100905-017	*Cryptops parisi*	Austria, NW Weinbachbauernhof 1 km NE Strobl, 8 km WNW Bad Ischl
39	GBOL14860	KU497156	ZSM-ART-JSP150124-074	*Cryptops parisi*	Austria, Kaltenbach NNE Ruine Wildenstein, 1 km SW Bad Ischl

L Nr	GBOL	GenBank	Voucher	Species	Locality
40	GBOL14861	KU497141	ZSM-ART-JSP150201-104	*Cryptops parisi*	Germany, W slope of Lercheck, 1 km NW Unterau, 5 km NE Berchtesgaden
41	GBOL02742	KU497140	ZSM-ART-JSP130522-015	*Cryptops parisi*	Germany, SW Grafenaschau, 8 km SW Murnau
42	ZFMK-DNA-112780073	KU342053	ZSM-ART-JSP100510-004	*Cryptops parisi*	Germany, Bad Toelz, Altjoch
43	ZFMK-TIS-2517130	KU342055	ZFMK-MYR 2470	*Cryptops parisi sebini*	Italy, Lombardia, Brescia, Pisogne, Type locality
	GBOL12332	KU497142	ZSM-ART-JSP141214-001	*Cryptops parisi*	UK, Wales, Aberbargoed,
44	ZFMK-TIS-1587	KM491706	ZFMK-MYR 4072	*Cryptops anomalans*	Germany, Leipzig, Pleißemühlgraben
45	ZFMK-TIS-18969	KM491703	ZFMK-MYR 1379	*Cryptops anomalans*	Germany, Bonn, Friesdorf
46	ZFMK-TIS-15751	KM491699	ZFMK-MYR 1047	*Cryptops anomalans*	Germany, Bonn - Bad Godesberg, Panoramapark
46	ZFMK-TIS-15752	KM491639	ZFMK-MYR 1048	*Cryptops anomalans*	Germany, Bonn - Bad Godesberg, Panoramapark
47	BCZSMMYR00489	JN266286	ZSM-ART-JSP100619-017	*Cryptops umbricus*	Germany, Langenaltheimer Haardt 1 km W Solnhofen, 4 km S Pappenheim
48	GBOL02745	KU497151	ZSM-ART-JSP130812-004	*Cryptops anomalans*	Germany, Hummelgraben, Stuttgart-Zuffenhausen
49	GBOL14852	KU497158	ZSM-ART-JSP110624-001	*Cryptops anomalans*	Germany, SW Stuttgart-Muehlhausen
50	GBOL14950	KU497159	ZSM-ART-JSP141105-017	*Cryptops anomalans*	Germany, Ailenberg SE Stuttgart-Obertuerkheim, 1 km WSW Ruedern
51	ZFMK-TIS-2517180	KU342049	ZFMK-MYR 3320	*Cryptops croaticus*	Austria, Leithagebirge, Zeiler Berg
52	ZFMK-TIS-9466	KU342048	ZFMK-MYR 1236	*Cryptops croaticus*	Austria, Leithagebirge I
53	ZFMK-TIS-9755	KM491620	ZFMK-MYR-1185	*Cryptops sp.*	Austria, Burgenland, Rosaliakapelle
54	GBOL14960	KU497153	ZSM-ART-JSP110425-008	*Cryptops sp.*	Croatia, NW Baci and Brestova, 10 km NE Labin
55	ZFMK-TIS-1434	KU342042	ZFMK-MYR 3662	*Cryptops sp.*	Germany, Saxony, Leipzig, Zoo, Gondwanaland
56	GBOL14857	KU497143	ZSM-ART-JSP150118-047	*Cryptops sp.*	Slovenia, Osojca 2 km NW Zagon, 5 km NW Postojna

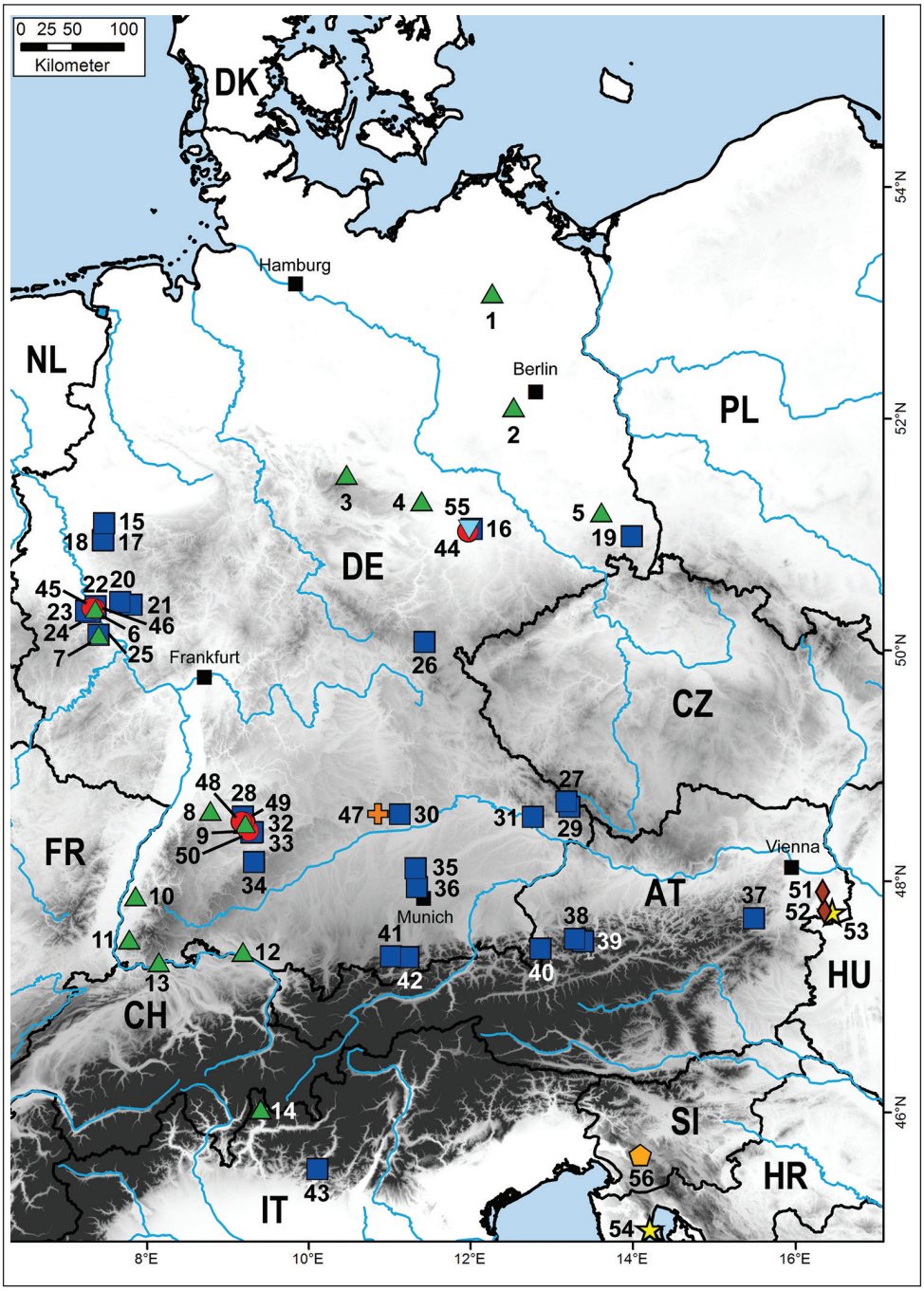

Figure 1. Distribution map of all successfully sequenced Central European specimens of *Cryptops*. Numbers refer to each specimen (see Table 1). Symbols and colours denote species. Blue rectangle = *C. parisi*; red circle = *C. anomalans*; green triangle = *C. hortensis*; brown diamond = *C. croaticus*; orange cross = *C. umbricus*; light blue, orange, and yellow symbols mark undetermined *Cryptops* species.

DNA extraction was attempted for 77 specimens of *Cryptops* as well as one each of *Scolopendra cingulata* and *Theatops erythrocephalus* as outgroups (See Table 1).

Maps were created with ArcGIS 10.

DNA extraction and sequencing

At the ZFMK, DNA was extracted from the tissue samples using the BioSprint96 magnetic bead extractor by Qiagen (Germany). After the extraction, samples were outsourced for PCR and sequencing (BGI China). For PCR and sequencing, the degenerated primer pair HCOJJ/LCOJJ (Astrin and Stüben 2008) was used, resulting in a success rate of >75% (38 of 49 extracted specimens).

At the ZSM, a single leg was removed from each specimen and sent in 96 well lysis plates to the Canadian Centre for DNA Barcoding (CCDB, Guelph, Canada) for standardized, high-throughput DNA extraction, PCR amplification and bidirectional Sanger sequencing (http://www.ccdb.ca/resources.php). For PCR and sequencing, a primer cocktail (Hebert et al. 2004, see Table 2) was used, resulting in a success rate of >90% (23 from 25 extracted specimens). All voucher information and the DNA barcode sequences, primer pairs and trace files were uploaded to BOLD (http://www.boldsystems.org).

Sequences were obtained for 61 *Cryptops* as well as the two outgroup specimens. The three available sequences of Central European *Cryptops* were added from a previously published dataset (Spelda et al. 2011). Sequence identities were confirmed with BLAST searches (Altschul et al. 1997). All 63 new sequences were deposited in GenBank (see Table 1 for accession numbers). In order to rule-out the accidental amplification of nuclear copies of the mitochondrial COI gene, the whole dataset was translated into amino acids (see Supplemental Material) following the 'invertebrate' code in MEGA6 (Tamura et al. 2013); internal stop codons were absent in our dataset. There were a total of 657 positions in the final dataset, gaps were absent.

Table 2. List of primers used for amplification and sequencing of the 5' part of the mitochondrial COI gene.

Primer name	Sequence	Publication	Used at
LCO1490	5'-GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994	CCDB for ZSM
HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994	CCDB for ZSM
LepF1	5'-ATTCAACCAATCATAAAGATATTGG	Hebert et. al. 2004	CCDB for ZSM
LepR1	5'-TAAACTTCTGGATGTCCAAAAAATCA	Hebert et. al. 2004	CCDB for ZSM
C_LepFolF	cocktail of LepF1 and LCO1490	www.boldsystems.org/index.php/Public_Primer_PrimerSearch	CCDB for ZSM
C_LepFolR	cocktail of LepR1 and HCO2198	www.boldsystems.org/index.php/Public_Primer_PrimerSearch	CCDB for ZSM
LCO1490-JJ	5'-CHACWAAYCATAAAGATATYGG	Astrin and Stüben 2008	ZFMK
HCO2198-JJ	5'-AWACTTCVGGRTGVCCAAARAATCA	Astrin and Stüben 2008	ZFMK

Phylogenetic analysis

Sequences were aligned by hand in Bioedit (Hall 1999). The final dataset included 66 nucleotide sequences with 657 positions (63 newly sequenced). Phylogenetic analyses were conducted in MEGA6 (Tamura et al. 2013). A Modeltest, as implemented in MEGA6 (Tamura et al. 2013), was performed to find the best fitting maximum likelihood substitution model. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the best substitution pattern. Included codon positions were 1st+2nd+3rd+Noncoding. Modeltest selected the General Time Reversible model (Nei and Kumar 2000) with gamma distribution and invariant sites as best fitting model (lnL -4725.286624, Invariant 0.505, Gamma 1.65919, R 3.11, Freq A: 0.2844, T: 0.3433, C: 0.2113, G: 0.1606). The tree with the highest log likelihood (-4725.2866) is used here to infer the genetic distances and evolutionary history of the analyzed specimens. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.6591)). The rate variation model allowed for some sites to be evolutionarily invariable ((+I), 50.5% sites). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the analyzed taxa. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Distance analysis

The number of base differences per site between sequences is shown in figures and tables (Fig. 3; Suppl. material 2). The analysis involved 66 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 657 positions in the final dataset. Evolutionary distance analyses were conducted in MEGA6 (Tamura et al. 2013). Two frequency distribution diagrams of all pair-wise intra- and inter-specific distances were produced to further evaluate species divergence in *Cryptops*. All samples of each species were grouped in the first analysis, while *Cryptops parisi* was split into the three separate clades *C. parisi* sensu stricto, *C. parisi sebini* and *C. parisi* lineage3 in the second analysis.

Results

Phylogenetic analysis

The monophyly of the genus *Cryptops* is strongly supported (97%) in our tree (Fig. 2). One undetermined *Cryptops* sp. collected from the tropical rainforest

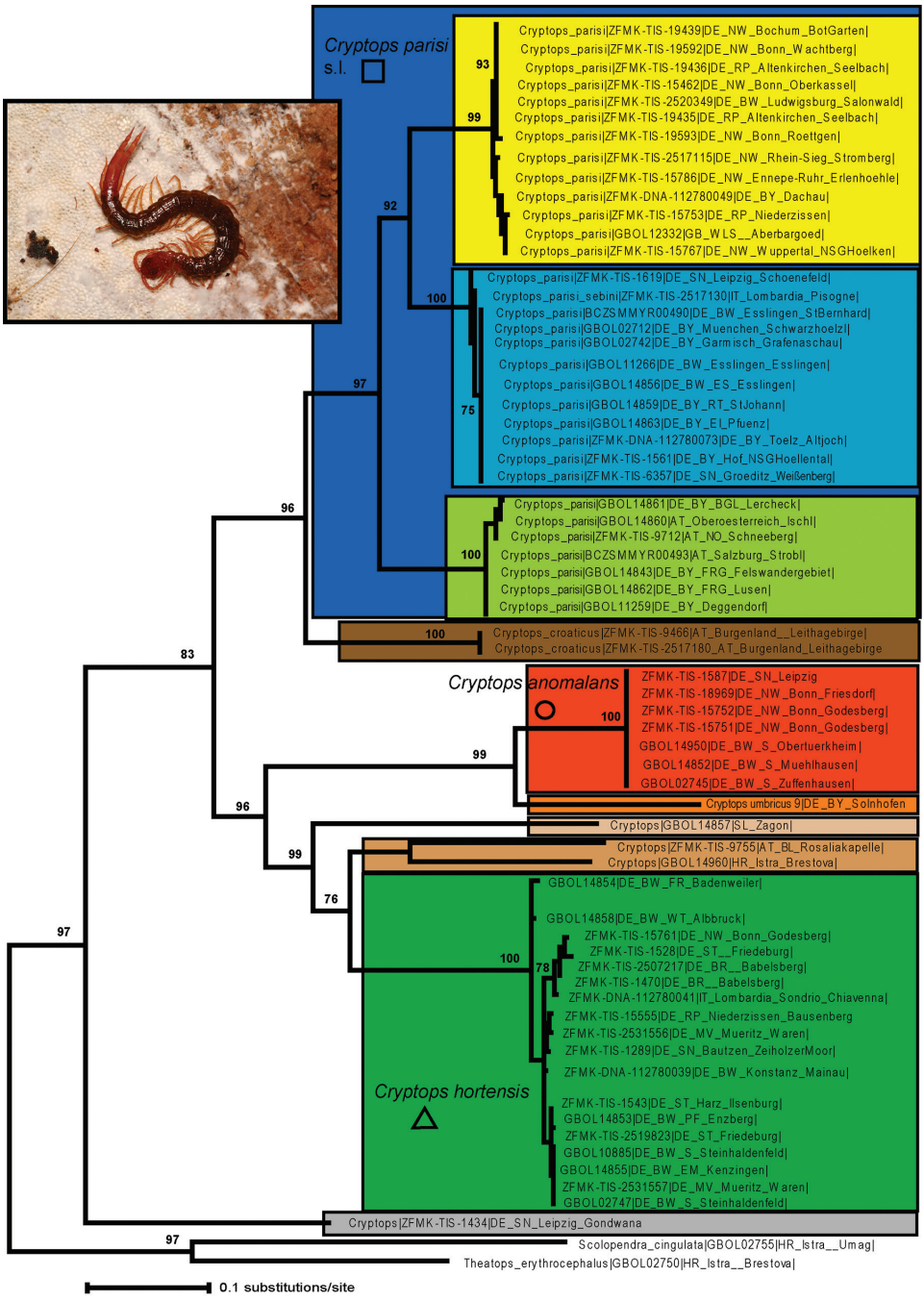

Figure 2. Maximum likelihood tree under the GTR+G+I model, 1000 bootstrap replicates. Colours and symbols correspond to Maps (Figs 1, 4). Country of origin given after specimen number: **AT** = Austria; **DE** = Germany; **GB** = Wales; **HR** = Croatia; **IT** = Italy; **SL** = Slovenia. Photograph shows a specimen of *Cryptops parisi* s.s. from Breckerfeld (photo A. Steiner), western Germany. For full data on all specimens, see Table 1.

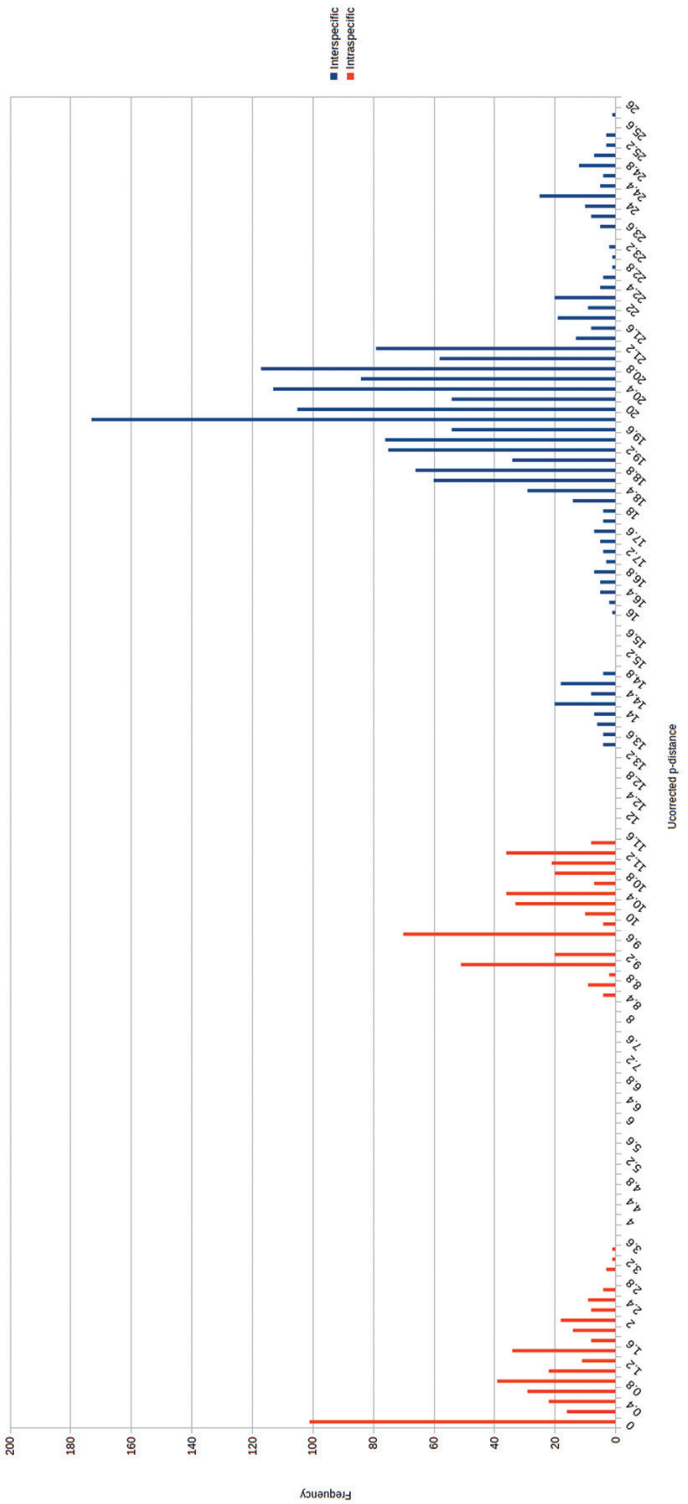

Figure 3. Frequency distribution of pairwise intraspecific (blue) and interspecific (red) distances. All lineages of *C. parisi* treated as one species, *C. parisi* sensu lato. Basic table see Suppl. material 1.

greenhouse at the Leipzig Zoo in eastern Germany (Fig. 1: 55) is in a basal position juxtaposed to all other *Cryptops* specimens (Fig. 2). The remaining Central European *Cryptops* are split into two clades, of which only the *C. parisi*/*C. croaticus* clade receives high statistical support (96%). The unsupported clade unites *C. umbricus* and *C. anomalans*, three specimens of uncertain identity, and *C. hortensis* (Fig. 2). *C. anomalans* is in a basal position regarding this second (unsupported) clade with a single haplotype spread all over Germany, forming a monophylum with *C. umbricus* from Solnhofen, Germany, representing the first record from this country (Fig. 1: 46). The uncertain *Cryptops* sp. from Slovenia is a sister group to a weakly supported clade (76% bootstrap support) uniting two unidentified *Cryptops* sp. specimens with *C. hortensis* (Fig. 2). The latter two unidentified *Cryptops* sp. specimens from eastern Austria and Croatia are grouped together, but this grouping is not statistically supported.

The monophyly of the 18 specimens of *C. hortensis* is strongly supported (100%). Of the shallow clades inside *C. hortensis* (Fig. 2), only one, a clade uniting five different haplotypes from Italy, eastern and western Germany (Fig. 4), receives some statistical support (78%). Interestingly, a second specimen from Friedeburg, Saxony-Anhalt, the same locality as one of the five haplotypes mentioned above (see Table 1), groups within a separate clade (Fig. 2).

The clade uniting *C. parisi* sensu lato and *C. croaticus* receives high statistical support (96%). While both specimens of *C. croaticus* show the same haplotype, the 32 specimens of *C. parisi* s. l. are separated into three statistically well-supported (99–100%) clades. The basalmost clade (Fig. 2) includes seven specimens and represents three different haplotypes from the eastern alpine region (Fig. 4: green). The remaining two clades of *C. parisi* are clearly related (92% support); one represents a western clade (Fig. 4: yellow) and the other is found slightly more to the east (Fig. 4: blue) and also includes the topotypoid of the subspecies *C. parisi sebini*.

Distance analysis

Cryptops specimens differ from the outgroups *Scolopendra* and *Theatops* by 19.8–25.7% (Supplementary Material 2). Interspecific and intraspecific distances of the different nominal *Cryptops* species show no overlap (Fig. 3). Interspecific distances lie between 13.4–21.1% (Fig. 3), with the lowest observed between *C. croaticus* and *C. parisi* s. l. (13.4–14.8%) as well as between *C. anomalans* and *C. umbricus* (13.9%). Otherwise, interspecific distances are always >16%, with the highest value of >20% observed between *C. anomalans* and *C. hortensis*, as well as between *C. parisi sebini* and *C. hortensis*. Intraspecific distances are between 0–11.3%. However, intraspecific distances are low, 0–3.3%, if we treat the three distinct lineages of *C. parisi* as distinct species (Fig. 5).

Discussion

Distance analysis

Clear intraspecific distances in German or even Central European *Cryptops* are low. The specimens filling the majority of our barcoding gap between 3 and 11.3% are the different lineages of *C. parisi*, which differ by 8.4–11.3% from one another (Fig. 3) and might represent distinguishable taxonomic units (see below). Two specimens directly at the edge between inter- and intraspecific distances (Fig. 5), the two *Cryptops* "sp. 2" specimens from Austria and Croatia (15.9%), require a careful re-study (see below).

The biogeographic and ecological pattern of *C. hortensis* and *C. parisi* in Central Europe

Cryptops parisi and *C. hortensis* belong to the South European and Central Asiatic European chorotypes respectively (Zapparoli 2006). In central Europe *C. hortensis* and *C. parisi* s. l. seem to exclude each other either geographically or ecologically. In the lowland areas of north-western Germany and in the Upper Rhine valley it is usually *C. hortensis* that occurs, while in the lower mountain ranges usually *C. parisi* is present. Nevertheless, *C. parisi* mainly avoids higher altitudes. In the eastern part of Germany *C. parisi* dominates.

Cryptops parisi is generally classified as a mesophilous woodland species (Spelda 1999, Minelli and Iovane 1987, Voigtländer et al. 1997), but may also occur outside of forests, especially in northern Germany where more anthropogenic influenced places are inhabited.

The two clearly differentiated genetical lineages in *C. parisi* s. s. in Germany (see below) are reflected in distinct ecological differences in the preferred habitats between the western and eastern parts of Germany. In the more Atlantic areas in the West, the species prefers woodland like in its main distribution area. In the more continental influenced East, *C. parisi* inhabits open-dry habitats such as dry meadows, mesoxeric meadows and their successional shrub-stages, as well as dwarf-shrub heaths (Voigtländer 2003a, 2003b, 2005).

A single haplotype in German *Cryptops anomalans*

C. anomalans is viewed as a species introduced to Germany and England (Eason 1964; Voigtländer 1988). Specimen records are rare, e.g. the species has only recently been recorded from Germany, where it only occurs in localized areas, usually in parks or gardens (Lindner 2010, Decker and Hannig 2011). Our findings show that a single haplotype (Fig. 2) is present in western, eastern and southern Germany (Fig. 1), while all other *Cryptops* (see Fig. 3), as well as Geophilomorpha species (Wesener et al. 2015)

show different haplotypes across a large geographical area. An identical haplotype from different localities might be interpreted as recent human introductions from a homogenous source population or a rapid spread of *C. anomalans* in Germany.

First record of *C. umbricus* in Germany

Our analyses first showed one outlier *C. anomalans* specimen from Solnhofen, Bavaria (Fig. 1), which strongly differs by 13.9% from the common German haplotype. This was the only specimen of *C. anomalans* in a previous analysis involving German centipedes (Spelda et al. 2011). A morphological check against similar species showed that it was indeed not *C. anomalans* but represents *C. umbricus*, a first record for Germany. This finding shows the usefulness of the barcoding method in detecting previously unrecorded species.

At least three undetermined *Cryptops* species in Central Europe

Cryptops sp. 1 is only represented in our dataset by a single specimen from Slovenia, which is unfortunately missing the pre-ultimate legs and can therefore not easily be determined morphologically.

Cryptops sp. 2 is represented by two specimens that are separated by a wide genetic distance of 15.9%. This distance usually falls right into the lower limit observed between different *Cryptops* species (Fig. 3). The two specimens are from the eastern lowlands of Austria (Burgenland) and Croatia (Brestova). Unfortunately, the Austrian specimen is heavily damaged with missing posterior segments, which prevents any determination. As both specimens of *Cryptops* sp. 2 are related, but potentially not conspecific, they are discussed here together.

These two specimens are similar to *C. hortensis*, but are missing the ventral furrow on the prefemora of the ultimate leg pair. An available name for one of these lines might be *C. rucneri* Matic, 1966. This species was synonymised with *C. hortensis* by Koren (1986), followed by Spelda (1999), but treated as a valid species later (Stoev (2002). The presently discovered genetic diversity brings this name into consideration again. One argument for the identity of one of our lines with *C. rucneri* is the configuration of the prefemur of the ultimate legpair, where Matic (1966) did not mention a ventral furrow. Although Matic (1966, 1972) did not describe and depict the poison gland in great detail, his figures clearly show that in both *C. hortensis* sensu Matic (1972a) and *C. rucneri*, the calyx of the poison glands lie mainly in the femur and tibia of the forcipule. Matic also records *C. rucneri* from Italy (Matic 1967), Austria: Carynthia (Matic 1972b), and Slovenia (Matic 1979).

Maybe this specimen is the same species to which Pichler (1987) refers to as *Cryptops* cf. *hortensis* from North Tyrol. The shape of the poison gland was not illustrated for *C.* cf. *hortensis*. The poison gland allows a clear separation from *C. parisi* even in

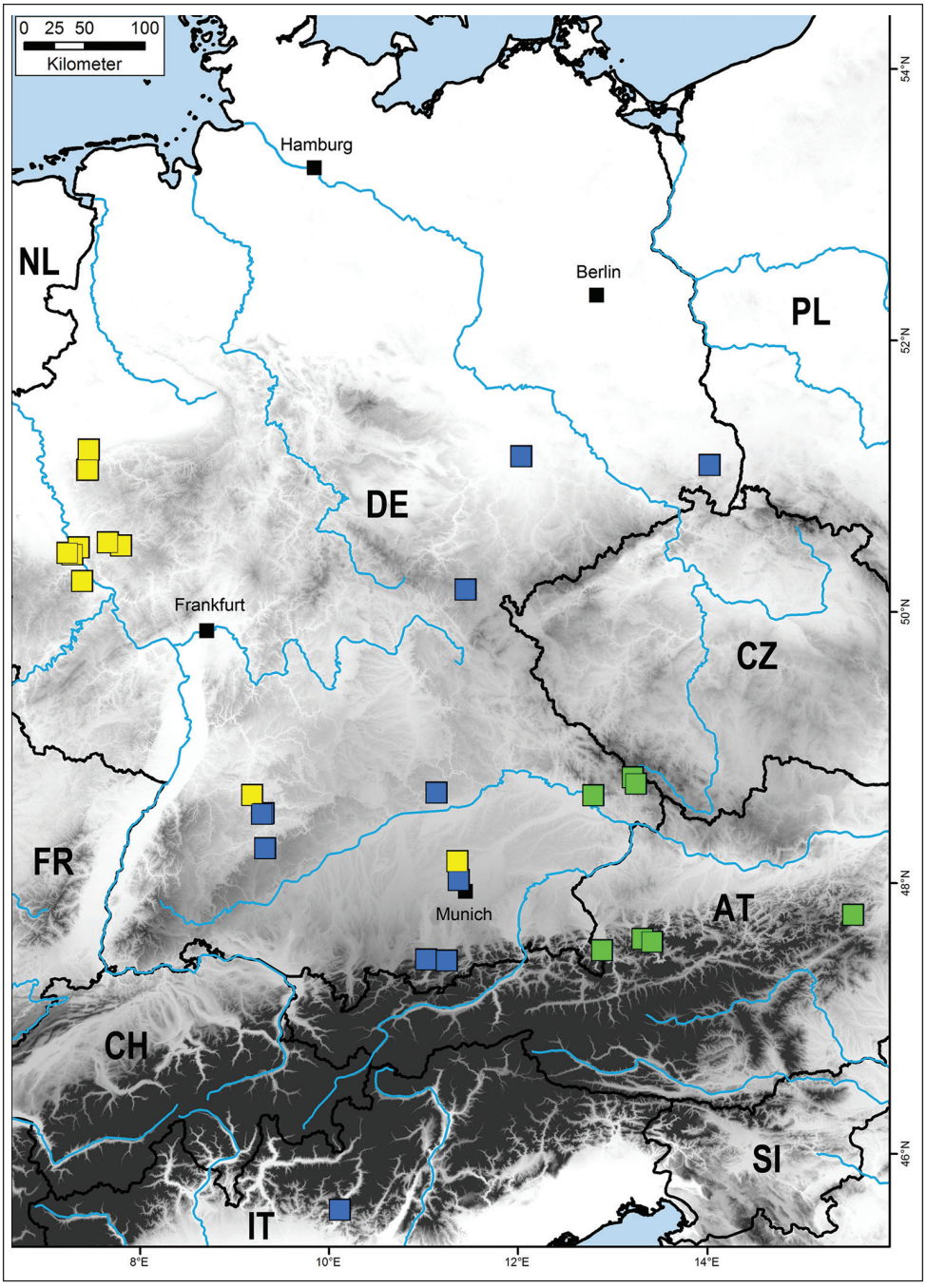

Figure 4. Distribution map of all successfully sequenced Central European specimens of *Cryptops parisi*. Different colours mark the three different clades. Yellow = *C. parisi* sensu stricto; blue = *C. parisi sebini*; green = *C. parisi* lineage 3 (potentially *C.* cf. *hortensis* sensu Pichler 1987).

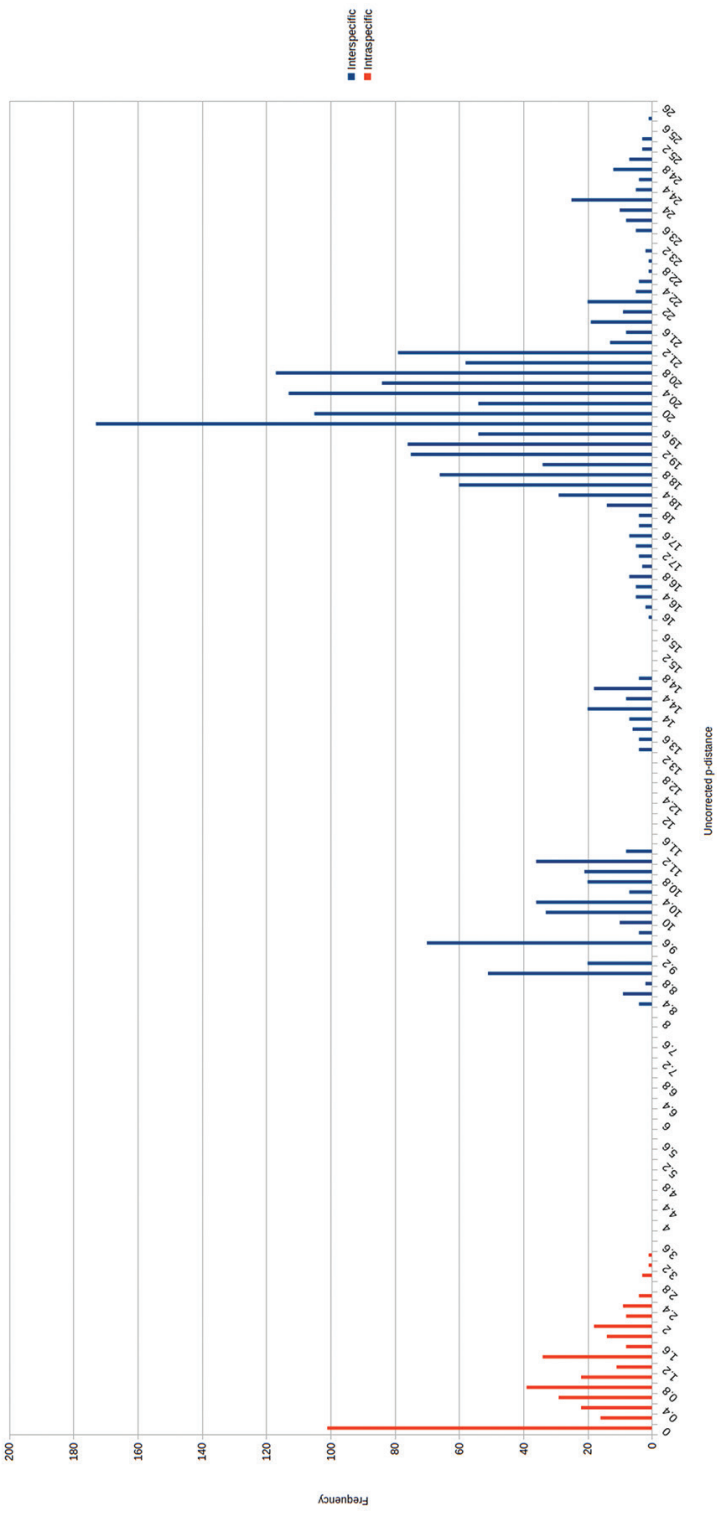

Figure 5. Frequency distribution of pairwise intraspecific (blue) and interspecific (red) distances. The three lineages of *C. parisi* treated as different species. Basic table see Suppl. material 1.

very early stages. Without checking the poison gland, juvenile specimens of *C. parisi*, which lack the characteristics of adult specimens (a central depression on the forcipular tergite and the pair of occipital sutures), can be easily mistaken for *C. hortensis*. Pichler (1987) records an unidentate labrum for *C.* cf. *hortensis*, as does Matic (1966) for *C. rucneri*. Pichler's (1987) fig. 18 of the 21st pleurocoxa corresponds to fig. 4 of Matic (1966) for *C. rucneri*.

Of the two specimens of *Cryptops* sp. 2, the one from Brestova is the most probable to represent *C. rucneri*. This specimen was collected only 30 kilometres distant from the type locality of *C. rucneri* and shows the characteristic elongated 20th leg pair, which is unfortunately missing in the other specimen (as well as in our *Cryptops* sp. 1). Nevertheless, while having only three sequences of these eastern *C. hortensis*-relatives and without being able to provide a revision of the *hortensis*/*rucneri*-complex we prefer at the moment to keep these specimens under the name *Cryptops* sp.

Cryptops sp. 3, previously determined as *C.* cf. *doriae* Pocock, 1891 is only known from the Leipzig Zoo in eastern Germany, where it was collected in a large tropical greenhouse (Decker et al. 2014). It was provisionally identified as *C. doriae*, a member of the *doriae*-group, which is characterized by having teeth on femur, tibia and tarsus of the ultimate legs (Lewis 2011). *C. doriae* was already reported from a tropical biome in England (Lewis 2007) and is so far the only introduced tropical *Cryptops* species with records in Europe (Stoev et al. 2010). A BLAST search of our specimen against the sequences of *C. doriae* already deposited on GenBank (11.2015) reveals a large genetic distance between our specimen and the ones from the Pacific, which is the reason we refer to our specimen as *Cryptops* sp. 3.

First record of *Cryptops croaticus* in Austria

Cryptops croaticus was originally described from Bakar (formerly Buccari) in Croatia (Verhoeff 1931) and subsequently recorded from other localities in Croatia, Slovenia and Bosnia-Herzegovina (Matic 1966, 1979, Kos 1992), Greece (Matic 1976), Bulgaria (Stoev 1997a, 2002), and Italy (Matic 1960, 1968, Matic and Darabantu 1971, Minelli 1985, 1992). Currently, *C. croaticus* seems to be absent or not yet found in Hungary (Dányi 2008). One subspecies (*C. croaticus burzenlandicus*) was described from Romania (Verhoeff 1931) and was subsequently synonymised with the nominal subspecies (Matic 1972a), another subspecies, *C. croaticus albanicus*, has been described from Albania (Verhoeff 1934) and was later synonymized under *C. anomalans* (Stoev 1997b). Several subspecies have been described from Italy, namely *C. croaticus bergomatius* (Verhoeff 1934), *C. croaticus longobardius* and *C. croaticus baldensis* (Manfredi 1948), subsequently cited by Conci (1951) and Boldori (1969). Based on this wide distribution, the occurrence of *C. croaticus* in Austria is not unexpected. In Austria, it is currently only known from a southern exposed slope, which is home to numerous relic species adapted to a warmer climate. *C. croaticus* shares its habitat with the recently rediscovered population of *Scolopendra cingulata* in Austria (Oeyen et al. 2014), as well

as the thermophilic beetle *Carabus hungaricus* and other thermophilic animals (Böhme et al. 2014). However, the determination of our specimens as *C. croaticus* is only based on the characters given in the original description (Verhoeff 1931) as no better description exists. Numerous important characters, such as the last leg pairs, are unfortunately missing in our specimens. A revision of *C. croaticus* is urgently needed (Matic 1966) as it may be that some of the nominal subspecies represent independent species. One way to clarify this is to collect and sequence topotypic material. Once *C. croaticus* has been properly revised, a re-evaluation of the Austrian specimens should be undertaken.

The three lineages of *Cryptops parisi* sensu lato

The three lineages of specimens placed in *C. parisi* by morphological characters differ 8.4–11.3% from one another, while their intra-lineage genetic distance is much lower at 0–1.1%. A large barcoding gap becomes clearly visible in our dataset when we treat the three different lineages of *C. parisi* as separate species (Figs 4, 5). Endosymbionts like *Wolbachia* (Hurst and Jiggins 2005) are an unlikely explanation for the different lineages, as such endosymbionts have never been recorded in the Myriapoda (Witzel et al. 2003).

One lineage clearly represents the *C. parisi* sensu stricto (Fig. 2: yellow). This group shows a western distribution in Germany, with a single specimen from southern Germany (Fig. 4). The type locality of *C. parisi* is, as the species epithet implies, Paris, France. Our only sample from Great Britain (Wales) also falls into this group. Intra-lineage variation is low with 0–1.7%. Inner structure of the lineage is limited due to the small genetic distances inside the group, but one group containing only few haplotypes differing in a single or two basepairs from one another is well-supported. This group contains specimens from western Germany, as well as a single specimen each from southwestern (ZFMK-TIS 2520349) and southeastern Germany (ZFMK-DNA-112780049), but these two were collected in a park and a garden.

A second distinct group (Fig. 2: Blue) contains the topotypic specimen of the subspecies *C. parisi sebini* Verhoeff, 1934. *C. parisi sebini* was recently synonymised under *C. parisi* because no morphological differences could be detected (Lewis 2011). However, the distinctiveness of the subspecies *C. parisi sebini* should be re-evaluated, as our genetic data supports this monophyletic subspecies (100% bootstrap support) with a high genetic distance to *C. parisi* s. s. (8.4–9.4%) in combination with low intra-lineage variation (0–0.6%) despite the large geographical distances between the analyzed specimens from Italy and eastern Germany. This *C. parisi* group 2 shows a distribution to the east of *C. parisi* s. s., with localities in eastern northern Italy and the eastern half as well as the south of Germany (Fig. 4). Another name potentially available for this clade is *C. parisi rhenanus* Verhoeff, 1931, which is characterized by its extremely elongated calyx of the poison gland (Verhoeff 1931). If both names turn out to represent the same species, this taxon would have priority over *C. parisi sebini*, with

which it is compared in the original description (Verhoeff 1934). Unfortunately, Verhoeff (1931) never designated a type for *C. parisi rhenanus*. The specimens represented in the Bavarian State Collection of Zoology originate from a large number of localities.

The specimens of *C. parisi* s. l. belonging to a third group (Fig. 2: green), referred here as *C. parisi* lineage 3, are morphologically and genetically distinct and may also be identical to the specimens of *C.* cf. *hortensis* in the literature (Pichler 1987, Lewis 2011). Our specimens of *C. parisi* lineage 3 come mainly from alpine habitats in Austria and Germany. In the most recent revision of the species group (Lewis 2011), these specimens were listed in the key under *C. parisi*, but with remarks concerning its unique morphology. Coxal pores are too numerous (~50) for *C. hortensis* and more closely resemble the lower end of *C. parisi*. Other morphological characters prompted Lewis (2011) to place these specimens in his key under *C. parisi*, an affinity confirmed here by our genetic analysis.

However, the large genetic distance of 10–11.3% between *C. parisi* lineage 3 and *C. parisi* s. s. as well as to the lineage containing *C. parisi sebini*, combined with a low intraspecific distance (0–1.1%) are clear indications that these specimens might represent a species of its own.

To find names for our two eastern lines of *C. parisi* one has to go back to C. L. Koch, who described three *Cryptops* species from around Regensburg, Germany: *C. ochraceus* C. L. Koch, 1844 from the Keilstein (a calcareous mountain east of Regensburg), *C. sylvaticus* C. L. Koch, 1844 from the Naab-valley (north of Regensburg) and *C. pallens* C. L. Koch, 1847 from the moat of Regensburg. More information on these species, such as the precise type localities and more detailed descriptions, are provided in Koch (1863), which has often resulted in these species erroneously being assigned to the date of this second publication.

Attems (1930) indicated that it would be impossible to assign these species to either *C. hortensis* or *C. parisi*, while Matic (1972a) simply synonymized them with *C. hortensis*. Both did not take note of the central depression, often darker than the adjacent parts of the tergite, as a character separating *C. parisi* from *C. hortensis*, at least for adult specimens from southern Germany (own observation, JS). This depression is also described by Attems (1930) as existing in some *C. parisi* specimens, but is not otherwise mentioned in the available keys separating the two species (Attems 1930, Brölemann 1930, Verhoeff 1931, Eason 1964, Matic 1972a, Koren 1982, 1986, Iorio and Geoffroy 2008). Verhoeff (1934) also described this character in *C. parisi sebini*. Koch (1863) clearly states and depicts the depression for his species *C. sylvaticus* and *C. ochraceus*. It seems only to be missing in *C. pallens*, which represents a juvenile specimen. Another argument against a synonymy of these species with *C. hortensis* is the absence of the latter species in our extensive collections from eastern Bavaria. Topotypoids of *C. ochraceus* have already been collected and might clarify this species in the near future.

It should be noted that Matic (1972a) depicts a *C. parisi* with a short poison gland. This specimen surely represents a different species.

Outlook/future studies

Future prospects should include the parallel sequencing of nuclear genes to confirm the relationships drawn from the mitochondrial barcoding fragment. To clarify the taxonomic relationships within *Cryptops parisi*, it would be important to collect further samples to enable an extensive morphological evaluation.

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Supplementary material 1

Full specimen information

Authors: homas Wesener, Karin Voigtländer, Peter Decker, Jan Philip Oeyen, Jörg Spelda

Data type: occurence

Explanation note: Full specimen information.

Supplementary material 2

Uncorrected P-Distance

Authors: homas Wesener, Karin Voigtländer, Peter Decker, Jan Philip Oeyen, Jörg Spelda

Data type: measurements

Explanation note: Uncorrected P-Distance between the 66 analyzed specimens.

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RESEARCH ARTICLE

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Larval descriptions of the family Porcellanidae: A worldwide annotated compilation of the literature (Crustacea, Decapoda)

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Abstract

For most of the family Porcellanidae, which comprises 283 species, larval development remains to be described. Full development has been only described for 52 species, while part of the larval cycle has been described for 45 species. The importance of knowing the complete larval development of a species goes beyond allowing the identification of larval specimens collected in the plankton. Morphological larval data also constitute a support to cladistic techniques used in the establishment of the phylogenetic status (see Hiller et al. 2006, Marco-Herrero et al. 2013). Nevertheless, the literature on the larval development of this family is old and widely dispersed and in many cases it is difficult to collect the available information on a particular taxon. Towards the aim of facilitating future research, all information available on the larval development of porcellanids has been compiled. Following the taxonomic checklist of Porcellanidae proposed by Osawa and McLaughlin (2010), a checklist has been prepared that reflects the current knowledge about larval development of the group including larval stages and the method used to obtain the larvae, together with references. Those species for which the recognised names have been changed according to Osawa and McLaughlin (2010) are indicated.

Keywords

Checklist, larval development, Anomura, zoea, megalopa

Introduction

Porcellanidae, commonly known as porcelain crabs, is a family of decapods belonging to the infraorder Anomura (Crustacea, Decapoda). The group comprises 283 species according to the classification proposed by Osawa and McLaughlin (2010). Like most decapods, their life cycle contains a planktonic larval phase presenting various morphological changes during ontogenic development; this produces different larval morphologies that vary even within the same species. This high inter- and intra-specific morphological diversity poses many difficulties both for the identification of specimens from plankton samples and for the taxonomic description of undescribed larval stages. Morphological studies are thus of crucial importance if such problems are to be overcome.

Although decapod larvae were first described almost 250 years ago (*Cancer pagurus*, described as *Cancer germanicus* by Linnaeus, 1767), the morphology of a porcellanid larva was not described until 1835, when J. Vaughan Thompson published a brief description of a larva of *Porcellana* reared from eggs of females collected in British waters. Eight years later, Dujardin (1843) presented for the first time a more comprehensive description of a porcellanid larva, describing the zoeal stage of *Pisidia longicornis* (as *Porcellana longicornis*). Numerous descriptions of the larval stages have been published during more than 170 years. The number of published descriptions of the larval morphology of porcellanids, and of other groups of decapods, has grown exponentially since the 1960's (Martin 1984, Rice 1993). Several researchers, including Gore (1968–1977) or the team constituted by Hernández, Bolaños and Graterol (see papers from 1996 to 2012), have made special contributions to knowledge of porcellanid larval morphology.

González-Gordillo et al. (2001) showed that, in addition to the limited number of descriptive studies on decapod larval morphology, a large percentage are based on organisms collected from plankton samples or reared under laboratory conditions from females that were not accurately identified. Furthermore, several published larval descriptions are brief or very general, with inadequate illustrations that are far from the well-accepted standard proposed by Clark et al. (1998).

In addition, the literature on larval descriptions is scattered or very old; since literature of this kind is often not available in digital formats for download or online request, or it has been published in local scientific journals ("grey" literature), it is complicated to access it using common bibliographic search engines. As a consequence, in studies requiring the identification of planktonic organisms (with the eventual need to present identification keys), or in morphological studies in which new larval stages are described, where it becomes necessary to compare results with those reported in previous publications of larval descriptions, the researcher has a difficult task in compiling the available information for the target taxon. Although this situation has yielded publication of several bibliographic compilations for brachyurans, like those of Gurney (1939), Soltanpour-Gargari et al. (1989), Martin (1984), Wear (1985), Wehrtman and Baez (1997) and González-Gordillo et al. (2001), there is still no published compilation on porcellanids on a worldwide basis.

Many larval publications first appeared more than 30 years ago; for example according to González-Gordillo et al. (2001), 86.6 % of the descriptions made for species of decapods from the Gibraltar Strait were published more than 25 years ago. The scientific name of a species described then could have changed, or two or more different species could have been reclassified as one species. This complicates even further the bibliographic search because a search using the current name of a target species will almost certainly omit old studies of that species under a name that has changed or been superseded.

Therefore, the objective of this study is three-fold: 1) to compile the available literature on porcellanid larval morphologies; 2) to record the possible changes in the nomenclature of species, or synonymies; and 3) to describe the state-of-the-art on the larval development of species belonging to the family Porcellanidae.

Methods

The data set of this study comprises a total of 133 entries obtained from 83 published papers (from 1835 to 2012). Search engines and scientific databases such as *Google Scholar, Scopus, Science Direct* and *Web of Science* have been used for the bibliographic compilation. The current total number of porcellanid species and the taxonomic classification used for the present checklist follow those of Osawa and McLaughlin (2010). The current validity of the species has been also checked by consulting the *World Register of Marine Species* (http://www.marinespecies.org).

In the checklist, the status of current knowledge of the larval development is specified for each species as follows: i) the author(s) and the date of publication of the larval description; ii) the specific larval stages described, using the following classification: prezoeal stage (PR), first to fifth zoeal stage (Z1-5), and megalopal stage (M); iii) the method used to obtain the larvae, according to the following designations: from plankton samples (Pl), larvae reared under laboratory conditions from an identified ovigerous females (Lab) and larvae obtained from plankton and by instar-to-instar laboratory rearing, from unknown parentage, but often a species recognizable from its postlarval or juvenile stages (P+L). Entries marked with asterisk mean that the larval description available, in our opinion, is accurate enough to establish comparisons with other species and have all stages fully described and illustrated. In the checklist, if the taxonomical name of the species described does not match the current taxonomic name according to Osawa and McLaughlin (2010), this is indicated by '*as*' followed by the name of the species cited in the description.

Results

The larval development of porcellanids usually consists of two zoeal stages and one megalopal stage, with the exception of *Petrocheles spinosus,* which has five zoeal stages.

Description of the larval development of porcellanids first appeared in 1843, when Dujardin published a description of the first zoeal stage of *Pisidia longicornis*, referred

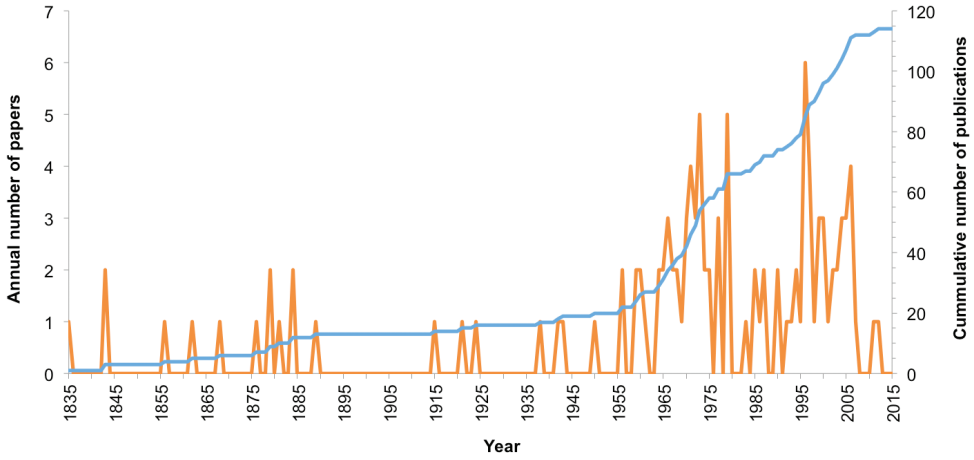

Figure 1. Number of papers describing the larval morphology of porcellanids. Number of publications per year (left-hand scale) and cumulative number of publications represented by the blue line (right-hand scale).

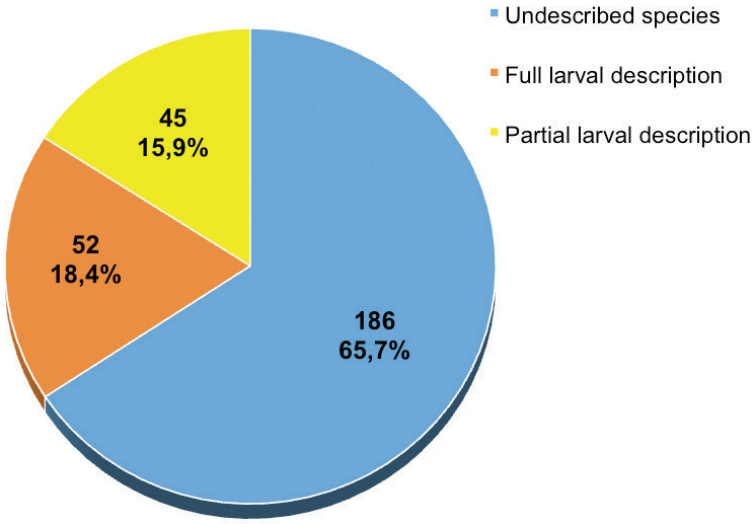

Figure 2. Number and proportion of porcellanid species (N = 283) for which undescribed species (blue sector), full larval description (orange sector) and partial larval description (yellow sector) exists.

to as *Porcellana longicornis*. The larval descriptions available were poor in number until the 1960's and 1970's, when an increasing trend in the number of publications is observed; this was possibly due to the increased number of scientists specializing in this area, to the increased facilities for cultivating larvae in laboratory conditions, and to the advances in microscope technology (Rice 1993). The historical peak for the number of publications per annum occurred in the late 1990's and at the beginning of the current century.

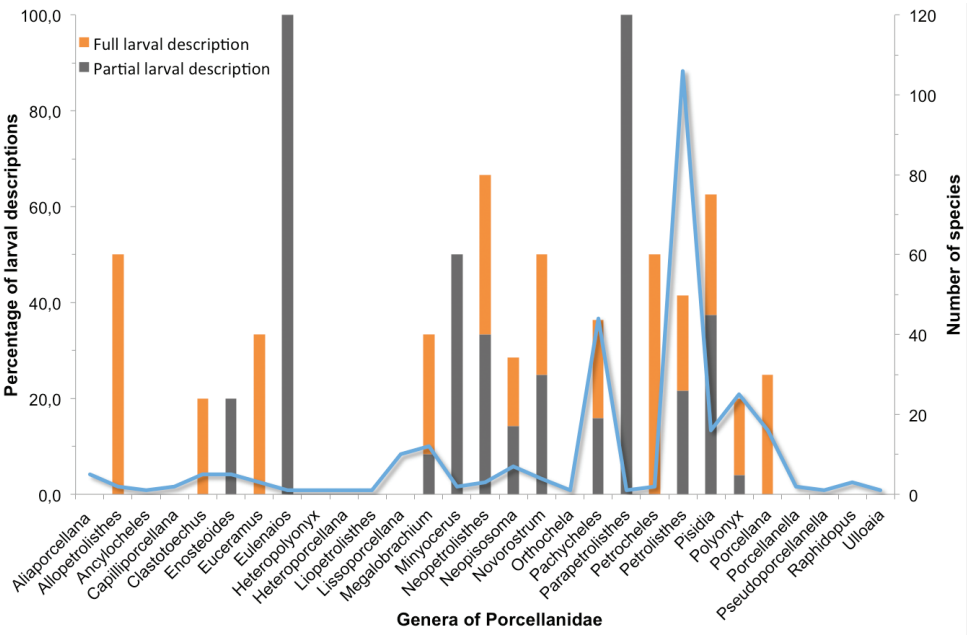

Figure 3. State of current knowledge of larval development of Porcellanidae, grouped by genus. Shown in orange is the percentage of species for which larval development has been completely described. Shown in grey is the percentage of species for which only some of the larval stages have been described (left-hand scale). The total number of species per genus is also represented with a solid blue line (right-hand scale).

Currently, the family Porcellanidae family consists of 283 species (Osawa and McLaughlin 2010). Complete larval development has been described for 52 species (18.4%), while only some larval stages have been described for another 45 species (15.9%). For the remaining 186 species (65.7%), none of the larval stages has been described.

The current knowledge of larval development by genus (percentages) and the number of species in each genus are shown in Figure 3. Although the family Porcellanidae consists of 29 genera, the larval stages have not been described for 12 genera. The genera with the most numerous species are *Petrolisthes* (106 species) and *Pachycheles* (44 species); however, the complete larval development has been described for only 21 species of *Petrolisthes* (19.8%) and only nine species of *Pachycheles* (20.4%).

Annotated bibliography of porcellanid larvae

Family Porcellanidae Haworth, 1825

Thompson (1935) as *Porcellana* sp; Z1: Lab

Webb (1921) as *Porcellana* sp; M: Pl

Gurney (1924) as Porcellanid larva; Z1, Z2: Pl

Aliaporcellana kikuchii Nakasone & Miyake, 1969: larvae undescribed

Aliaporcellana pygmaea (De Man, 1902): larvae undescribed
Aliaporcellana taiwanensis Dong, Li & Chan, 2011: larvae undescribed
Aliaporcellana suluensis (Dana, 1852): larvae undescribed
Aliaporcellana telestophila (Johnson, 1958): larvae undescribed
Allopetrolisthes angulosus (Guérin, 1835): full larval description
*Wehrtmann et al. (1996); PR, Z1, Z2, M: Lab
Allopetrolisthes punctatus (Guérin, 1835): larvae undescribed
Ancylocheles gravelei (Sankolli, 1963): larvae undescribed
Capilliporcellana murakamii (Miyake, 1942): larvae undescribed
Capilliporcellana wolffi Haig, 1981: larvae undescribed
Clastotoechus diffractus (Haig, 1957): larvae undescribed
Clastotoechus gorgonensis Werding & Haig, 1983: larvae undescribed
Clastotoechus hickmani Harvey, 1999: larvae undescribed
Clastotoechus lasios Harvey, 1999: larvae undescribed
Clastotoechus nodosus (Streets, 1872): full larval description
*Hernández et al. (2003); Z1, Z2, M: Lab
Enosteoides lobatus Osawa, 2009: larvae undescribed
Enosteoides melissa (Miyake, 1942): larvae undescribed
Enosteoides ornatus (Stimpson, 1858): partial larval description
Sankolli (1967) as *Porcellana ornata*; PR, Z1: Lab
Ko (2000); Z1: Lab
Enosteoides palauensis (Nakasone & Miyake, 1968): larvae undescribed
Enosteoides philippinesnsis Dolorosa & Werding, 2014: larvae undescribed
Euceramus panatelus Glassell, 1938: larvae undescribed
Euceramus praelongus Stimpson, 1860: full larval description
Roberts (1968); Z1, Z2, M: Lab
Maris (1983); Z1, Z2: Pl
Euceramus transversilineatus (Lockington, 1878): larvae undescribed
Eulenaios cometes (Walker, 1887): partial larval description
Ng and Nakasone (1993); Z1: Lab
Heteropolyonyx biforma Osawa, 2001: larvae undescribed
Heteroporcellana corbicola (Haig, 1960): larvae undescribed
Liopetrolisthes mitra (Dana, 1852): larvae undescribed
Lissoporcellana demani Dong & Li, 2014: larvae undescribed
Lissoporcellana flagellicola Osawa & Fujita, 2005: larvae undescribed
Lissoporcellana furcillata (Haig, 1965): larvae undescribed
Lissoporcellana miyakei Haig, 1981: larvae undescribed
Lissoporcellana monodi Osawa, 2007: larvae undescribed
Lissoporcellana nakasonei (Miyake, 1978): larvae undescribed
Lissoporcellana nitida (Haswell, 1882): larvae undescribed
Lissoporcellana pectinata Haig, 1981: larvae undescribed
Lissoporcellana quadrilobata (Miers, 1884): larvae undescribed
Lissoporcellana spinuligera (Dana, 1853): larvae undescribed

Megalobrachium erosum (Glassell, 1936): larvae undescribed
Megalobrachium festae (Nobili, 1901): larvae undescribed
Megalobrachium garthi Haig, 1957: larvae undescribed
Megalobrachium mortenseni Haig, 1962: partial larval description
Kraus (2006); Z1, Z2: Pl
Megalobrachium pacificum Gore & Abele, 1974: larvae undescribed
Megalobrachium peruvianum Haig, 1960: larvae undescribed
Megalobrachium poeyi (Guérin-Méneville, 1855): full larval description
*Gore (1971b); Z1, Z2, M: Lab
Megalobrachium roseum (Rathbun, 1900): full larval description
*Hernández et al. (2002); Z1, Z2, M: Lab
Megalobrachium sinuimanus (Lockington, 1878): larvae undescribed
Megalobrachium smithi (Glassell, 1936): larvae undescribed
Megalobrachium soriatum (Say, 1818): full larval description
*Gore (1973b); Z1, Z2, M: Lab
Megalobrachium tuberculipes (Lockington, 1878): larvae undescribed
Minyocerus angustus (Dana, 1852): partial larval description
Hernández et al. (1996); Z1: Lab
Minyocerus kirki Glassell, 1938: larvae undescribed
Neopetrolisthes alobatus (Laurie, 1926): larvae undescribed
Neopetrolisthes maculatus (H. Milne Edwards, 1837): partial larval description
Fujita and Osawa (2003); Z1, Z2: Lab
Kraus (2006); Z1: Pl
Neopetrolisthes spinatus Osawa & Fujita, 2001: partial larval description
Fujita and Osawa (2003); Z1, Z2: Lab
Neopisosoma angustifrons (Benedict, 1901): full larval description
*Gore (1977); Z1, Z2, M: Lab
Neopisosoma bicapillatum Haig, 1960: larvae undescribed
Neopisosoma curacaoense (Schmitt, 1924): larvae undescribed
Neopisosoma dohenyi Haig, 1960: larvae undescribed
Neopisosoma mexicanum (Streets, 1871): larvae undescribed
Neopisosoma neglectum Werding, 1986: full larval description
*Werding and Müller (1990); Z1, Z2, M: Lab
Neopisosoma orientale Werding, 1986: larvae undescribed
Novorostrum decorocrus Osawa, 1998: full larval description
*Fujita and Osawa (2005); Z1, Z2, M: Lab
Novorostrum indicum (De Man, 1893): partial larval description
Osawa (2000); Z1, Z2: Lab
Novorostrum phuketense Osawa, 1998: larvae undescribed
Novorostrum securiger (Melin, 1939): larvae undescribed
Orthochela pumila Glassell, 1936: larvae undescribed
Pachycheles sp.
Williamson (1970) as *Pachycheles* nrs39; Z2: Pl

Pachycheles ackleianus A. Milne-Edwards, 1880: larvae undescribed
Pachycheles attaragos Harvey & de Santo, 1997: larvae undescribed
Pachycheles barbatus A. Milne-Edwards, 1878: larvae undescribed
Pachycheles bellus (Osorio, 1887): larvae undescribed
Pachycheles biocellatus (Lockington, 1878): larvae undescribed
Pachycheles calculosus Haig, 1960: larvae undescribed
Pachycheles chacei Haig, 1956: partial larval description
Kraus (2006); Z1, Z2, M: Pl
Pachycheles chubutensis Boschi, 1963: partial larval description
González et al. (2006); Z1: Lab
Pachycheles crassus (A. Milne-Edwards, 1869): larvae undescribed
Pachycheles crinimanus Haig, 1960: larvae undescribed
Pachycheles cristobalensis Gore, 1970: larvae undescribed
Pachycheles garciaensis (Ward, 1942): partial larval description
Osawa (1997a); Z1: Lab
Pachycheles granti Haig, 1965: larvae undescribed
Pachycheles greeleyi (Rathbun, 1900): larvae undescribed
Pachycheles grossimanus (Guérin, 1835): larvae undescribed
Pachycheles hertwigi Balss, 1913: partial larval description
Ko (1999); Z1: Lab
Pachycheles holosericus Schmitt, 1921: larvae undescribed
Pachycheles johnsoni Haig, 1965: larvae undescribed
Pachycheles laevidactylus Ortmann, 1892: full larval description
*Boschi et al. (1967) as *Pachycheles haigae*; Z1, Z2, M: Lab
Pachycheles marcortezensis Glassell, 1936: larvae undescribed
Pachycheles monilifer (Dana, 1852): full larval description
*Gore (1973a); Z1, Z2, M: Lab
Pachycheles natalensis (Krauss, 1843): full larval description
Sankolli (1967); Z1: Lab
*Shenoy and Sankolli (1973a); Z1, Z2, M: Lab
*Yaqoob (1979d); Z1, Z2, M: Lab
Pachycheles panamensis Faxon, 1893: larvae undescribed
Pachycheles pectinicarpus Stimpson, 1858: larvae undescribed
Pachycheles pilosus (H. Milne Edwards, 1837): full larval description
*Piñate et al. (2005); Z1, Z2, M: Lab
Kraus (2006); Z1: Pl
Pachycheles pisoides (Heller, 1865): larvae undescribed
Pachycheles pubescens Holmes, 1900: full larval description
*McMillan (1972); Z1, Z2, M: Lab
*Gonor and Gonor (1973); PR, Z1, Z2, M: P+L
Pachycheles riisei (Stimpson, 1859): partial larval description
Kraus (2006); Z1: Pl
Pachycheles rudis Stimpson, 1859: full larval description

Knight (1966); Z1, Z2: Lab
*Gonor and Gonor (1973); PR, Z1, Z2, M: Lab
Pachycheles rugimanus A. Milne-Edwards, 1880: larvae undescribed
Pachycheles sahariensis Monod, 1933: larvae undescribed
Pachycheles sculptus (H. Milne Edwards, 1837): partial larval description
Osawa (1997a); Z1: Lab
Pachycheles serratus (Benedict, 1901): full larval description
*Rodríguez et al. (2004); Z1, Z2, M: Lab
Kraus (2006); Z1: Pl
Pachycheles setiferous Yang, 1996: larvae undescribed
Pachycheles setimanus (Lockington, 1878): larvae undescribed
Pachycheles spinidactylus Haig, 1957: larvae undescribed
Pachycheles spinipes (A. Milne-Edwards, 1873): larvae undescribed
Pachycheles stevensii Stimpson, 1858: full larval description
Kurata (1964); Z1, Z2: Pl
*Konishi (1987); Z1, Z2, M: Lab
Pachycheles subsetosus Haig, 1960: larvae undescribed
Pachycheles susanae Gore & Abele, 1974: partial larval description
Kraus (2006); Z1, Z2: Pl
Pachycheles tomentosus Hendersson, 1893: full larval description
* Tirmizi and Yaqoob (1979); Z1, Z2, M: Lab
Pachycheles trichotus Haig, 1960: larvae undescribed
Pachycheles velerae Haig, 1960: larvae undescribed
Pachycheles vicarius Nobili, 1901: larvae undescribed
Parapetrolisthes tortugensis (Glassell, 1945): partial larval description
Kraus (2006); Z1: Pl
Petrocheles australiensis (Miers, 1876): larvae undescribed
Petrocheles spinosus (Miers, 1876): full larval description
Gurney (1924); Z1: Pl
*Wear (1965); Z1-Z5: Pl; M: P+L
Wear (1966); PR: Lab
Petrolisthes aegyptiacus Werding & Hiller, 2007: larvae undescribed
Petrolisthes agassizii Faxon, 1893: larvae undescribed
Petrolisthes amoenus (Guérin Méneville, 1855): larvae undescribed
Petrolisthes armatus (Gibbes, 1850): full larval description
Lebour (1943); Z1: Lab
Lebour (1950); Z2: Pl
*Gore (1970); Z1, Z2, M: Lab
*Gore (1972a); Z1, Z2, M: Lab
Maris (1983); Z1, Z2: Pl
Petrolisthes artifrons Haig, 1960: larvae undescribed
Petrolisthes asiaticus (Leach, 1820): partial larval description
Osawa (1997b); Z1, Z2: Lab

Petrolisthes bifidus Werding & Hiller, 2004: larvae undescribed
Petrolisthes bispinosus Borradaile, 1900: larvae undescribed
Petrolisthes bolivarensis Werding & Kraus, 2003: full larval description
Kraus (2006); Z1, Z2, M: Pl
Petrolisthes borradailei Kropp, 1984: larvae undescribed
Petrolisthes boscii (Audouin, 1826): full larval description
*Yaqoob (1979a); Z1, Z2, M: Lab
Petrolisthes brachycarpus Sivertsen, 1933: larvae undescribed
Petrolisthes cabrilloi Glassell, 1945: larvae undescribed
Petrolisthes caribensis Werding, 1983: partial larval description
Kraus et al. (2004); Z1, Z2: Lab
Kraus (2006); Z1, Z2: Pl
Petrolisthes carinipes (Heller, 1861): larvae undescribed
Petrolisthes celebesensis Haig, 1981: larvae undescribed
Petrolisthes cinctipes (Randall, 1840): full larval description
*Gonor and Gonor (1973); PR, Z1, Z2, M: Lab
Petrolisthes coccineus (Owen, 1839): partial larval description
Osawa (1995); Z1, Z2: Lab
Petrolisthes cocoensis Haig, 1960: larvae undescribed
Petrolisthes columbiensis Werding, 1983: partial larval description
Kraus (2006); Z1: Pl
Petrolisthes crenulatus Lockington, 1878: larvae undescribed
Petrolisthes decacanthus Ortmann, 1897: larvae undescribed
Petrolisthes desmarestii (Guérin, 1835): larvae undescribed
Petrolisthes dissimulatus Gore, 1983: partial larval description
Kraus (2006); Z1: Pl
Petrolisthes donadio Hiller & Werding, 2007: larvae undescribed
Petrolisthes donanensis Osawa, 1997: larvae undescribed
Petrolisthes edwardsii (de Saussure, 1853): partial larval description
Kraus (2006); Z1: Pl
Petrolisthes eldredgei Haig & Kropp 1987: larvae undescribed
Petrolisthes elegans Haig, 1981: larvae undescribed
Petrolisthes elegantissimus Werding & Hiller, 2015: larvae undescribed
Petrolisthes elongatus (H. Milne Edwards, 1837): full larval description
*Wear (1964a); Z1: Lab; Z2, M: P+L
*Greenwood (1965); PR, Z1: Lab; Z2, M: P+L
Petrolisthes eriomerus Stimpson, 1871: full larval description
Forss and Coffin (1960); Z1, M: Lab
*Gonor and Gonor (1973); PR, Z1, Z2, M: Lab
Petrolisthes extremus Kropp & Haig, 1994: larvae undescribed
Petrolisthes fimbriatus Borradaile, 1898: larvae undescribed
Petrolisthes galapagensis Haig, 1960: larvae undescribed
Petrolisthes galathinus (Bosc, 1802): partial larval description

Kraus (2006); Z1: Pl
Petrolisthes gertrudae Werding, 1996: larvae undescribed
Petrolisthes glasselli Haig, 1957: larvae undescribed
Petrolisthes gracilis Stimpson, 1859: larvae undescribed
Petrolisthes granulosus (Guérin, 1835): full larval description
*Saelzer et al. (1986); PR, Z1, Z2, M: Lab
Petrolisthes haigae Chace, 1962: partial larval description
Hernández et al. (2007); Z1: Lab
Kraus (2006); Z1: Pl
Petrolisthes haplodactylus Haig, 1988: larvae undescribed
Petrolisthes hastatus Stimpson, 1858: partial larval description
Osawa (1997b); Z1, Z2: Lab
Petrolisthes haswelli Miers, 1884: larvae undescribed
Petrolisthes heterochrous Kropp, 1986: larvae undescribed
Petrolisthes hians Nobili, 1901: larvae undescribed
Petrolisthes hirtipes Lockington, 1878: larvae undescribed
Petrolisthes hirtispinosus Lockington, 1878: larvae undescribed
Petrolisthes hispaniolensis Werding & Hiller, 2005: larvae undescribed
Petrolisthes holotrichus Nobili, 1901: larvae undescribed
Petrolisthes inermis (Heller, 1862): larvae undescribed
Petrolisthes japonicus (De Haan, 1849): partial larval description
Muraoka and Konishi (1987); Z1: Lab
Osawa (1995); Z1, Z2: Lab
Petrolisthes jugosus Streets, 1872: partial larval description
Kraus (2006); Z1: Pl
Petrolisthes kranjiensis Johnson, 1970: larvae undescribed
Petrolisthes laevigatus (Guérin, 1835): full larval description
*Albornoz and Wehrtmann (1996); PR, Z1, Z2, M: Lab
Petrolisthes lamarckii (Leach, 1820): full larval description
Sankolli (1967); Z1: Lab
*Shenoy and Sankolli (1975); Z1, Z2, M: Lab
*Yaqoob (1979c); Z1, Z2, M: Lab
Petrolisthes leptocheles (Heller, 1861): larvae undescribed
Petrolisthes lewisi (Glassell, 1936): partial larval description
Kraus (2006); Z1: Pl
Petrolisthes limicola Haig, 1988: larvae undescribed
Petrolisthes lindae Gore & Abele, 1974: larvae undescribed
Petrolisthes magdalenensis Werding, 1978: full larval description
Müller and Werding (1990); Z1, Z2, M: Lab
*Hernández and Magan (2012); Z1, Z2, M: Lab
Petrolisthes manimaculis Glassell, 1945: larvae undescribed
Petrolisthes marginatus Stimpson, 1859: partial larval description
Kraus (2006); Z1: Pl

Petrolisthes masakii Miyake, 1943: larvae undescribed
Petrolisthes melini Miyake & Nakasone, 1966: partial larval description
Osawa (1995) as *Petrolisthes carinipes*; Z1, Z2: Lab
Petrolisthes mesodactylon Kropp, 1984: larvae undescribed
Petrolisthes militaris (Heller, 1862): larvae undescribed
Petrolisthes miyakei Kropp, 1984: larvae undescribed
Petrolisthes moluccensis (De Man, 1888): partial larval description
Osawa (1997b); Z1, Z2: Lab
Petrolisthes monodi Chace, 1956: partial larval description
Lebour (1959); M: Pl
Petrolisthes nanshensis Yang, 1996: larvae undescribed
Petrolisthes nigrunguiculatus Glassell, 1936: larvae undescribed
Petrolisthes nobilii Haig, 1960: partial larval description
Hernández et al. (2007); Z1: Lab
Petrolisthes novaezelandiae Filhol, 1885: full larval description
*Wear (1964b); Z1, Z2, M: P+L
*Greenwood (1965); PR, Z1: Lab; Z2, M: P+L
Petrolisthes obtusifrons Miyake, 1937: larvae undescribed
Petrolisthes ornatus Paulson, 1875: full larval description
*Yaqoob (1977b); Z1, Z2, M: Lab
Petrolisthes ortmanni Nobili, 1901: partial larval description
Kraus (2006); Z1: Pl
Petrolisthes perdecorus Haig, 1981: larvae undescribed
Petrolisthes platymerus Haig, 1960: full larval description
*Gore (1972b); Z1, Z2, M: Lab
Petrolisthes politus (Gray, 1831): full larval description
*Hernández et al. (2000); Z1, Z2, M: Lab
Petrolisthes polymitus Glassell, 1937: larvae undescribed
Petrolisthes pubescens Stimpson, 1858: partial larval description
Osawa (1995); Z1, Z2: Lab
Petrolisthes quadratus Benedict, 1901: partial larval description
Kraus (2006); Z1: Pl
Petrolisthes rathbunae Schmitt, 1921: larvae undescribed
Petrolisthes robsonae Glassell, 1945: full larval description
*García-Guerrero et al. (2005); Z1, Z2, M: Lab
Petrolisthes rosariensis Werding, 1982: partial larval description
Kraus (2006); Z1, Z2: Pl
Petrolisthes rufescens (Heller, 1861): full larval description
*Yaqoob (1974); Z1, Z2, M: Lab
Petrolisthes sanfelipensis Glassell, 1936: larvae undescribed
Petrolisthes sanmartini Werding & Hiller, 2002: larvae undescribed
Petrolisthes scabriculus (Dana, 1852): larvae undescribed
Petrolisthes schmitti Glassell, 1936: larvae undescribed

Petrolisthes squamanus Osawa, 1996: larvae undescribed
Petrolisthes teres Melin, 1939: larvae undescribed
Petrolisthes tiburonensis Glassell, 1936: larvae undescribed
Petrolisthes tomentosus (Dana, 1852): partial larval description
Osawa (1997b); Z1, Z2: Lab
Petrolisthes tonsorius Haig, 1960: full larval description
*Pellegrini and Gamba (1985); Z1, Z2, M: Lab
Kraus (2006); Z1: Pl
Petrolisthes tridentatus Stimpson, 1859: full larval description
*Gore (1971c); Z1, Z2, M: Lab
Kraus (2006); Z1: Pl
Petrolisthes trilobatus Osawa, 1996: partial larval description
Ko (2004); Z1: Lab
Petrolisthes tuberculatus (Guérin, 1835): larvae undescribed
Petrolisthes tuberculosus (H. Milne Edwards, 1837): larvae undescribed
Petrolisthes tuerkayi Naderloo & Apel, 2014: larvae undescribed
Petrolisthes unilobatus Henderson, 1888: full larval description
*Fujita et al. (2002); Z1, Z2, M: Lab
Petrolisthes uruma Osawa & Uyeno, 2013: larvae undescribed
Petrolisthes violaceus (Guérin, 1831): full larval description
Faxon (1879) as *Porcellana macrocheles*; Z2: Pl
*Wehrtmann et al. (1997); PR, Z1, Z2, M: Lab
Petrolisthes virgatus Paulson, 1875: larvae undescribed
Petrolisthes zacae Haig, 1968: full larval description
*Gore (1975); Z1, Z2, M: Lab
Pisidia sp
Barnich (1996); Z1, Z2: Pl
Rice and Williamson (1977) as *Pisidia* sp asm10; Z1, Z2, M: Pl
Pisidia bluteli (Risso, 1816): full larval description
Bourdillon-Casanova (1956) as *Porcellana bluteli*; Z1, Z2, M: Pl
Bourdillon-Casanova (1960) as *Porcellana bluteli*; M: Pl
Kaya and Özel (1992); Z1, Z2, M: Pl
Pisidia brasiliensis Haig, in Rodrigues da Costa, 1968: partial larval description
Hernández et al. (1996); Z1: Lab
Kraus (2006); Z1: Pl
Pisidia dehaanii (Krauss, 1843): full larval description
*Yaqoob (1979b); Z1, Z2, M: Lab
Pisidia delagoae (Barnard, 1955): larvae undescribed
Pisidia dispar (Stimpson, 1858): full larval description
Sheperd (1969); PR, Z1: Lab; Z2, M: P+L
Pisidia gordoni (Johnson, 1970): larvae undescribed
Pisidia inaequalis (Heller, 1861): partial larval description
Gurney (1938) as *Porcellana inaequalis*; PR, Z1, Z2: Pl

Pisidia longicornis (Linnaeus, 1767): full larval description
Dujardin (1843) as *Porcellana longicornis*; Z1: Lab
Gosse (1856) as *Galathea*; Z2: Pl
Bate (1879) as *Porcellana longicornis*; Z1: Pl
Hesse (1884) as *Porcellana platycheles*; Z1: Pl
Sars (1889) as *Porcellana longicornis*; Z1, Z2: P+L
Williamson (1915) as *Porcellana longicornis*; Z1, M: Pl
Webb (1921) as *Porcellana longicornis*; Z1, Z2: Pl
Gurney (1942) as *Porcellana* sp; Z2: Pl
*Lebour (1943) as *Porcellana longicornis*; Z1, Z2, M: P+L
Kurian (1956) as *Porcellana longicornis*; Z1, Z2, M: Pl
Lebour (1959) as *Porcellana longicornis*; M: Pl
Le Roux (1966) as *Porcellana longicornis*; Z1, Z2, M: P+L
Pisidia longimana (Risso, 1816): partial larval description
Kaya and Özel (1992); Z1, Z2: Pl
Pisidia magdalenensis (Glassell, 1936): larvae undescribed
Pisidia serratifrons (Stimpson, 1858): partial larval description
Sankolli (1967) as *Pisidia spinulifrons*; PR, Z1: Lab
Kim and Ko (2011); Z1, Z2: Lab
Pisidia streptocheles (Stimpson, 1858): larvae undescribed
Pisidia streptochiroides (Johnson, 1970): partial larval description
Sheperd (1969); PR, Z1: Lab; Z2: P+L
Pisidia striata Yang and Sun, 1990: larvae undescribed
Pisidia vanderhorsti (Schmitt, 1924): partial larval description
*Schoppe (1994) as *Clastotoechus vanderhorsti*; PR, Z1, Z2: Lab
Pisidia variabilis (Yang & Sun, 1985): larvae undescribed
Polyonyx biunguiculatus (Dana, 1852): larvae undescribed
Polyonyx boucheti Osawa, 2007: larvae undescribed
Polyonyx bouvieri Saint Joseph, 1900: larvae undescribed
Polyonyx confinis Haig, 1960: larvae undescribed
Polyonyx gibbesi Haig, 1956: partial larval description
Gore (1968); PR, Z1, Z2: Lab
Maris (1983); Z1, Z2: Pl
Polyonyx haigae McNeil, 1968: larvae undescribed
Polyonyx hendersoni Southwell, 1909: full larval description
Sankolli (1967); PR, Z1: Lab
*Shenoy and Sankolli (1973b): Z1, Z2, M: Lab
Polyonyx loimicola Sankolli, 1965: full larval description
*Shenoy and Sankolli (1973b): Z1, Z2, M: Lab
Polyonyx maccullochi Haig, 1965: larvae undescribed
Polyonyx nitidus Lockington, 1878: larvae undescribed
Polyonyx obesulus Miers, 1884: larvae undescribed
Polyonyx pedalis Nobili, 1905: larvae undescribed

Polyonyx plumatus Yang & Xu, 1994: larvae undescribed
Polyonyx quadratus Chace, 1956: larvae undescribed
Polyonyx quadriungulatus Glassell, 1935: full larval description
*Knight (1966); Z1, Z2, M: Lab
Polyonyx senegalensis Chace, 1956: larvae undescribed
Polyonyx sinensis Stimpson, 1858: larvae undescribed
Polyonyx spina Osawa, 2007: larvae undescribed
Polyonyx splendidus Sankolli, 1963: larvae undescribed
Polyonyx thai Werding, 2001: larvae undescribed
Polyonyx transversus (Haswell, 1882): full larval description
Sheperd (1969); PR, Z1: Lab; Z2, M: P+L
Polyonyx triunguiculatus Zehntner, 1894: larvae undescribed
Polyonyx tulearis Werding, 2001: larvae undescribed
Polyonyx utinomii Miyake, 1943: larvae undescribed
Polyonyx vermicola Ng & Sasekumar, 1993: larvae undescribed
Porcellana africana Chace, 1956: larvae undescribed
Porcellana cancrisocialis Glassell, 1936: full larval description
*García-Guerrero et al. (2006); Z1, Z2, M: Lab
Porcellana caparti Chace, 1956: larvae undescribed
Porcellana corbicola Haig, 1960: larvae undescribed
Porcellana curvifrons Yang and Sun, 1990: larvae undescribed
Porcellana elegans Chace, 1956: larvae undescribed
Porcellana foresti Chace, 1956: larvae undescribed
Porcellana habei Miyake, 1961: larvae undescribed
Porcellana hancocki Glassell, 1938: larvae undescribed
Porcellana lillyae Lemaitre & Campos, 2000: larvae undescribed
Porcellana paguriconviva Glassell, 1936: larvae undescribed
Porcellana persica Haig, 1966: larvae undescribed
Porcellana platycheles (Pennant, 1777): full larval description
Couch (1843); Z1: Lab
Faxon (1879) as *Porcellana* (*Polyonyx*) *macrocheles*; Z2: Pl
Williamson (1915); Z1, M: Pl
Webb (1921); Z1: Pl
Lebour (1943); Z1, Z2, M: Lab
Le Roux (1961); PR, Z1, Z2, M: Lab
Kaya and Özel (1992); Z1, Z2, M: Pl
Barnich (1996); Z1, Z2: Pl
*González-Gordillo et al. (1996); Z1, Z2, M: Lab
Porcellana pulchra Stimpson, 1858: larvae undescribed
Porcellana sayana (Leach, 1820): full larval description
Brooks and Wilson (1881) as *Porcellana ocellata*; PR, Z1: Lab
Hernández et al. (1998); Z1, Z2, M: Lab
Porcellana sigsbeiana A. Milne-Edwards, 1880: full larval description

*Gore (1971a); Z1, Z2, M: Lab
Maris (1983); Z1, Z2: Pl
Porcellanella haigae Sankarankutty, 1963: larvae undescribed
Porcellanella triloba White, 1852: larvae undescribed
Pseudoporcellanella manoliensis Sankarankutty, 1961: larvae undescribed
Raphidopus ciliatus Stimpson, 1858: larvae undescribed
Raphidopus indicus Henderson, 1893: larvae undescribed
Raphidopus johnsoni Ng & Nakasone, 1994: larvae undescribed
Ulloaia perpusillia Glassell, 1938: larvae undescribed

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Species delimitation in northern European water scavenger beetles of the genus *Hydrobius* (Coleoptera, Hydrophilidae)

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Abstract

The chiefly Holarctic *Hydrobius* species complex (Coleoptera, Hydrophilidae) currently consists of *H. arcticus* Kuwert, 1890, and three morphological variants of *H. fuscipes* (Linnaeus, 1758): var. *fuscipes*, var. *rottenbergii* and var. *subrotundus* in northern Europe. Here molecular and morphological data are used to test the species boundaries in this species complex. Three gene segments (COI, H3 and ITS2) were sequenced and analyzed with Bayesian methods to infer phylogenetic relationships. The Generalized Mixed Yule Coalescent (GMYC) model and two versions of the Bayesian species delimitation method BPP, with or without an *a priori* defined guide tree (v2.2 & v3.0), were used to evaluate species limits. External and male genital characters of primarily Fennoscandian specimens were measured and statistically analyzed to test for significant differences in quantitative morphological characters. The four morphotypes formed separate genetic clusters on gene trees and were delimited as separate species by GMYC and by both versions of BPP, despite specimens of *H. f.* var. *fuscipes* and *H. f.* var. *subrotundus* being sympatric. *H. arcticus* and *H. f.* var. *rottenbergii* could only be separated genetically with ITS2, and were delimited statistically with GMYC on ITS2 and with BPP on the combined data. In addition, six or seven potentially cryptic species of the *H. fuscipes* complex from regions outside northern Europe were delimited genetically. Although some overlap was found, the mean values of six male genital characters were significantly different between the morphotypes ($p < 0.001$). Morphological characters previously presumed to be diagnostic

were less reliable to separate *H. f.* var. *fuscipes* from *H. f.* var. *subrotundus*, but characters in the literature for *H. arcticus* and *H. f.* var. *rottenbergii* were diagnostic. Overall, morphological and molecular evidence strongly suggest that *H. arcticus* and the three morphological variants of *H. fuscipes* are separate species and *Hydrobius rottenbergii* Gerhardt, 1872, **stat. n.** and *Hydrobius subrotundus* Stephens, 1829, **stat. n.** are elevated to valid species. An identification key to northern European species of *Hydrobius* is provided.

Keywords

GMYC, species complex, BPP, guide tree, Fennoscandia, morphometrics, Bayesian, genitalia, molecular phylogeny, species boundaries, morphology, cryptic species, integrative taxonomy, DNA barcoding, identification key, taxonomy, checklist

Introduction

The chiefly Holarctic genus *Hydrobius* Leach, 1815 (Hydrophilidae, Hydrophilinae) has nine species (Short and Fikáček 2011), including *H. orientalis* Jia and Short, 2009, recently described from a part of China belonging to the Oriental Region. The recent study of hydrophilid phylogeny made by Short and Fikáček (2013) indicated that *Hydrobius* as currently delimited in fact may be paraphyletic. The morphologically variable and strictly Holarctic *H. fuscipes* (Linnaeus, 1758) is seemingly closely related to the two genera *Ametor* Semenow, 1900, and *Sperchopsis* LeConte, 1861, known from North America, the East Palearctic and adjacent parts of the Oriental Region. The Nearctic *H. melaenus* (Germar, 1824), representing the more convex and less elongate species, was not close to *H. fuscipes* but had a more uncertain and not well supported placement within Hydrobiusini.

The circumpolar *H. fuscipes* group poses some severe problems when it comes to species delimitation, by tradition paid most attention to in West Europe so far, but including also three named species in the East Palearctic. In Europe only the two species, *Hydrobius fuscipes* and *H. arcticus* Kuwert, 1890, are recognized in current taxonomic works (de Jong 2011; Hansen 1987; Löbl and Smetana 2004).

Traditionally, however, three morphological variants of *Hydrobius fuscipes* have been recognized in Europe: *H. fuscipes* var. *fuscipes*, *H. fuscipes* var. *subrotundus* Stephens, 1829 and *H. fuscipes* var. *rottenbergii* Gerhardt, 1872. These taxa have different distributions. *H. f.* var. *rottenbergii* is distributed in coastal areas of southern and central parts of Fennoscandia and Central Europe, *H. f.* var. *subrotundus* is known from Fennoscandia and Central Europe, while *H. f.* var. *fuscipes* has the largest distribution and is found in large parts of the Holarctic region. *Hydrobius arcticus* is distributed in the northern parts of Fennoscandia and European Russia (Hansen 1987; 1991). The taxa also have different habitat preferences with *H. arcticus* being a typical tundra species and *H. f.* var. *rottenbergii* inhabiting rock pools with brackish water or rain water near tidal zones. *H. f.* var. *subrotundus* and *H. f.* var. *fuscipes* have more similar, but yet distinct, habitat preferences where the former prefer colder and more shady habitats and is often found in more acidic waters and near edges of running water. *H. f.* var. *fuscipes* seems to prefer sun-exposed eutrophic stagnant ponds and can be found in

temporary ponds and pools in open landscape (Hansen 1987). Despite different habitat preferences, *H. f.* var. *fuscipes* can be found living in sympatry with *H. arcticus* in northern parts of Fennoscandia, and in sympatry with *H. f.* var. *subrotundus* in parts of their common distribution range. *H. f.* var. *rottenbergii* has on the other hand not been found in sympatry with the other species and variants (Balfour-Browne 1910; Hansen 1987; Schneider 1907).

The different variants of *H. fuscipes* have previously been considered separate species, but based on morphological studies that view has changed over time (e.g. Balfour-Browne 1910; 1958; Kuwert 1890; Rey 1885; Seidlitz 1891; Stephens 1839). All morphological variants were originally described as new taxa on the species-level, but a variable degree of synonymization has later occurred. *Hydrobius fuscipes* has more than 20 synonyms worldwide (Hansen 1999; Löbl and Smetana 2004), where only *H. f.* var. *rottenbergii*, *H. f.* var. *subrotundus* and *H. f.* var. *fuscipes* currently are considered different enough to be regarded as distinct morphological variants (Hansen 1987). *Hydrobius arcticus* has fewer species synonyms worldwide, but was earlier considered as a morphological variant or as a subspecies of *H. fuscipes* (Hansen 1999).

The most recent study of the species complex involved morphological studies of approximately 400 specimens from Sweden and Finland and argued that the three variants of *H. fuscipes* are separate species based on morphological differences (Lindberg 1943). However, Lindberg (1943) did not include *H. arcticus* in his study and this makes his results and subsequent conclusion inadequate (Hansen 1987). Because of this, Hansen (1987) treated *H. f.* var. *subrotundus* and *H. f.* var. *rottenbergii* as intraspecific variation of *H. fuscipes*. This was later implemented in the world catalogue of Hydrophilidae (Hansen 1999) and in the catalogue of Palearctic Coleoptera (Löbl and Smetana 2004). No secondary sexual characters have been described in *Hydrobius*, and comparative genitalia studies have never been conducted on the northern European species (Balfour-Browne 1910; Hansen 1987).

Species-level documentation of biological diversity and analyses of species boundaries have increased with the availability of genetic data and new methodological approaches (Carstens et al. 2013). While many morphological studies delimit species by use of discrete characters or continuous quantitative characters without overlap between species, both quantitative body- and male genitalia characters have been used to delimit species within species complexes of beetles (e.g. Bergsten et al. 2012b; Drotz et al. 2001; Nilsson 1987; 1994; Nilsson and Ribera 2007; Tocco et al. 2011). Usually the molecular loci used in species delimitation studies are neutral markers and not directly involved in the actual emergence of reproductive barriers between incipient species. Molecular methods developed for identification purposes like a 10x barcode-gap threshold (Hebert et al. 2004) are clearly inadequate for some organism groups, especially as it fails to recognize young species (Hickerson et al. 2006). Also the expectation of reciprocal monophyly in genealogies has limitations as the process of lineage sorting can take considerable time and is dependent on the effective population size (Bergsten et al. 2012a). Recently, more sophisticated statistical methods have been developed to delimit species using molecular data. These methods can be categorized into two

groups based on whether or not sample assignment is required (Carstens et al. 2013). Discovery methods are methods where data are analyzed without *a priori* partitioning of samples. Validation methods, however, require *a priori* partitioning of samples and should only be used in situations where either existing knowledge of the taxonomy or other characters can be used to make a testable hypothesis for delimitation, or where populations are clearly delineated (Carstens et al. 2013).

The Generalized Mixed Yule Coalescent (GMYC) model (Pons et al. 2006) is a discovery method that applies the phylogenetic species concept with assumed reciprocal monophyly in gene trees. It has increasingly been used in recent times to delimit closely related species (e.g. Cornils and Held 2014; Hjalmarsson et al. 2013; Pardo et al. 2014; Rodriguero et al. 2013; Zhang et al. 2014). Analyses are based on ultrametric single-locus genealogies as input, where the rate of branching is expected to be higher between specimens of the same species than between specimens of different species. The method attempts to model the transition point where there is a shift in the branching rate. This shift reflects the transition from between-species processes (e.g. speciation and extinction) to within-species processes (coalescence).

The Bayesian species delimitation method BPP (Bayesian Phylogenetics and Phylogeography) as originally presented is a validation method that applies reversible jump Markov chain Monte Carlo iterations (rjMCMC) to estimate the posterior probability of different hypotheses of species delimitation (Rannala and Yang 2003; 2013; Yang and Rannala 2010; 2014). The method estimates ancestral population sizes (within species) and species divergence times (between species) and can be used in species delimitation using multi-locus sequence data from closely related species. It required a guide tree as input in earlier versions (e.g. BPP v2.2), in which a species tree where the topology and the assignment of terminals into proposed species, are defined before analysis. However, version 3.0 (Yang and Rannala 2014) has overcome the need for a guide tree and estimates the species tree with a Nearest-Neighbor Interchange (NNI) algorithm simultaneously as species are delimited. This is a significant advantage over the old version since misspecifications of the guide tree can affect how many species are delimited and give misleading results (Leache and Fujita 2010). In principal if each specimen is assigned to a separate population, BPP version 3.0 also makes redundant the *a priori* assignment of specimen to (maximally subdivided) potential species and truly becomes a discovery method (Yang and Rannala 2010; 2014). However, such analyses are discouraged, except for very small datasets, because of the size of parameter space and computational complexity (Yang and Rannala 2014). The species delimitation algorithm computes the posterior probabilities of each node in the evaluated species tree (or guide tree in older versions) representing a speciation event by allowing the rjMCMC to sample all the possible ways of collapsing nodes in the species tree (or guide tree) into fewer species. BPP uncouples gene trees and species trees and therefore has the benefit of allowing the gene tree coalescences to be older than species tree coalescences. This accommodates the issue of gene trees and species trees often not being the same (Rannala and Yang 2003; 2013; Yang and Rannala 2010; 2014). BPP is increasingly used to delimit species (e.g. Bochkov et al. 2014; De Crop et al. 2014;

Derkarabetian and Hedin 2014; Guillin et al. 2014; Hamback et al. 2013), but as of to date few studies have used the guide tree-free BPP v3.0 on empirical data.

The mitochondrial gene cytochrome c oxidase subunit I (COI) is the standard genetic marker used to identify animal species with DNA Barcoding (Hebert et al. 2003). High substitution rates and deep divergences between closely related species in many animal groups have contributed in making COI the primary marker for the Barcode of Life Initiative. However, mitochondrial DNA (mtDNA) is maternally inherited in insects, thus occurrence of heteroplasmy (e.g. Magnacca and Brown 2010), male-killing or cytoplasmic incompatibility-inducing symbionts (e.g. *Wolbachia*; Werren et al. 2008) or introgressive hybridization (Ballard and Whitlock 2004) can produce misleading results in conflict with patterns based on nuclear DNA (e.g. Shaw 2002). Because of this, it is an advantage to use both mitochondrial and nuclear loci when analyzing species boundaries.

The main objective of this study was to statistically test species boundaries in the northern European *Hydrobius fuscipes* group using both molecular (three gene segments: COI, H3 and ITS2) and morphological data (both external and male genital characters).

Material and methods

Specimens

For the sake of simplicity, *Hydrobius arcticus* and the different variants of *H. fuscipes* will from here on be referred to as "morphotypes" and listed with subspecies terminology.

Adult specimens of the four morphotypes were obtained from expeditions throughout the Palearctic and Nearctic regions, with the most extensive sampling being in Norway and Sweden. The specimens were collected at various localities using an aquatic net in shallow vegetation along the edges of lakes, ponds and pools. The specimens were immediately stored in 70–96% ethanol after capture to keep optimal preservation conditions. Additional specimens from the Palearctic and Nearctic regions were obtained on loan from natural history museums and other institutions in Europe (Table S1 in Suppl. material 3). Type specimens of the different species and variants were borrowed and examined morphologically when possible, but we were unable to examine the type of *H. arcticus* (Table 1). The type of *H. fuscipes* was not examined, but the Linnean Society of London made an image available for examination. Specimens used in DNA extraction were dried and glued on mounting cards after measurements were taken. Specimens were identified with the use of appropriate identification keys and diagnostic characters (Hansen 1987).

In total, 62 *H. arcticus*, 100 *H. f. subrotundus*, 97 *H. f. rottenbergii* and 130 *H. f. fuscipes* specimens were examined in this study. The specimens used were chosen pseudo-randomly depending on distribution and availability with the intent to cover all morphotypes from most of their distribution area with a clear focus on the morpho-

Table 1. Examined type specimens of *Hydrobius*. † Specimen not examined, an image of the specimen was used in morphological analyses.

Variant of *Hydrobius fuscipes*	Type	Type locality	Storing institution
fuscipes (Linnaeus, 1758)	Holotype†	Europe	Linnean Society of London, UK
subrotundus Stephens, 1829	Possible syntype	British Isles	Natural History Museum, London, UK
rottenbergii Gerhardt, 1872	3x syntypes	Germany or Poland	Bavarian State Collection of Zoology, Munich, Germany

types of *Hydrobius* in northern Europe. Detailed morphological measurements and molecular analyses were conducted on a subsample of these specimens (approximately 30 of each morphotype, Suppl. material 1).

DNA extraction, amplification and sequencing

Most specimens used in the molecular analyses were relatively fresh (0-11 years old) and stored in 70-96% ethanol prior to the extraction; the oldest successfully extracted specimens had been pinned for 15 years before extraction. Whole specimens were used to extract DNA, but lysis was done non-destructively to preserve the exoskeleton for morphological analysis. The second or third abdominal ventrite of the specimens was punctured with sharp sterile forceps to facilitate lysis and diffusion of DNA out of the specimens. The forceps were cleaned between handling of different specimens with DNA AWAY™ Surface Decontaminant (Thermo Scientific, Wilmington, USA) and 80% ethanol. Beetles were placed in 100 µL Lysis Buffer (Mole Genetics, Lysaker, Norway) and 4 µL QIAGEN® Proteinase-K (QIAGEN, Venlo, Netherlands) and incubated overnight at 56 °C for 7-12 hours. The lysate was transferred to sample tubes after lysis and MoleStrips™ DNA Tissue (Mole Genetics) was used to extract DNA using a GeneMole® robot (Mole Genetics). Either 100 µL or 200 µL elution buffer was used for elution; 100 µL elution buffer used for older specimens. A selection of the specimens (n = 5) went through the DNA extraction process twice to be used as controls.

Three presumed unlinked gene segments were analyzed, one protein-coding mitochondrial gene segment (COI), one protein-coding nuclear gene segment (Histone H3; abbr. H3), and one non-functional nuclear rDNA segment (Internal transcribed spacer 2; abbr. ITS2) (Table 2). Each PCR reaction mixture contained 2 or 3µl DNA template (3µl for concentrations < 10 ng/µl, else 2µl), 1 µl of forward and reverse primer (10µM), a mixture with Taq polymerase, and molecular grade water (ddH_2O) for a total reaction volume of 25µl. Two different Taq polymerase mixtures were used: HotStarTaq® DNA Polymerase (QIAGEN) and premixed illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare, Uppsala, Sweden). The HotStarTaq® mixture contained 2.5µl 10x PCR-buffer, 2.0µl $MgCl_2$ (25mM), 2.0µl dNTPs (5mM each)

Table 2. Primers used in PCR and sequence reactions.

Gene	Forward primer	Sequence	Reference
COI	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer et al. (1994)
H3	HexAF	5'-ATGGCTCGTACCAAGCAGACGGC-3'	Ogden and Whiting (2003)
ITS2	CAS5p8sFc	5'-TGAACATCGACATTTYGAACGCACAT-3'	Ji et al. (2003)

Gene	Reverse primer	Sequence	Reference
COI	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer et al. (1994)
H3	HexAR	5'-ATATCCTTGGGCATGATGGTGAC-3'	Ogden and Whiting (2003)
ITS2	CAS28sB1d	5'-TTCTTTTCCTCCSCTTAYTRATATGCTTAA-3'	Ji et al. (2003)

and 0.2 µl HotStarTaq® DNA Polymerase. While both reaction mixtures were able to successfully amplify the gene segments, the Ready-To-Go PCR Beads had a higher success rate than the HotStarTaq® mixture for all gene segments.

All PCR reactions were performed with a C1000™ Thermal Cycler (Bio-Rad Laboratories, Foster City, USA). Blank samples with molecular grade water (ddH_2O) instead of DNA template were used as control-samples in all PCR-runs. The following PCR conditions were used in the amplification of the COI Barcode segment with the HotStarTaq® mixture: initial denaturation for 5 min at 95 °C; 60 s at 94 °C; 5 cycles of 30 s at 94 °C, 30 s at 45 °C, 60 s at 72 °C; 35 cycles of 30 s at 94 °C, 30 s at 51 °C, 60 s at 72 °C; ending with a final elongation for 5 min at 72 °C. Amplification of the COI Barcode segment with the Ready-To-Go PCR Beads: initial denaturation for 5 min at 95 °C; 42 cycles of 30 s at 95 °C, 30 s at 45 °C, 60 s at 72 °C; ending with a final elongation for 8 min at 72 °C. Amplification of H3 with HotStarTaq® mixture and Ready-To-Go PCR Beads: initial denaturation for 5 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 50 °C, 60 s at 72 °C; ending with a final elongation for 8 min at 72 °C. Amplification of ITS2 with HotStarTaq® mixture and Ready-To-Go PCR Beads: initial denaturation for 5 min at 95 °C; 35 cycles of 40 s at 94 °C, 30 s at 55 °C, 40 s at 72 °C; ending with a final elongation for 7 min and 45 s at 72 °C.

Aliquots of the PCR-products selected for sequencing were purified with illustra™ ExoStar™ 1-Step (GE Healthcare) or with illustra™ ExoProStar™ 1-Step (GE Healthcare) following the producers recommendation. Samples were sequenced in both directions by cycle sequencing technology using dideoxy chain termination/cycle sequencing on ABI 3730XL sequencing machines at Eurofins Genomics (Germany).

In cases where DNA was extracted twice from the same specimens, both replicates were sequenced if successfully amplified with PCR. The replicates were used as controls and were expected to yield the same sequence.

Sequenced specimens are kept as DNA vouchers at their respective institutions, labeled with the IDs listed in Suppl. material 1.

Molecular analysis

Editing and alignment of sequences

DNA Baser Sequence Assembler v4.10.1.13 (2012, Heracle BioSoft SRL, http://www.DnaBaser.com) was used to assemble and edit DNA sequences. The forward and reverse sequences were automatically assembled by the software and the contig was inspected and edited manually. When base calls were ambiguous, the appropriate International Union of Pure and Applied Chemistry (IUPAC) codes were used to represent this. In a few cases the chromatogram was only readable in one direction. Sequences with very low quality were not used in downstream analysis.

Sequences are available in the BOLD project FENHY (http://www.boldsystems.org/index.php/MAS_Management_OpenProject?code=FENHY) and submitted to GenBank under accession numbers KU380492–KU380737. Additional COI Barcodes were also downloaded from BOLD (Ratnasingham and Hebert 2007) and used in downstream analyses (Suppl. material 1), including sequences from Hendrich et al. (2015) and Pentinsaari et al. (2014). The following acronyms were used for the geographical locations of the samples in the phylogenetic trees: CAN = Canada, FIN = Finland, GER = Germany, GREECE = Greece, ITA = Italy, NOR = Norway, POR = Portugal, RUS = Russia, SPA = Spain, SWE = Sweden, UK = United Kingdom, and USA = United States of America.

MEGA v6.06 (Tamura et al. 2013) was used to align the edited nucleotide contigs. All segments were aligned with MUSCLE (Edgar 2004) under default settings, where the COI and H3 segments were aligned as amino acids, whereas ITS2 was aligned as DNA. The ends of all three alignments were trimmed to remove low quality parts of sequences and primers. BLAST (Altschul et al. 1990) was used on irregular sequences to identify and remove contaminants.

Phylogenetic analyses

Bayesian methods were used to find the phylogenetic relationship between specimens of different morphotypes. Analyses of both single locus datasets and a concatenated dataset were conducted. The concatenated dataset combined all three gene segments (COI, H3 and ITS2), removing any samples that lacked sequences from one or two genes to avoid large sections of missing data in the matrix. *Hydrobius convexus* was used as outgroup in all phylogenetic analyses.

Bayesian information criterion (BIC) was used within PartitionFinder v1.1.1 (Lanfear et al. 2012) to find and select the best fit substitution model and partition scheme for use in Bayesian analyses.

MrBayes v3.2 (Ronquist et al. 2012) was used for Bayesian phylogenetic inference of sequence data. The best partition schemes and corresponding substitution models from PartitionFinder were used in two simultaneous but independent analyses using

Metropolis-coupled Markov chain Monte Carlo (MCMCMC) iterations each with four chains (nchains = 4). The number of generations run for each analysis was dependent on the size of the dataset and whether or not convergence was easy to obtain, but a minimum of 2,500,000 generations were always run (ngen ≥ 2,500,000). Heating of chains was set to 0.2 (temp = 0.2). Sampling frequency was set to every 1000 generation (samplefreq = 1000). Trace plots were used to determine the required burnin and the first 25% of sampled trees were discarded as burn-in trees (relburnin = yes burninfrac = 0.25). Standard deviation of split frequencies (≤ 0.01), effective sample sizes (ESS) and trace plots visualized with Tracer v1.6 (Rambaut et al. 2013) were used as convergence diagnostics. A 50% majority rule consensus tree (contype = halfcompat) was calculated from the remaining sampled trees after the removal of burn-in.

Species delimitation

The maximum likelihood based GMYC model (Pons et al. 2006) and the Bayesian method applied in BPP v3.0 and BPP v2.2 (Rannala and Yang 2013; Yang and Rannala 2010; 2014) were used to evaluate species delimitations.

The GMYC analyses were conducted in the statistical software R v3.0.3 (R Core Team 2014), with the use of ape, MASS, gee, paran and splits packages. The input for the GMYC was an ultrametric single locus gene tree with multiple individuals per species for multiple potential species. To test if a strict molecular clock could be appropriate to infer the ultrametric trees, stepping-stone sampling was used in MrBayes v3.2 (Ronquist et al. 2012) to find the marginal model likelihoods for a model with a strict molecular clock and for a time-free model. The tests were run 5 times for each model and averages of these runs were used to compare the models in a Bayes factor test. The marginal likelihood of the models with a strict molecular clock were higher for all three gene segments than the time-free models, thus implementing a strict molecular clock was justified.

The ultrametric trees, one for each gene segment, were made with BEAST v2.1.3 and corresponding user interface (BEAUti 2) (Bouckaert et al. 2014). The best partition schemes and corresponding substitution models found in PartitionFinder were used with sites unlinked, while the clock and tree models were linked. A strict clock model was implemented and a Coalescent Constant Population prior was used as the tree prior. The numbers of generations were 10 million for H3 and ITS2 data and 20 million for COI data. Sampling of parameters and trees was set to every 1000 (H3 and ITS2 data) or 2000 (COI) generations. Effective sample sizes (ESS) and trace plots estimated with Tracer v1.6 (Rambaut et al. 2013) were used as convergence diagnostics. Sampled trees from two independent runs were pooled together after manually discarding 15% (H3 and ITS2) or 20% (COI) of the trees as burn-in (determined by examining trace plots). Ultrametric maximum clade credibility (MCC) trees were computed using the mean node heights with TreeAnnotator v2.0.3 (Drummond and Rambaut 2007) for each gene segment. The arbitrary time scales of the trees were rescaled so that the root had an age of 1.

The GMYC analyses were conducted with the single-threshold version, since Fujisawa and Barraclough (2013) found it to outperform the multiple-thresholds version on simulated data. The maximum likelihood of the GMYC model was tested with a likelihood ratio test against a one-species null model (where the entire tree is considered as a single coalescent).

Comparison and selection of the best models were performed with the method described by Powell (2012), where Akaike Information Criterion values taking sample size into account (AICc) of the different models are compared. Models with Δ AICc-values from 0 to 2 are considered the best explanations of the data among the models compared, models with Δ AICc-values from 4 to 7 are generally considered to have little support from the data, whereas models with Δ AICc-values >10 are considered to have essentially no support from the data compared to the other models (Burnham and Anderson 2002). Support values of the GMYC-delimited species (GMYC-support; Fujisawa and Barraclough 2013), defined as the sum of Akaike weights of candidate delimitation models in which a specific node is included, were calculated using models within the 95% confidence set.

The Bayesian species delimitation methods in BPP v3.0 and BPP v2.2 (Rannala and Yang 2013; Yang and Rannala 2010; 2014) were used with multi-locus data (COI, H3 and ITS2). All analyses included *H. convexus* as outgroup, as Rannala and Yang (2013) showed that including a closely related outgroup may increase the statistical power of BPP. Five different species scenarios with a total of 4 guide trees were used in BPP v2.2. The assignment of specimens to potential species for both BPP versions, and the topologies used in the guide trees in BPP v2.2, were chosen based on taxonomical knowledge (from morphological studies), the species delimited with GMYC and based on the topology and clusters found in the phylogenetic trees. The four known northern European morphotypes of *Hydrobius* were the main focus of the species delimitation tests.

Each theta (Θ, ancestral population size) and tau (τ, species divergence time) parameters in the BPP analyses (both versions) used priors specified with a gamma distribution with mean α/β. Only the root in the species tree (τ_0) was given as a tau prior whereas other τ parameters were generated with the Dirichlet distribution with default settings in BPP. $\alpha = 1$ was used as a diffuse prior in all analyses, while different combinations of β were tested for Θ and τ_0. Multiple initial runs with different combinations of β were used to find combinations of β that made the means (α/β) be within an order of magnitude from the posterior estimates of Θ and τ_0, as recommended by Zhang et al. (2011). The dataset had a posterior estimate of $\Theta \approx 0.01$ and a posterior estimate of $\tau_0 \approx 0.03$. The following four combinations of gamma distributions were used in both BPP versions; 1: Θ: G(1, 50), τ_0: G(1, 20); 2: Θ: G(1, 50), τ_0: G(1, 200); 3: Θ: G(1, 500), τ_0: G(1, 20); and 4: Θ: G(1, 500), τ_0: G(1, 200). The combinations include the posterior estimates of Θ and τ_0 and the means (α/β) are within an order of magnitude of these estimates.

All BPP-analyses were run for 100,000 generations with sampling every two generations (nsample = 50,000 and sampfreq = 2), after discarding an initial burn-in of 40,000 generations (burnin = 40,000). Heredity scalars were set to 0.25 for COI and

1.0 for H3 and ITS2. Automatic adjustments of finetune parameters were used while making sure that the acceptance proportions were within the range of 0.2–0.7 as recommended by Yang and Rannala (2010). Every analysis was run twice with different starting species trees to check for convergence between runs and agreement on the posterior probability of the species delimitation models. Both algorithm "0" and algorithm "1" (see Yang and Rannala 2010) were tested and gave very similar results, and thus primarily results obtained with algorithm 0 will be reported.

Morphological analysis

Specimens were examined with a Leica MZ16 stereomicroscope (Leica Microsystems, Wetzlar, Germany) in reflected light using the measurement module of the software Leica Application Suite 3.2 (Leica Microsystems).

Detailed morphological measurements were conducted after results from the molecular analyses were obtained. A total of 21 *H. arcticus*, 33 *H. f. subrotundus*, 26 *H. f. rottenbergii* and 33 *H. f. fuscipes* specimens were measured, selected primarily based on the presence of molecular data, to link morphological and molecular divergence patterns. Some specimens that were not included in the molecular analyses were also measured to increase the sample size, especially specimens of *H. arcticus* and *H. f. rottenbergii*. These specimens were selected based on morphology and geographical locality, making sure they were of the correct species/variant. Characters that seemed to have very high intraspecific variation or were prone to high amounts of measurement errors were excluded from statistical analyses. The measurements of the first 10 specimens were repeated at a later stage to detect potential errors and ensure repeatability in measurements. A large selection of presumably diagnostic and informative external body characters were measured and analyzed. Genitalia were dissected in male specimens and genital characters examined and measured at approximately 60x magnification. A total of 15 *H. arcticus*, 16 *H. f. subrotundus*, 15 *H. f. rottenbergii* and 16 *H. f. fuscipes* had their genitalia measured, including type specimens of *H. f. rottenbergii* and *H. f. subrotundus*. For pinned specimens, the genitalia were dissected after softening of the specimens in warm water for 10-20 minutes. A hooked needle was used to bring the genital capsule out from the abdomen, before the genitalia was separated from the genital capsule with two needles while placed in ethanol under a stereomicroscope. The abdomen and genitalia were placed on the same mounting card after measurements were conducted.

Characters

A total of 29 characters was examined and measured, 14 male genital characters and 15 external body characters (Suppl. material 2). The following six genital and four body characters were most informative:

Male genital characters (Fig. 1)

The mean of the left and right paramere character were used as one character for characters measured in dorsal view.

1.1) *Length of parameres*: dorsal view. Measured as the total length from the tip of the paramere to the bottom part of the paramere where it overlaps with the basal piece of the aedeagus.

1.2) *Width of parameres*: dorsal view. Measured as the width of the paramere at the narrowest part.

1.3) *Robustness of parameres*: dorsal view. Measured as a ratio between the lengths of the parameres (character 1.1) divided by the narrowest width of paramere (character 1.2). A low value means that the paramere is more robust.

1.4) *Ratio between paramere length and penis length*: dorsal view. Measured as the length of the paramere (character 1.1) divided by the length of the sclerotized part of the penis.

1.5) *Width of paramere*: lateral view. Measured as width of the paramere at the narrowest part.

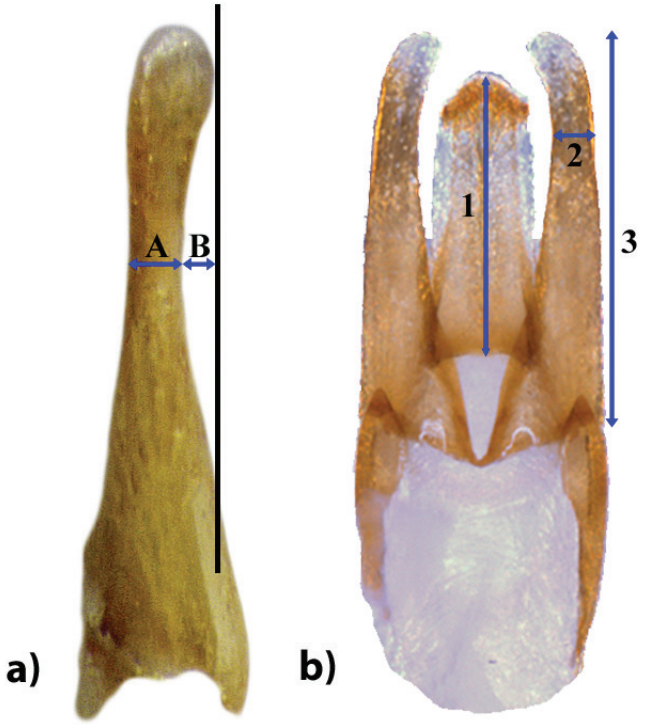

Figure 1. Measurements of *Hydrobius* male genitalia. **a** Paramere in lateral view. A: width of paramere (character 1.5). Curvature of paramere tip (character 1.6) = A+B **b** Genitalia in dorsal view. 1: Length of sclerotized part of penis. 2: Width of narrowest part of paramere (character 1.2). 3: Length of paramere (character 1.1). Robustness of paramere (character 1.3) = 3 / 2. Paramere length relative penis length (character 1.4) = 3/1. Images of *Hydrobius fuscipes rottenbergii*.

1.6) *Curvature of paramere tip*: lateral view. Measured as length from dorsal side of the narrowest part of the paramere to a vertical line from the tip of paramere on the ventral side, parallel to the dorsal line.

Body characters:

2.1) *Relative position of trichobothria (systematic punctures) in relation to the 3rd and 5th row of elytral serial punctures*: previously used to separate variants of *H. fuscipes* (Hansen 1987). Quantified and measured as a ratio between the length from the 3th or 5th row of serial punctures to the first 20 trichobothria posterior to scutellum, divided by the length from the 3rd or 5th row to the 2nd or 4th row, respectively (Fig. 2). A low value means that the trichobothria are close to the 3rd or 5th row of serial punctures, while a higher value, e.g. 0.5, means that they are positioned in the elytral intervals.

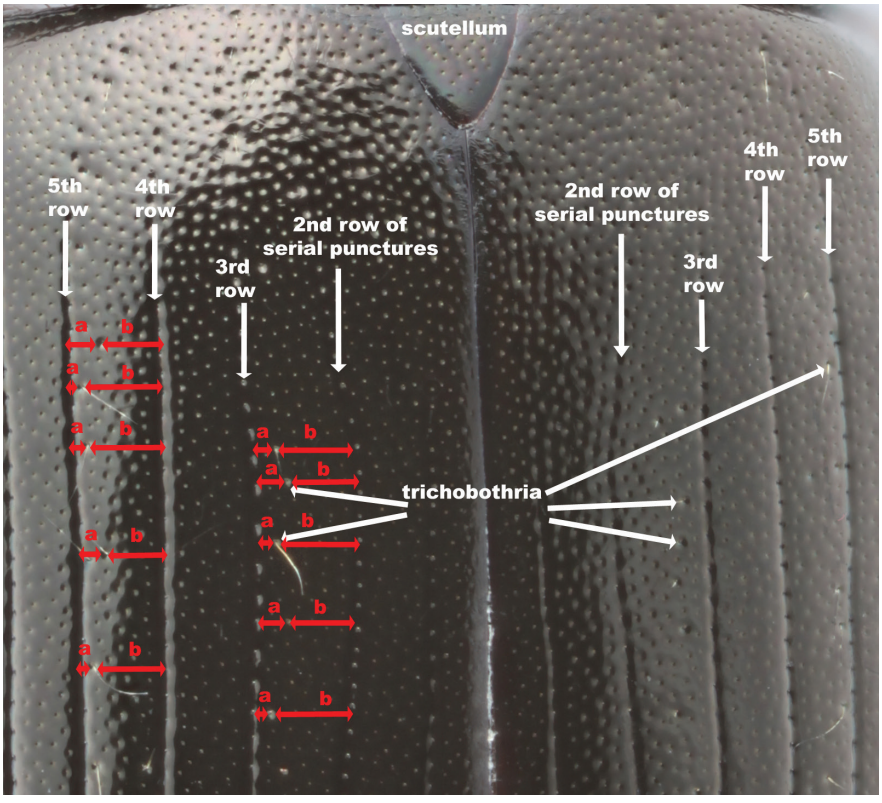

Figure 2. Measurement of the relative position of trichobothria on the elytra (character 2.1). Dorsal view of anterior part of the elytra, showing how several trichobothria encountered posterior to the scutellum were measured. Each relative position of a trichobothrium was measured by dividing the length from the 3rd row of serial punctures to the trichobothrium (a) by the length from the 3rd row to the 2nd row (a+b). The same was done with trichobothria in or near the 5th row of serial punctures. Image of *Hydrobius fuscipes fuscipes*.

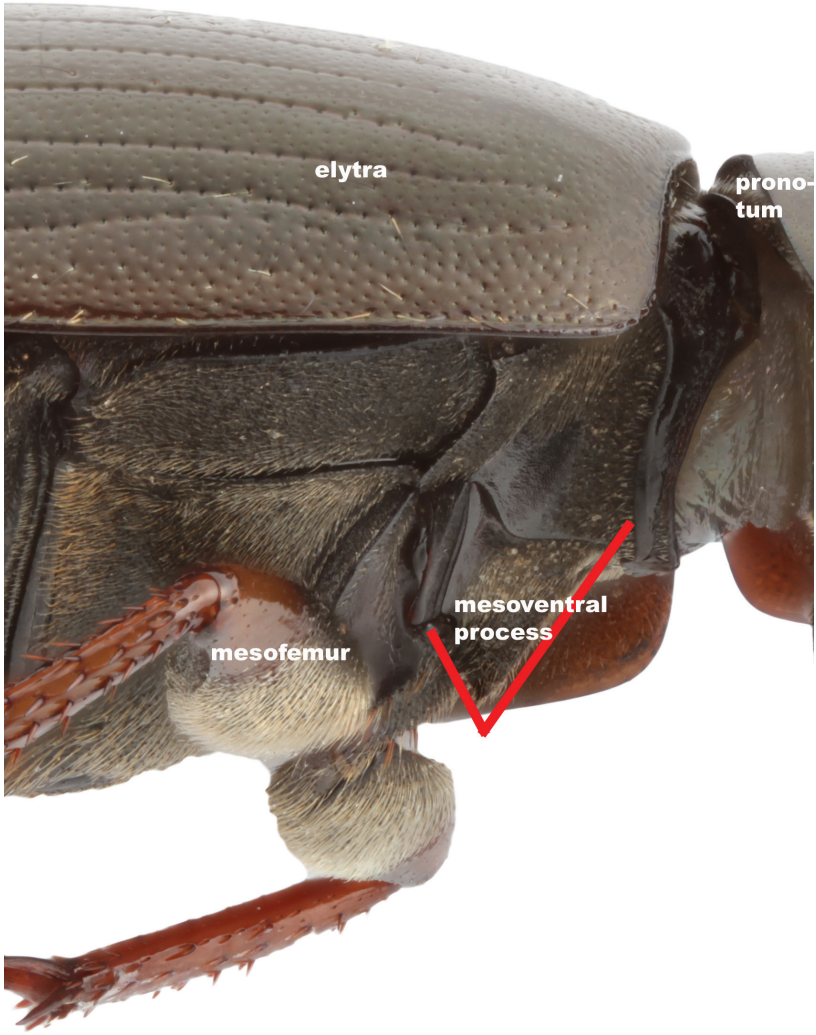

Figure 3. The shape of the mesoventral process (character 2.2). Measured in lateral view as an angle (indicated by red lines). Image of *Hydrobius fuscipes fuscipes*.

2.2) *Shape of mesoventral process*: previously used to separate *H. fuscipes* from *H. arcticus* (Hansen 1987). Measured in lateral view as an angle (Fig. 3). A low value means that the mesoventrite has a relatively strong acute process.

2.3) *Color of legs*: previously used to separate variants of *H. fuscipes* (Hansen 1987). The colors of the tibiae and femora were examined qualitatively.

2.4) *Body shape*: previously used to separate variants of *H. fuscipes* (Hansen 1987). Quantified with the *Elytral Index* (EI), where the length of the elytra is divided by the maximum width of the elytra, when both elytra are in focus (Fig. 4). A low value means that the body shape is shorter and more convex.

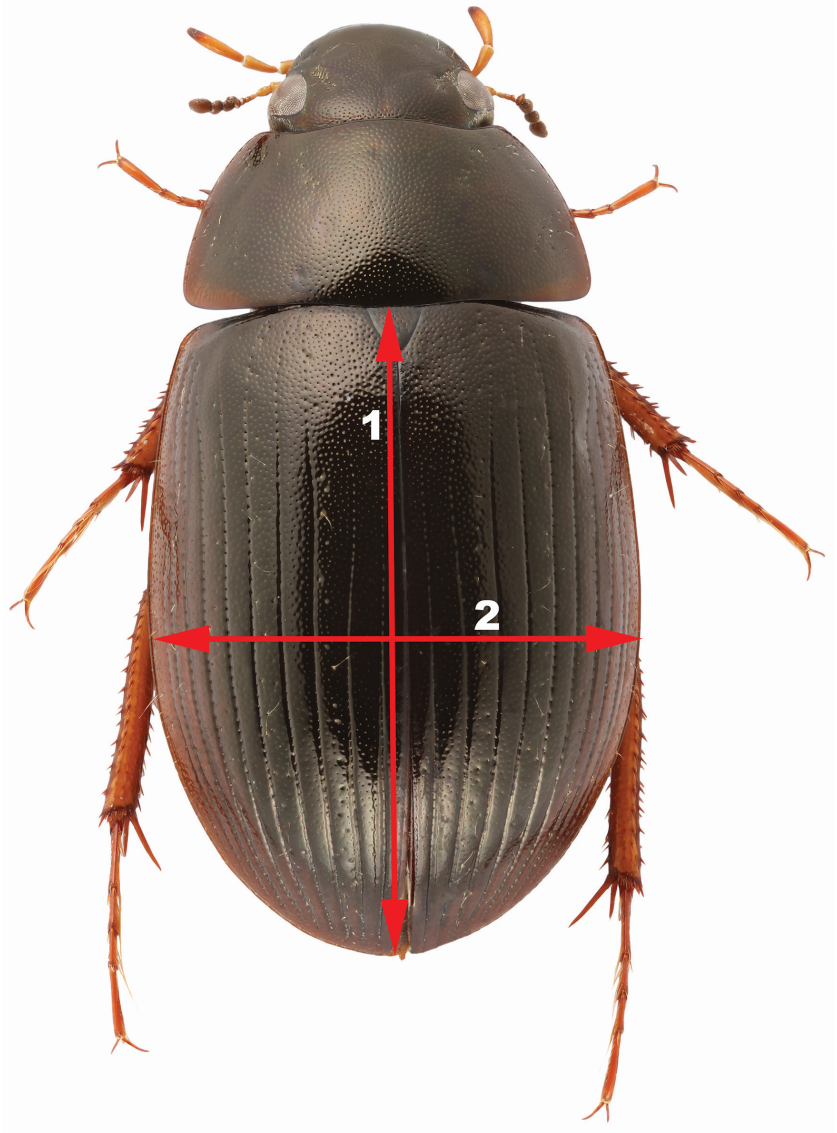

Figure 4. Measurement of Elytral Index (EI). **1** Length of elytra **2** Maximum width of elytra. EI (character 2.4) = 1 / 2. Image of *Hydrobius fuscipes fuscipes*.

Statistical analysis of morphological characters

In order to find a reliable estimate of body size, repeated measurements of the total body length, measured from the anterior margin of the labrum to the posterior elytral apex, were compared to the combined length of elytra and the length of pronotum in 19 specimens. The sum of the elytra and the pronotum lengths was found to be less variable between repeated measurements than the complete body length and was

therefore used as a more reliable and reproducible estimate of body size in all analyses. A potential bias towards one side (left or right) of assumed symmetric characters was examined using a Student's t-test to see if the means of right and left structures were statistically different. A visual comparison of the differences by using a histogram showing the differences between the left and right structure was also conducted.

To test if the morphotypes were significantly different in the measured characters, an analysis of covariance (ANCOVA) was used with log-transformed character values as the response variable, the morphotypes as a predictor variable and a log-transformed estimate of body size as a covariate. The estimated body size was used to control for any confounding allometric relationships between the morphological character and body size. The models were reduced, by comparing the models' adjusted R^2 values and AIC-values, to only include statistically significant effects, including reduction to an analysis of variance (ANOVA) in cases where body size was non-significant. Post hoc comparison of the morphotypes was performed with Tukey's HSD (honestly significant difference) test with adjusted p-values. Non-log-transformed variables were used in cases where the models without log-transformed variables had a greater R^2 value than the models with log-transformed variables. Characters that are ratios were not log-transformed, neither did body size in these analyses, as the allometric relationship for ratios are less predictable. A selection of interesting male genital characters were plotted against each other and a Convex Hull (de Berg et al. 2000) was used to illustrate the overlap of different morphotypes with regard to the characters of interest. All statistical analyses were performed with the statistical software R v3.0.3 (R Core Team 2014).

Results

Additional tables (S2–S10) and figures (S1–S6) are available in Supplementary material 3.

Molecular analyses

A total of 86 specimens from the four morphotypes was successfully sequenced for at least one gene segment (Table 3). Due to availability of fresh material, the number of successfully sequenced *H. arcticus* specimens (11) was considerably lower than the specimens of *H. fuscipes* variants (Table 3). There seem to be no clear differences in sequencing success among gene segments, but H3 amplified for a few more samples.

Sequence composition and alignment

The alignments were unproblematic as there were very few insertions or deletions (indels) (Table 4). Neither COI nor H3 had any indels, whereas the ingroup had one indel of 2–4 bases for ITS2. COI was the most variable segment with 21.3% variable and

Table 3. Number of successfully sequenced gene segments from *Hydrobius* morphotypes. †COI sequences from BOLD are not included.

Gene segment	Morphotype				Sum
	H. arcticus	***H. f. fuscipes***	***H. f. rottenbergii***	***H. f. subrotundus***	
COI	7	29	14	30	80†
H3	9	30	14	31	84
ITS2	9	27	14	29	79
Specimens with at least one segment	11	30	14	31	86†
Specimens with all three segments	5	27	14	29	75

Table 4. Basic statistics on gene segments used in molecular analyses of the *Hydrobius* species complex. Unique sites refer to variable but parsimony uninformative sites. † Only specimens with all three gene segments were included in the concatenated dataset.

	COI (incl./excl. outgroup)	**H3** (incl./excl. outgroup)	**ITS2** (incl./excl. outgroup)	**Concatenated dataset** † (incl./excl. outgroup)
Length of segment (bp)	658/658	328/328	405/405	1391/1391
Length used in analyses, incl. gaps (bp)	611/611	306/306	412/389	1329/1306
Indels in aligned segment	0/0	0/0	3/1	3/1
Conserved sites (bp)	446/481	247/278	338/362	1041/1131
Variable sites (bp)	165/130	59/28	51/27	265/175
Parsimony informative sites (bp)	116/113	22/20	26/26	154/149
Unique sites (bp)	49/17	37/8	25/1	111/26
A (%)	30.4/30.4	25.7/25.8	16.6/16.6	25.2/25.2
C (%)	17.3/17.3	30.9/30.9	29.6/29.6	24.1/24.1
G (%)	16.3/16.3	24.1/24.0	32.5/32.5	22.9/22.9
T (%)	36.0/36.0	19.3/19.3	21.3/21.4	27.8/27.8
Number of unique haplotypes	49/48	18/17	12/11	37/36

18.5% parsimony informative sites in the ingroup. H3 had 9.15% variable and 6.54% parsimony informative sites, while ITS2 had 6.94% variable and 6.68% parsimony informative sites (Table 4). The length of COI used in analyses was 1.5 to 2 times more than the other segments, and the number of unique haplotypes was also proportionally higher for COI compared to the other two segments (Table 4).

Best fit substitution models and partition schemes

There was large agreement between the best partition schemes and substitution models for the single locus gene segments compared to the concatenated dataset, although for

example codon position 3 of H3 is assigned a K80+I model when using H3 data and a K80 model when using the concatenated data (Table S2 in Suppl. material 3). Less complex substitution models were most fit when using the H3 and ITS2 datasets without outgroups than when outgroups were included (Table S2 in Suppl. material 3).

Phylogenetic analyses

Up to eleven different genetically divergent clades, one of which is represented by a singleton, were found in the phylogenetic trees, although with different amount of consistency and support between the different gene segments analyzed. Highest resolutions were found in the trees resulting from analyses of COI and the concatenated dataset (Fig. 5 and Fig. S1 in Suppl. material 3), presumably as these datasets show the most variation. Some geographical structuring was found among the clades (Table 5). Within northern Europe, four clades (*H. arcticus*, *H. f. rottenbergii*, *H. f. fuscipes* and *H. f. subrotundus*) are found, which correspond well with the respective described morphospecies. *Hydrobius f. fuscipes* and *H. f. subrotundus* have the widest distribution, whereas *H. arcticus* and *H. f. rottenbergii* are only found in Norway and Sweden among included material. Clades I-III, VI and VII are central and southern European clades, whereas IV and V are North American clades (Table 5).

Concatenated data (COI, H3 and ITS2 combined)

Nine monophyletic clades are found in the phylogenetic tree of the concatenated data from MrBayes (Fig. 5). All clades except the *H. f. fuscipes* clade (posterior probability = 0.67) have strong support. There is strong support for Clade I and Clade II as sisters, strong support for the *H. f. rottenbergii* and *H. arcticus* clades as sisters, and moderate to strong support for the relationship (Clade III, (Clade IV, (*H. arcticus*, *H. f. rottenbergii*))). Specimens that were identified as different morphotypes (*H. f. fuscipes* or *H. f. subrotundus*) but were collected in sympatry at Rinnleiret (Nord-Trøndelag, Norway) or Motzen (Brandenburg, Germany) clustered within corresponding *H. f. fuscipes* or *H. f. subrotundus* clades rather than together based on locality.

Mitochondrial COI data

Ten monophyletic groups, all of which have moderate to strong support, are found in the phylogenetic tree of COI from MrBayes (Fig. S1 in Suppl. material 3). The *H. arcticus* and *H. f. rottenbergii* clades are clustered together with moderate to strong support as a single monophyletic group. There is moderate to strong support for the relationship (*H. f. fuscipes*, (Clade I, Clade II)), and as in the concatenated tree (Fig. 5), strong support for Clade I and Clade II as sisters. As in the concatenated tree (Fig. 5),

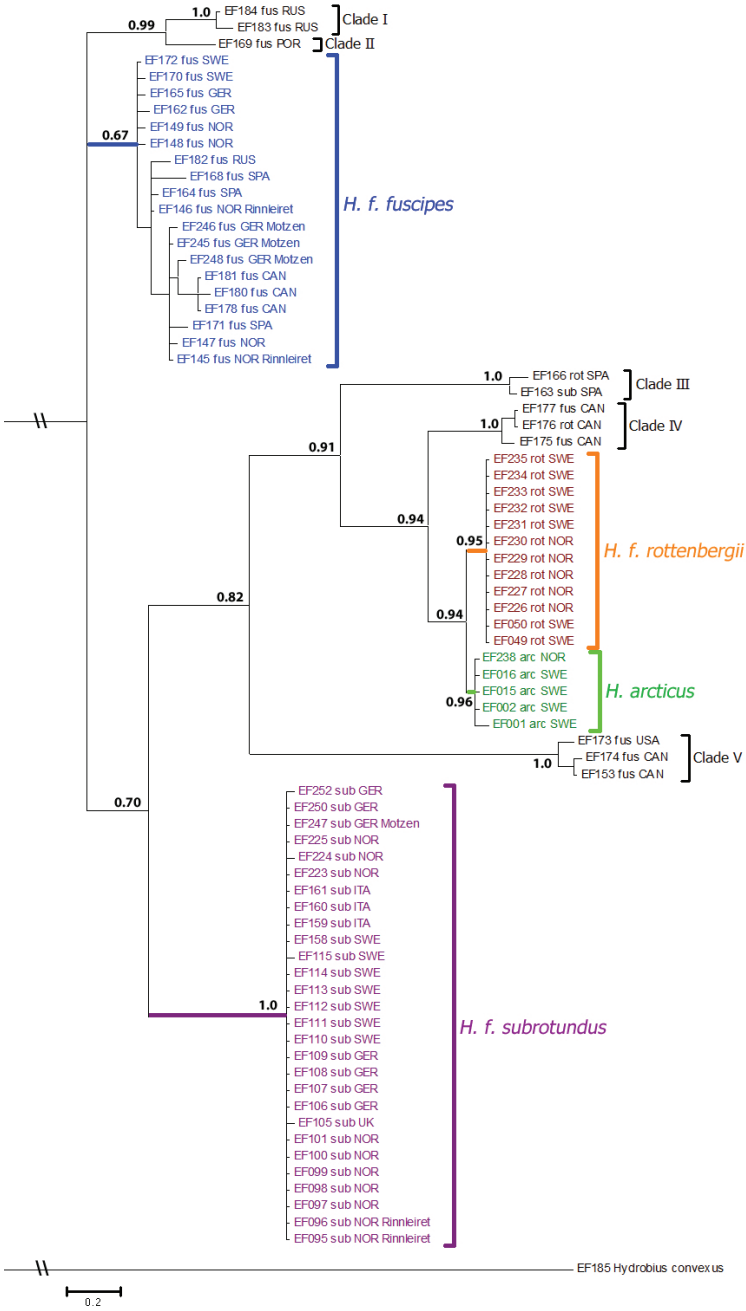

Figure 5. Majority-rule consensus tree from time-free Bayesian analysis of the concatenated data. Branch support values are posterior probabilities. Samples are labeled with ID-numbers, identified morphotypes and country of origin. Specimens collected in sympatry are also labeled with locality name (Rinnleiret or Motzen). Scale bar indicates expected number of nucleotide substitutions per site. Branches with "\\" have been manually cut. Abbreviations for morphotypes: arc = *arcticus*, fus = *fuscipes*, rot = *rottenbergii*, sub = *subrotundus*.

Table 5. Genetically divergent clades and their localities, including corresponding BOLD BINs. Clades primarily found on COI and concatenated tree. † Only COI data available (from Hendrich et al. 2015).

Clade name	Localities	BOLD BIN
H. arcticus	Norway and Sweden	BOLD:AAC5901
H. f. rottenbergii	Norway and Sweden	BOLD:AAC5901
H. f. fuscipes	Norway, Sweden, Finland, Germany, Spain, Russia and Canada	BOLD:AAC5900
H. f. subrotundus	Finland, Germany, Sweden, Norway, Italy and UK	BOLD:AAC5899
Clade I	Russia and Germany	BOLD:AAP9350
Clade II (singleton)	Portugal	BOLD:ACN8707
Clade III	Spain and Germany	BOLD:ACB2991
Clade IV	Canada	BOLD:AAH2906
Clade V	Canada and USA	BOLD:AAH0085
Clade VI	Greece †	BOLD:ACO5185
Clade VII	Germany †	BOLD:AAC5901

different morphotypes collected in sympatry cluster within the corresponding *H. f. fuscipes* or *H. f. subrotundus* clades rather than together based on locality (Fig. S1 in Suppl. material 3).

Nuclear H3 data

Clade III, Clade V and *H. f. subrotundus* form reciprocal monophyletic groups with moderate to strong support in the phylogenetic tree of H3 from MrBayes (Fig. S2 in Suppl. material 3). *H. arcticus*, *H. f. rottenbergii* and Clade IV cluster together as a single monophyletic group with strong support, whereas Clade I, Clade II and *H. f. fuscipes* are paraphyletic groups. As in the concatenated and COI trees (Fig. 5 and Fig. S1 in Suppl. material 3), different morphotypes collected in sympatry cluster with samples of the corresponding *H. f. fuscipes* or *H. f. subrotundus* clades rather than together based on locality (Fig. S2 in Suppl. material 3).

Nuclear ITS2 data

Multiple reciprocally monophyletic groups are found in the phylogenetic tree of ITS2 from MrBayes (Fig. S3 in Suppl. material 3). Clade III, Clade IV and Clade V have strong support, *H. f. subrotundus* has moderate support, while the *H. arcticus* clade has low to moderate support. Clade I and Clade II cluster together to form a monophyletic group with strong support. The *H. f. rottenbergii* clade is a basal paraphyletic group, although all samples are identical haplotypes. The *H. f. fuscipes* clade is paraphyletic, but as in the concatenated, COI and H3 trees (Fig. 5, Figs S1–S2 in Suppl. material 3), different morphotypes collected in sympatry cluster with samples of the corresponding *H. f. fuscipes* or *H. f. subrotundus* clades rather than together based on locality (Fig. S3 in Suppl. material 3).

Conflict between gene trees

The three gene trees differ in the relationships between Clade I, Clade II, Clade IV and *H. f. fuscipes*, *H. arcticus* and *H. f. rottenbergii* (Figs S1–S3 in Suppl. material 3). Clade I and Clade II are reciprocally monophyletic groups in the COI tree, but in trees based on nuclear gene segments the two clades are either paraphyletic (H3) or their members group as a single monophyletic unit (ITS2, Fig. S3 in Suppl. material 3). The *H. f. fuscipes* clade has the most variation in the COI gene segment and is paraphyletic for the nuclear gene segments (H3 and ITS2) where specimens are split in two groups. The two subgroups of *H. f. fuscipes* in the H3 tree (Fig. S2 in Suppl. material 3) differ from the two subgroups in the ITS2 tree (Fig. S3 in Suppl. material 3). Clade IV, *H. arcticus* and *H. f. rottenbergii* are closely related in the COI and H3 trees, while they are more basal in the ITS2 tree. Clade IV is separated genetically from the other two groups in the COI and ITS2 trees, but not in the H3 gene tree, whereas *H. arcticus* and *H. f. rottenbergii* are only possible to separate genetically with ITS2 data (Fig. S3 in Suppl. material 3).

Species delimitation analysis

GMYC

The ultrametric maximum clade credibility (MCC) tree from BEAST based on COI data (Fig. 6) is concordant with the non-ultrametric COI gene tree (Fig. S1 in Suppl. material 3) and supports the same clades. A GMYC model delimiting nine species with a single threshold was the maximum likelihood solution, but models delimiting eight or ten species also fall within two ΔAICc of the best GMYC model (Table 6), indicating that all three models are about equally good at explaining the data among the models compared. The log likelihood of the GMYC model at the optimal threshold (670.5) was also significantly better than the null model of a single coalescent (logL = 660.6) in a likelihood ratio test ($p < 0.001$). Most clades have GMYC-support values higher than 0.9 (Fig. 6), meaning that the probability of the clades being delimited as separate GMYC-species among the alternative models of delimitation (within a 95% confidence set) is higher than 0.9. Clade I and Clade II are by some models considered the same GMYC-species (GMYC-support = 0.20), but there is higher support for them being separate GMYC-species (GMYC-support = 0.80). Clade VII, *H. arcticus* and *H. f. rottenbergii* are considered the same species by a majority of the models (GMYC-support = 0.70), but Clade VII is considered a separate species under some models (GMYC-support = 0.30).

The ultrametric MCC tree from BEAST based on H3 data (Fig. S4 in Suppl. material 3) is concordant with the non-ultrametric H3 gene tree (Fig. S2 in Suppl. material 3) and supports the same clades. The GMYC model that is the maximum likelihood solution (logL = 506.1) delimited 20 species but was not significantly different from the one-species null model (lnL = 504.7) in a likelihood ratio test ($p = 0.23$).

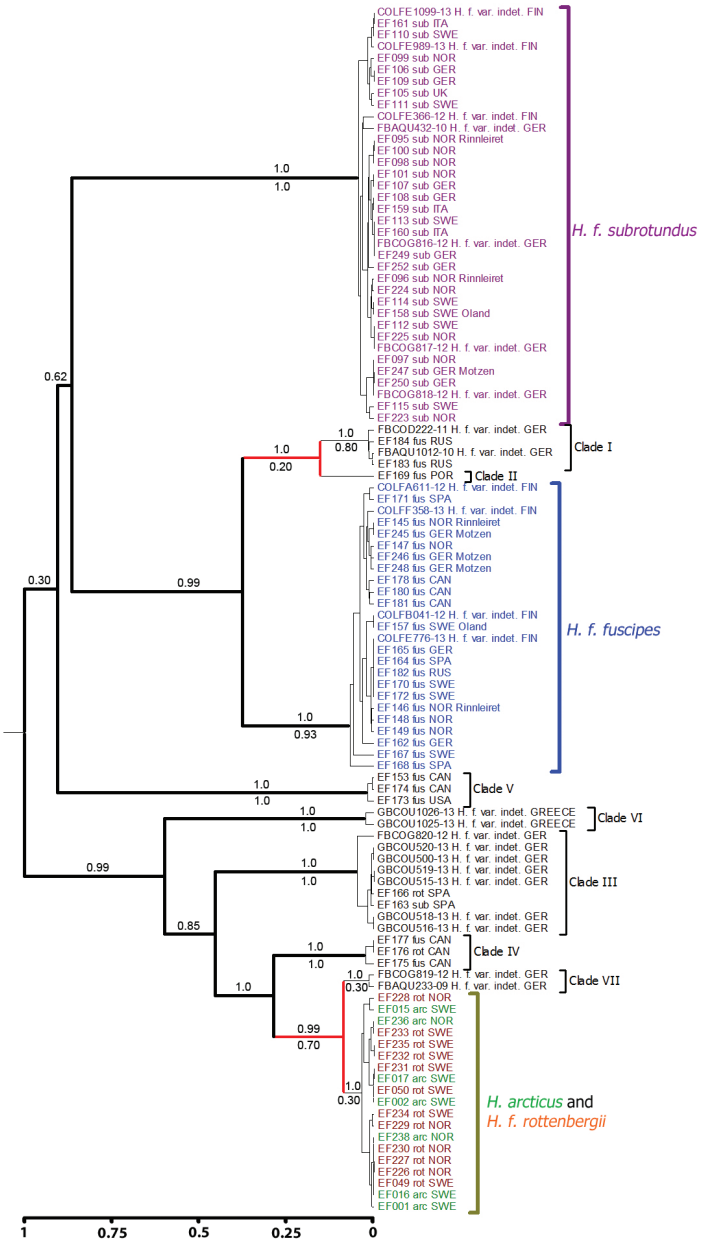

Figure 6. Ultrametric (strict clock) maximum clade credibility (MCC) tree used in GMYC analysis of COI. Terminal names and abbreviations as in Fig. 5. Samples from BOLD are marked with BOLD Sequence ID. Values above branches show Bayesian posterior probability support; values below branches show GMYC-support, i.e. support for the node as a GMYC-species among the alternative models of delimitation considered (95% confidence set). GMYC-support < 0.1 not shown. Splits of thick branches represent speciation events, splits of thin branches indicate within-species coalescent events and splits of red branches depend on the models considered (Table 6). Scale bar represents an artificial time scale with the root at time 1.

Table 6. Model selection in GMYC. Only models within 3 Δ AICc shown. Sorted by Δ AICc. All samples are considered the same species under the null coalescent model, whereas all samples are considered separate species under the null Yule model.

Gene segment	Model	Number of clusters	Number of singletons	Log likelihood	AICc	Δ AICc	Akaike weights
COI	9 species-model	8	1	670.5	-1330.302	0.000	0.463
	10 species- model	9	1	669.7	-1328.735	1.567	0.212
	8 species-model	8	0	669.6	-1328.508	1.794	0.189
H3	Null coalescent model	1	0	504.7	-1005.209	0.000	0.174
	Null Yule model	0	84	504.5	-1004.906	0.303	0.150
ITS2	Null Yule model	0	79	465.7	-927.2162	0.000	0.172
	Null coalescent model	1	0	465.3	-926.4433	0.773	0.117
	5 species-model	5	0	468.1	-925.3851	1.831	0.0688
	9 species-model	9	0	467.7	-924.5404	2.676	0.0451

The model had a ΔAICc = 3.69, which is higher than both the one-species null model and the Yule null model (where all samples are different species) (Table 6), meaning that the null models are the best explanations of the data among the models compared.

The ultrametric MCC tree from BEAST based on ITS2 data (Fig. 7) is concordant with the non-ultrametric ITS2 gene tree (Fig. S3 in Suppl. material 3) and supports the same clades. A GMYC model with 5 delimited species was the maximum likelihood solution, but both the one-species null model and the Yule null model have a lower ΔAICc, whereas a 9-species model also fall within 3 ΔAICc of the best null model (Table 6). The 5-species model's log likelihood (468.1) was not significantly different from the log likelihood of the one-species null model (465.3) in a likelihood ratio test ($p = 0.061$). All clades except Clade III (GMYC-support = 0.93) have low GMYC-support values (Fig. 7). There is higher support for *H. f. rottenbergii*, *H. arcticus* and Clade IV being separate species (GMYC-support >0.25) than for them being the same species (GMYC-support < 0.10).

BPP

BPP analyses without guide tree (BPP v3.0) were mostly conclusive and in agreement, independent of prior-combinations, parameter settings, algorithm (0 or 1), multiple runs or *a priori* sample assignments, and delimited most genetically divergent clades with posterior probabilities of 1.0 (Fig. 8 and Table 7). The largest uncertainty was whether Clade I and Clade II should be considered different species, but the posterior probability (PP) is higher for them as separate species (PP: 0.541–0.623) than for them as the same species (PP: 0.377–0.459). Clade VII was delimited as a separate species different from *H. arcticus* and/or *H. f. rottenbergii* only when it was *a priori* assigned as a potential separate species. Assigning Clade VII specimens as either *H. arcticus* or

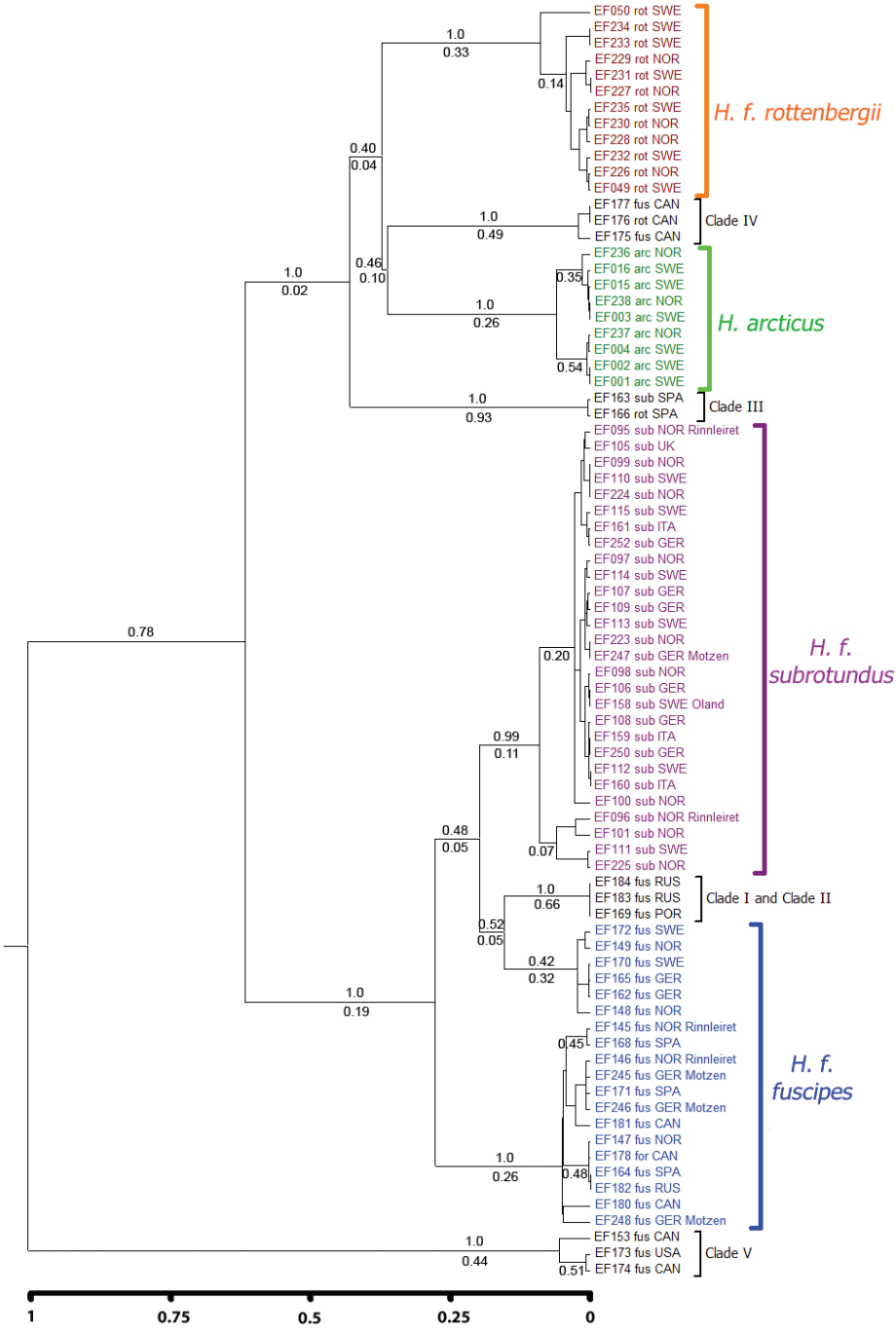

Figure 7. Ultrametric (strict clock) maximum clade credibility (MCC) tree used in GMYC analysis of ITS2. Terminal names and abbreviations as in Fig. 5. Values above branches show Bayesian posterior probability support (nodes with PP < 0.4 not shown); values below branches show GMYC-support. Scale bar represents an artificial time scale with the root at time 1.

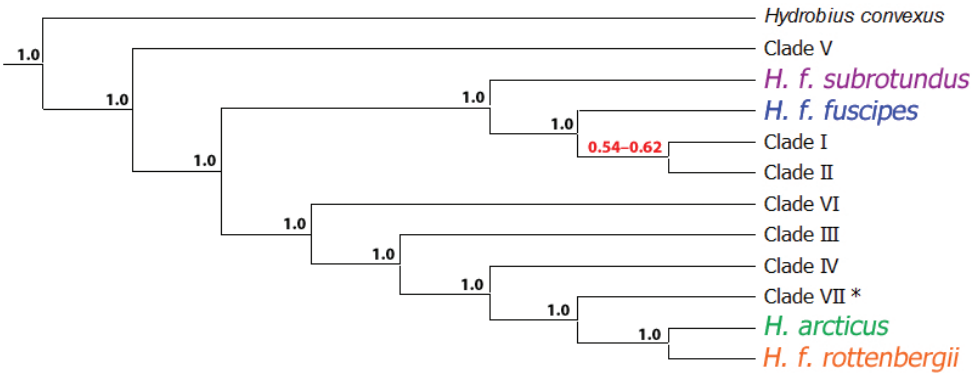

Figure 8. Species tree with the largest posterior probability from BPP v3.0 analyses conducted on *Hydrobius* specimens. Multi-locus data (COI, H3 and ITS2) used with *H. convexus* included as outgroup. Values above branches indicate range of split posterior probabilities, i.e. the probability for the node representing a speciation event, from four different prior-combinations. Values in red have split probabilities < 1.0. *Clade VII only delimited when specimens from Clade VII were *a priori* assigned as a potential species separate from *H. arcticus* and *H. f. rottenbergii*.

Table 7. Posterior probabilities (PP) of delimited species from BPP v3.0, based on multi-locus data (COI, H3 and ITS2) from 111 *Hydrobius* specimens. PP range from four prior-combinations and multiple runs with different starting trees and algorithms (0 vs 1). Species delimited with PP < 0.01 are not reported. †Only delimited when specimens from Clade VII were *a priori* assigned as a potential separate species from *H. arcticus* and *H. f. rottenbergii*.

Delimited species	Posterior probability (range)
H. convexus	1.0
H. arcticus	1.0
H. f. rottenbergii	1.0
H. f. fuscipes	1.0
H. f. subrotundus	1.0
Clade III	1.0
Clade IV	1.0
Clade V	1.0
Clade VI	1.0
Clade VII †	1.0
Clade I	0.541–0.623
Clade II	0.541–0.623
Clade I and Clade II	0.377–0.459

H. f. rottenbergii did not affect their posterior probability as separate species. The species trees with the highest posterior probability (Fig. 8 and Fig. S5 in Suppl. material 3) generally had similar topologies as the phylogenetic trees based on the concatenated dataset and the COI data (Fig. 5 and Fig. S1 in Suppl. material 3). Prior settings had an effect on the posterior probability of Clade I and Clade II as separate species, with

a strong tendency of increasing values of tau (τ_0) resulting in lower posterior probabilities and a weak tendency of increasing values of theta (Θ) resulting in higher posterior probabilities (Table S3 in Suppl. material 3).

The results from BPP v2.2 with a guide tree were very similar to the results from BPP v3.0, independent of prior-combinations, parameter settings, algorithm (0 or 1), multiple runs, guide tree topologies or *a priori* sample assignments (Fig. S6 and Table S4 in Suppl. material 3). Similar to the results from BPP v3.0, the best models delimited 11 or 12 species (including the outgroup *H. convexus*) depending on the *a priori* assignment of specimens of Clade VII. As in BPP v3.0, uncertainty was found in whether Clade I and Clade II should be considered different species, with them being separate species having a bit higher posterior probability than them being the same species. Prior settings had an effect on the split probability of Clade I and Clade II, with increased value of tau (τ_0) resulting in lower split probabilities (Table S5 in Suppl. material 3). Theta (Θ) did not seem to affect the split probabilities.

Morphological analyses

Only characters found to be significantly different between morphotypes are reported and discussed here. Measurements are available in Suppl. material 4.

Genital morphometrics

Male genitalia of the *Hydrobius* morphotypes were generally similar and morphometric measurements of characters overlapped to different degrees between morphotypes (Figs 9, 10).

Width of parameres (in logarithmic scale) in dorsal view was the most informative character and separated all morphotypes from each other, where the morphotypes explained 80.0% of the variation in the character (Table 8 and Fig. 11A). Neither body size nor an interaction between body size and morphotype were statistically significant (interaction effect: $df_N = 3$, $df_D = 54$, $F = 0.0871$, $p = 0.967$; effect of body size: $df_N = 1$, $df_D = 57$, $F = 0.166$, $P = 0.685$), meaning that body size did not affect the character. All morphotypes mean ln width of parameres were significantly different from each other, with the largest difference being *H. arcticus* having a mean that was 6.64% larger than the mean of *H. f. fuscipes* (Tables S6–S7 in Suppl. material 3). The *H. f. rottenbergii* type specimen had a width of paramere that is closer to the mean of the *H. arcticus* morphotype than the *H. f. rottenbergii* morphotype, whereas the *H. f. subrotundus* type and sympatric specimens of *H. f. fuscipes* and *H. f. subrotundus* had values within their respective morphotypes rather than based on locality (Fig. 11A).

Two characters, robustness of parameres and ratio between paramere length and penis length, separated *H. arcticus* and *H. f. rottenbergii* from *H. f. subrotundus* and *H. f. fuscipes*. The morphotypes explained 81.1% of the variation in robustness of para-

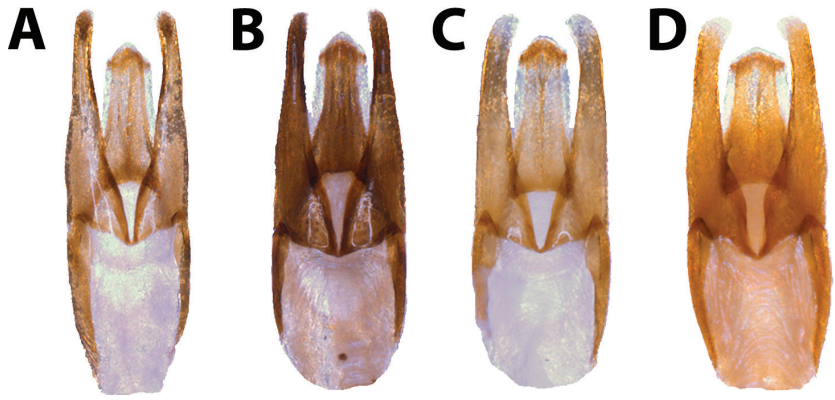

Figure 9. Male genitalia of *Hydrobius* morphotypes in dorsal view. **A** *H. fuscipes fuscipes* **B** *H. f. subrotundus* **C** *H. f. rottenbergii* **D** *H. arcticus*.

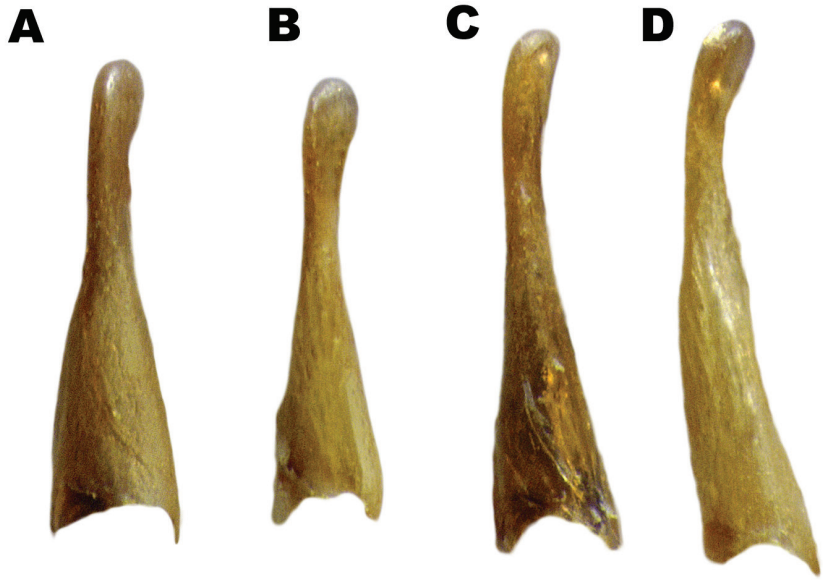

Figure 10. Male genitalia of *Hydrobius* morphotypes in lateral view. **A** *H. arcticus* **B** *H. fuscipes rottenbergii* **C** *H. f. fuscipes* **D** *H. f. subrotundus*.

meres (Table 8 and Fig. 11B). Neither body size nor an interaction between body size and morphotype were statistically significant (interaction effect: $df_N = 3$, $df_D = 52$, $F = 0.395$, $p = 0.757$; effect of body size: $df_N = 1$, $df_D = 55$, $F = 1.97$, $p = 0.166$), meaning that the character was not affected by body size. *H. arcticus* and *H. f. rottenbergii* had significantly more robust parameres, represented by approximately 20–25% lower mean robustness of paramere values than *H. f. fuscipes* and *H. f. subrotundus* (Tables S6–S7 in Suppl. material 3). All type specimens examined and sympatric specimens of

Table 8. ANOVA/ANCOVA for effect of body size and morphotypes on different male genital characters in *Hydrobius*. Only significant effects are shown. df=degrees of freedom. ln = natural logarithm. See Material and Methods for details on character measurements.

Character (unit)	Effect	df	Mean square	F-value	p-value
Width of parameres, dorsal view (ln(µm))	Morphotype	3	0.177	79.5	< 0.001
	Residuals	58	0.00222		
Robustness of parameres	Morphotype	3	41.9	79.8	< 0.001
	Residuals	56	0.525		
Ratio between paramere length and penis length	Morphotype	3	0.0990	20.9	< 0.001
	Residuals	56	0.00474		
Width of parameres, lateral view (ln(µm))	Morphotype	3	0.122	12.6	< 0.001
	Residuals	55	0.00965		
Curvature of paramere tip (µm)	Morphotype	3	1008	22.1	< 0.001
	Residuals	56	104.5		
Length of parameres (ln(µm))	Morphotype	3	0.0534	21.9	< 0.001
	ln (body size)	1	0.0122	5.03	0.0289
	Residuals	55	0.00243		

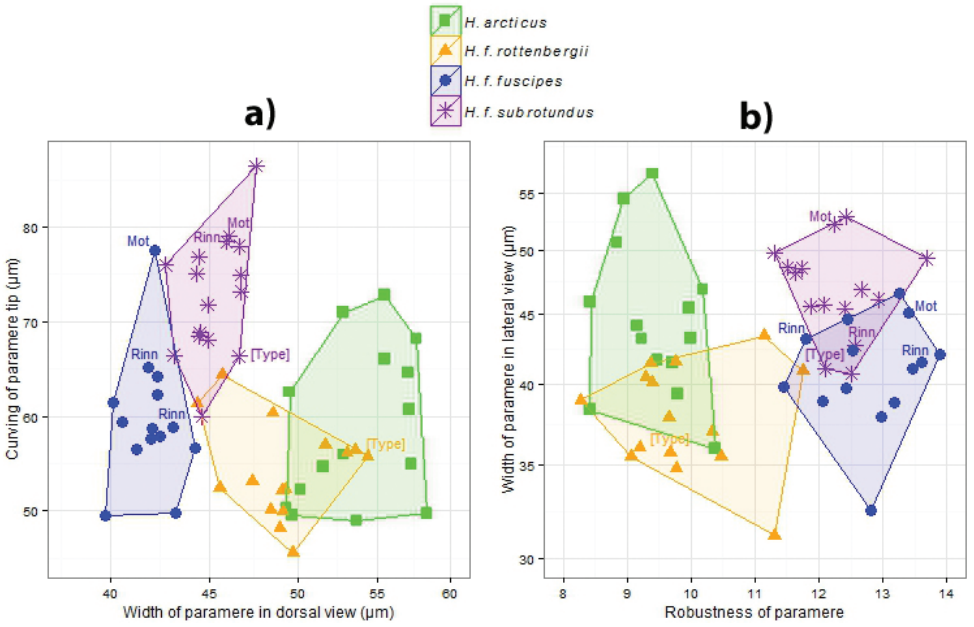

Figure 11. Morphometric differences between 60 (in a) and 59 (in **b**) specimens of *Hydrobius*. Two characters are plotted against each other in each figure with convex hulls used to show overlap in the data between morphotypes. Type specimens and specimens of *H. f. subrotundus* and *H. f. fuscipes* collected in sympatry (Rinn = locality Rinnleiret (Norway) and Mot = Motzen (Germany)) are labeled. **a** Curvature of paramere tip plotted against width of paramere in dorsal view. X-axis is in logarithmic scale **b** Width of paramere in lateral view plotted against the ratio robustness of paramere in dorsal view. Y-axis is in logarithmic scale.

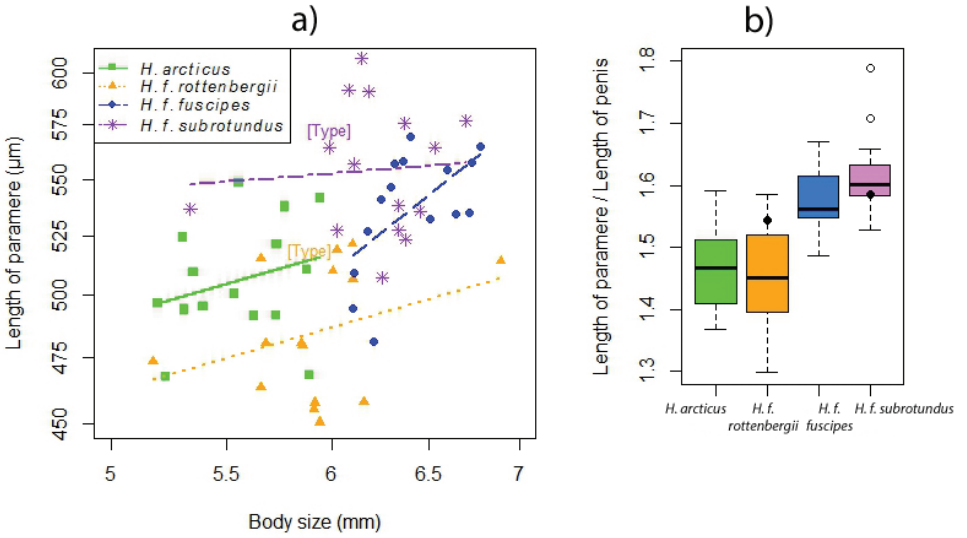

Figure 12. Morphometric differences between 60 specimens of *Hydrobius*. **a** Differences between morphotype and effect of body size on paramere length. Both axes are in logarithmic scale. Independently fitted lines for each morphotype are shown, slopes not significantly different. Type specimens of *H. f. subrotundus* and *H. f. rottenbergii* are labeled **b** Box- and whisker-plot showing differences between morphotypes on the ratio length of paramere / length of penis. Top and bottom of boxes represent first and third quartile; dark bands represent the second quartile (median); whiskers show the maximum and minimum values not including outliers (white points). Black points represent type specimens.

H. f. fuscipes and *H. f. subrotundus* had mean robustness values within their respective morphotypes.

The morphotypes explained 52.8% of the variation in the ratio between paramere length and penis length (Table 8 and Fig. 12B). Neither body size nor an interaction between body size and morphotype were statistically significant (interaction effect: $df_N = 3$, $df_D = 52$, $F = 0.1.36$, $p = 0.264$; effect of body size: $df_N = 1$, $df_D = 55$, $F = 0.145$, $p = 0.705$). The mean of *H. arcticus* and *H. f. rottenbergii* were significantly different, being approximately 7–10% lower, than the mean of *H. f. fuscipes* and *H. f. subrotundus* (Tables S6–S7 in Suppl. material 3). The *H. f. subrotundus* type specimen had a value between the first and third quartile of its morphotype, whereas the *H. f. rottenbergii* type specimen did not (Fig. 12B).

Hydrobius arcticus is separated from *H. f. rottenbergii* and *H. f. fuscipes* is separated from *H. f. subrotundus* with the character width of parameres in lateral view in logarithmic scale, and the morphotypes explain 40.8% of the variation in the character (Table 8 and Fig. 11B). Neither body size nor an interaction between body size and morphotype were statistically significant (interaction effect: $df_N = 3$, $df_D = 51$, $F = 0.1874$, $p = 0.905$; effect of body size: $df_N = 1$, $df_D = 54$, $F = 0.785$, $p = 0.380$). The mean of *H. f. subrotundus* was the largest and approximately 3–6% larger than the mean of *H. f. rottenbergii* and *H. f. fuscipes*, whereas the mean of *H. arcticus* was 4.39%

larger than the mean of *H. f. rottenbergii*, and these differences were significant (Tables S6–S7 in Suppl. material 3). The *H. f. rottenbergii* type specimen had a value close to the mean of other *H. f. rottenbergii*, whereas the type specimen of *H. f. subrotundus* and sympatric specimens of *H. f. fuscipes* and *H. f. subrotundus* generally had somewhat overlapping values.

The *H. f. subrotundus* morphotype had a significantly larger curving of the paramere tip than the other morphotypes, and the morphotypes explained 54.2% of the variation in the character (Table 8, Figs 10, 11A). Neither body size nor an interaction between body size and morphotype were statistically significant (interaction effect: $df_N = 3$, $df_D = 52$, $F = 0.144$, $p = 0.933$; effect of body size: $df_N = 1$, $df_D = 55$, $F = 1.67$, $p = 0.202$). *H. f. subrotundus* mean curvature was significantly different, by being approximately 22–34% larger, than the mean of the other morphotypes (Tables S6–S7 in Suppl. material 3). The type specimens of *H. f. subrotundus* and *H. f. rottenbergii* were largely within their respective morphotypes, although the former had a somewhat low value. All sympatric specimens of *H. f. fuscipes* and *H. f. subrotundus* had values within their respective morphotypes rather than based on locality, except for a *H. f. fuscipes* specimen from Motzen (Germany) which was a clear outlier (Fig. 11A).

Hydrobius f. rottenbergii had significantly lower length of parameres than the other morphotypes, but body size did also have an effect on the character (Table 8, Fig. 12A and Table S9 in Suppl. material 3). The best model was in log-log scale and explained 56.3% of the variation in length of parameres. No statistically significant interaction was found between the morphotypes and body size ($df_N = 3$, $df_D = 52$, $F = 0.842$, $p = 0.477$), meaning that body size has the same effect on each morphotype. The common slope of the morphotypes (0.300 ± 0.134) was significantly different from zero ($df = 55$, $t = 2.24$, $p = 0.0289$). The intercept of *H. f. rottenbergii* was significantly different, being approximately 1–2% lower, than the intercepts of the other morphotypes (Tables S8 and S9 in Suppl. material 3). This can be interpreted as *H. f. rottenbergii*, on average, having significantly shorter parameres than the other morphotypes, given the same body size. The type specimen of *H. f. rottenbergii* had somewhat longer parameres than what is expected for a specimen of its size, while the type specimens of *H. f. subrotundus* had length of parameres close to the mean of other *H. f. subrotundus* specimens of its size (Fig. 12A).

Body characters

Shape of mesoventral process

All morphotypes except *Hydrobius arcticus* had a strong or rather strong acute dentiform mesoventral process. Measurements of 10 randomly chosen specimens from each morphotype confirm this, with *H. arcticus* having higher non-overlapping values than the other morphotypes (Figs 13A, 14). The examined type specimens had the shape that is expected for their respective morphotype.

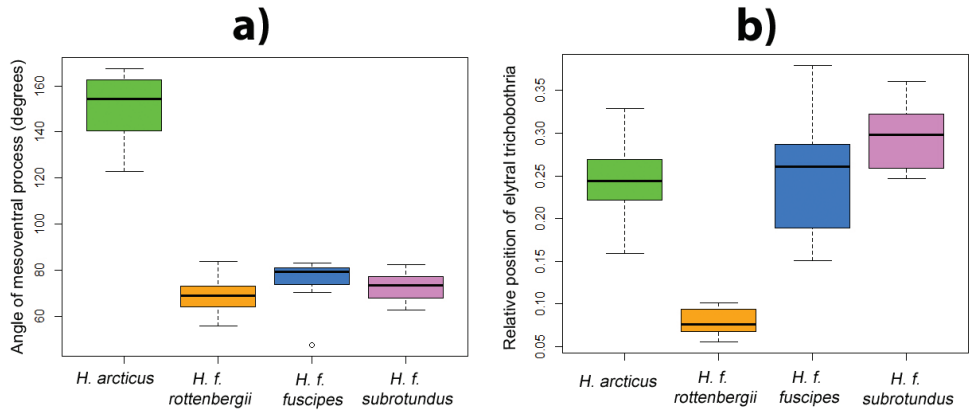

Figure 13. Box- and whisker-plot showing morphometric differences between morphotypes of *Hydrobius*. Top and bottom of boxes represent first and third quartile; dark bands represent the second quartile (median); whiskers show the maximum and minimum values not including outliers (white points). **a** Shape of mesoventral process. *H. arcticus* is the only morphotype with a blunt process (indicated by the higher values) **b** Relative position of trichobothria in relation to the 3rd and 5th row of elytral serial punctures. The trichobothria of *H. f. rottenbergii* are positioned closer to the serial punctures than in other morphotypes (indicated by lower values).

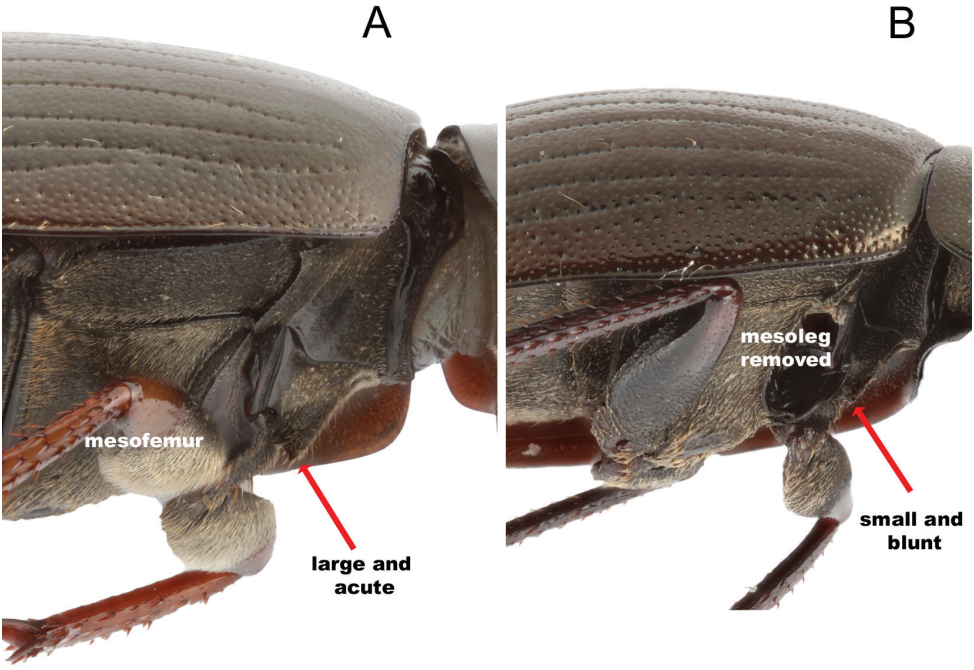

Figure 14. Comparison of the mesoventral process in *Hydrobius*. **A** Large and acute process found in all northern European variants of *H. fuscipes*, here represented by a specimen of *H. f. fuscipes* B Small and blunt process characteristic of *H. arcticus*.

Relative position of trichobothria in relation to the rows of elytral serial punctures

Fennoscandian specimens of *H. f. rottenbergii* had trichobothria positioned close or very close to the elytral serial punctures compared to the other morphotypes that had trichobothria located further into the elytral intervals (Fig. 15). This was only consistent for trichobothria located anteriorly on the elytra posterior to the scutellum. Trichobothria located laterally to the scutellum were generally close to the elytral serial punctures for all morphotypes, whereas trichobothria located on the posterior half of the elytra tended to be positioned further into the elytral intervals in all morphotypes. Some trichobothria deviated in relative position within the specimens, but an average of the position of several trichobothria was consistent for the morphotypes. Initial measurements of the position of trichobothria within the appropriate area showed that the average position of the Fennoscandian specimens of *H. f. rottenbergii* were non-overlapping with the other morphotypes (Fig. 13B), thus the relative position was not measured more thoroughly. This pattern was not as apparent for specimens collected outside of Fennoscandia, where some specimens identified as the *H. f. rottenbergii* morphotype had a relatively larger proportion of trichobothria located in the intervals than the Fennoscandian *H. f. rottenbergii* specimens. The examined type specimens had trichobothria located as expected for their respective morphotype.

Color of legs

On average *H. f. subrotundus* had darker femora and tibiae than the other morphotypes, but some overlap was found between the color of *H. f. subrotundus* and *H. f. fuscipes*. Color differences were more consistent for the femora than for the tibiae, although color of the femora often became lighter towards the trochanter. Specimens with entirely dark legs were always of the *H. f. subrotundus* morphotype, but overlap was found when comparing *H. f. subrotundus* specimens with less dark legs with the *H. f. fuscipes* specimens with the darkest legs. On the other hand, entirely yellow legs are common in *H. f. fuscipes*, but are never found in *H. f. subrotundus*. Specimens of *H. f. subrotundus* collected in sympatry with specimens of *H. f. fuscipes* had darker legs than the *H. f. fuscipes* specimens. The type specimen of *H. f. subrotundus* had dark legs, whereas type specimens of other morphotypes had lighter legs.

Body shape (Elytral Index)

Both morphotypes and body size had a significant effect on the Elytral Index (EI = length of the elytra / maximum width of elytra), with the best model explaining 51.0% of the variance in EI (Table 9 and Fig. 16). No statistically significant interaction was found between the morphotypes and body size (df_N = 3, df_D = 105, F = 2.56, p = 0.0591), meaning that body size affect each morphotype in the same way (i.e. the mor-

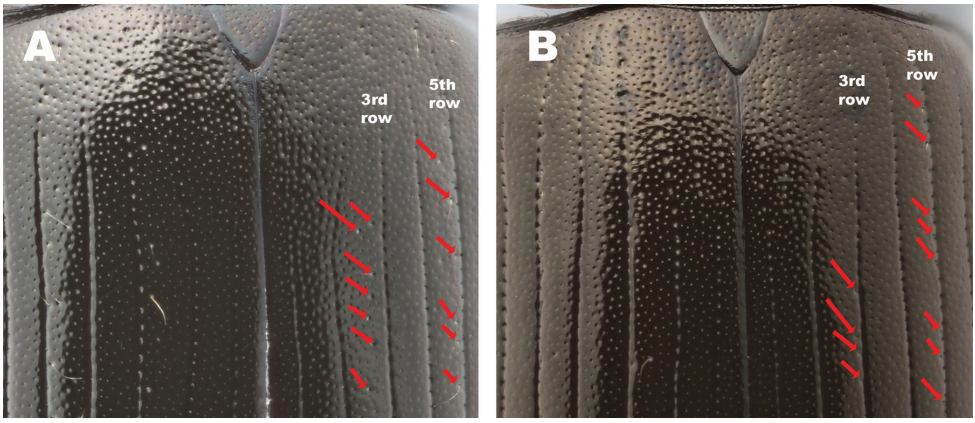

Figure 15. Comparison of the relative position of trichobothria (red arrows) on the elytra of *Hydrobius*. **A** Trichobothria positioned in the intervals between the 2nd and 3rd row of serial punctures, and between the 4th and 5th row. Typical positioning of trichobothria in *H. arcticus*, *H. fuscipes fuscipes* and *H. f. subrotundus*, here represented by a specimen of *H. f. fuscipes* **B** Trichobothria positioned in or very close to the 3rd and 5th row of serial punctures, which is characteristic of *H. f. rottenbergii*.

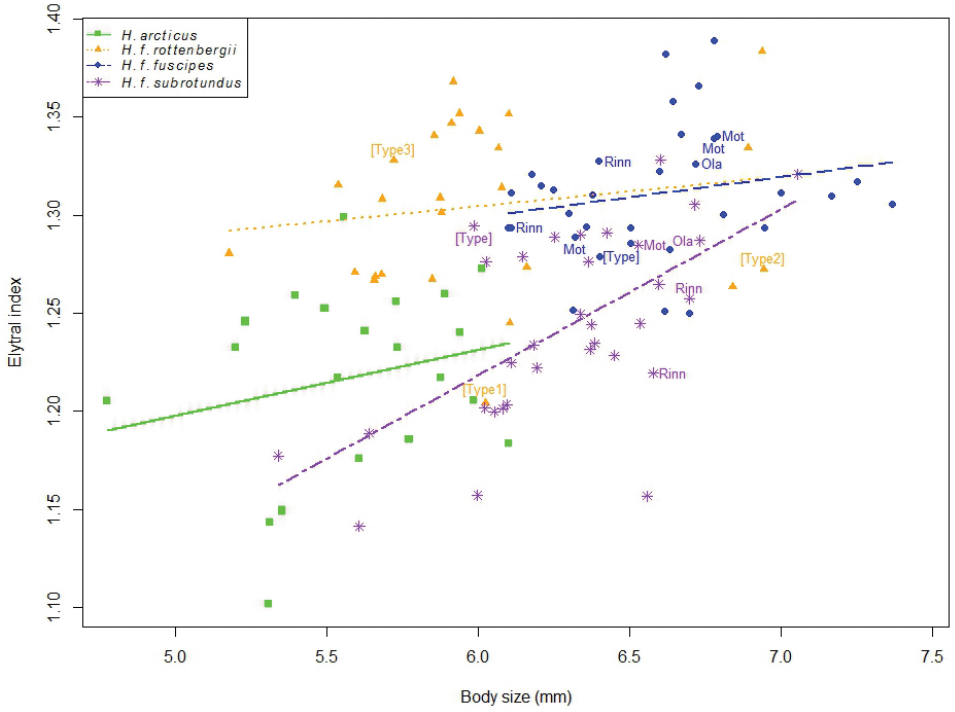

Figure 16. Morphometric differences between morphotypes and effect of body size on Elytral Index (EI) of *Hydrobius*. EI = length of the elytra / maximum width of elytra. 113 specimens measured. Independently fitted lines for each morphotype are shown, slopes not significantly different. Type specimens and specimens of *H. f. subrotundus* and *H. f. fuscipes* collected in sympatry (Rinn = locality Rinnleiret (Norway), Mot = Motzen (Germany) and Ola = Öland (Sweden)) are labeled.

Table 9. ANCOVA for effect of body size and morphotypes on Elytral Index (EI) in *Hydrobius*. Only significant effects are shown. df=degrees of freedom. See Material and Methods for details on character measurements.

Effect	df	Mean square	F-value	p-value
Morphotype	3	0.0558	33.188	< 0.001
Body size	1	0.0212	12.615	< 0.001
Residuals	108	0.00168		

photypes have a common slope). The common slope (0.0381 ± 0.0107) was significantly different from zero (df = 108, t = 3.55, p < 0.001) and means that EI increases by 0.0381 for each mm increase in body size in all morphotypes.

The intercepts of *H. f. subrotundus* and *H. arcticus* were significantly different, being approximately 5–7% lower, than the intercepts of *H. f. fuscipes* and *H. f. rottenbergii* (Tables S8 and S10 in Suppl. material 3). This can be interpreted as *H. arcticus* and *H. f. subrotundus* having on average an EI value that is 5–7% lower than the values of *H. f. fuscipes* and *H. f. rottenbergii*, given that the individuals being compared have identical body size. This means that *H. arcticus* and *H. f. subrotundus* generally have a more convex body than *H. f. fuscipes* and *H. f. rottenbergii* (Fig. 17). Sympatric specimens of *H. f. fuscipes* and *H. f. subrotundus* generally had EI-values within their respective morphotypes rather than based on locality (Fig. 16). The type specimen of *H. f. subrotundus* had an EI value above what is expected for a specimen of its size, while one of the type specimens of *H. f. rottenbergii* had an EI value below most *H. f. rottenbergii* specimens measured (Fig. 16). The type specimen of *H. f. fuscipes* had an EI-value close to what is expected for a specimen of its size, although somewhat low.

Discussion

Phylogenetic relationships

The nuclear gene segments H3 and ITS2 had comparatively low genetic variation (Table 4) and results based on these are therefore sensitive to editing and sequencing errors. However, subsamples of all markers were sequenced twice with the same result and all troublesome sequences were checked multiple times to eliminate the effect of wrong base calls. The low variation in the nuclear gene segments may have resulted in overparameterising of the phylogenetic models and explain why some expected clades in the H3 and ITS2 trees are basal paraphyletic groups without a common node (e.g. *H. f. rottenbergii* specimens with identical haplotypes in ITS2, Fig. S3 in Suppl. material 3). Since COI data are the most variable, the concatenated dataset and corresponding tree (Fig. 5) is highly affected by the COI data. However, Clade III, Clade V and the *H. f. subrotundus* clade were supported as reciprocal monophyletic groups by all markers, suggesting that there is informative data in the nuclear gene segments.

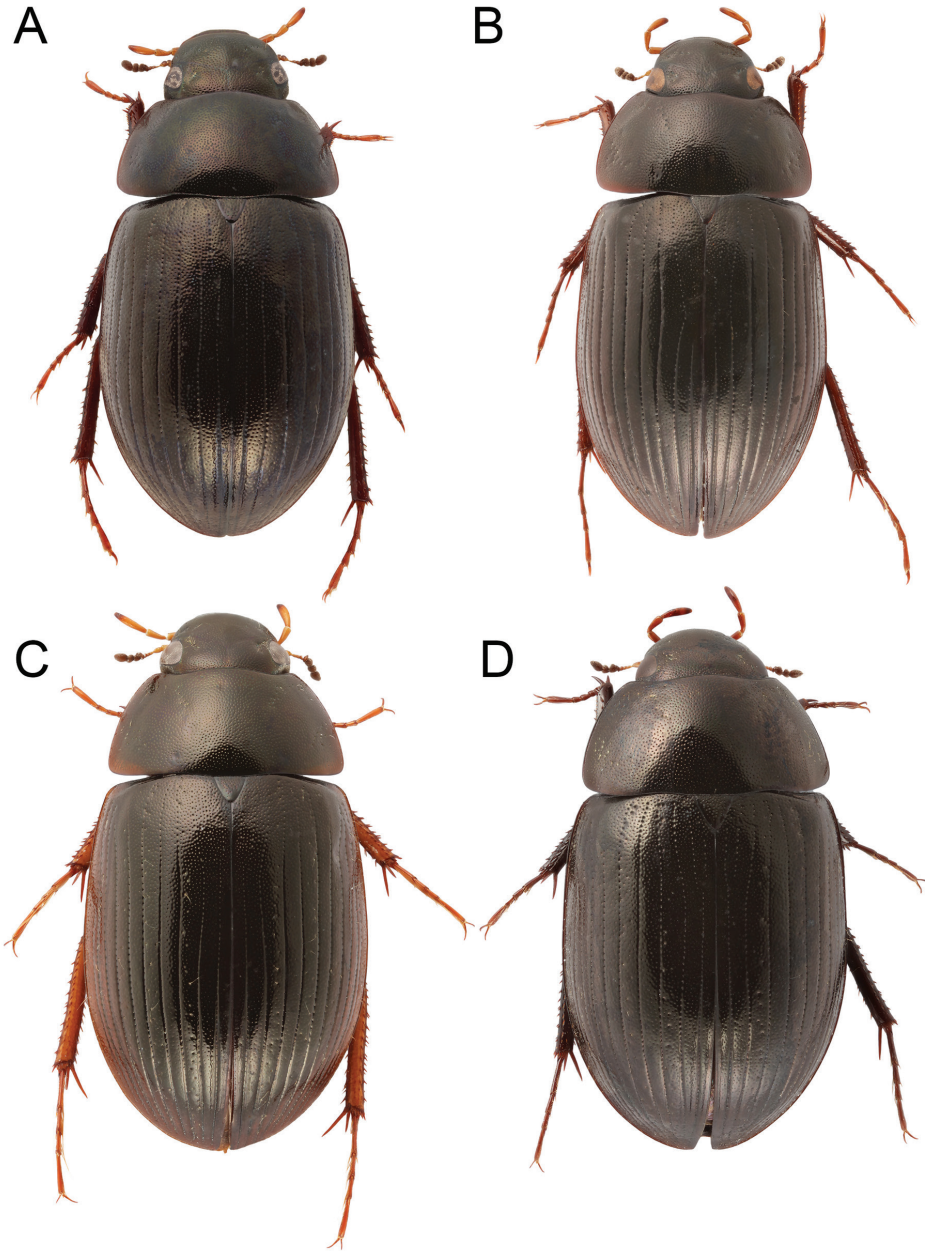

Figure 17. Habitus of *Hydrobius* morphotypes in dorsal view. **A** *H. arcticus* **B** *H. fuscipes rottenbergii* **C** *H. f. fuscipes* **D** *H. f. subrotundus*.

The ITS2 results differ from the other gene trees by the placement of *H. f. rottenbergii*, *H. arcticus* and Clade IV basally in the tree (Fig. S3 in Suppl. material 3). This is possibly due to the outgroup *Hydrobius convexus* having very divergent ITS2 sequences

(Table 4, Fig. S3 in Suppl. material 3) and that the substitution model best fit for the ingroup was unfit to use on the complete dataset (Table S2 in Suppl. material 3). As a strict clock model was preferred in the Bayes factor test using stepping stone sampling, the root inferred in the ultrametric tree (Fig. 7) is more appropriate than the root inferred by outgroup comparison under a non-clock model.

Interestingly, a more complex partition scheme and substitution model was also found for the H3 dataset when including as opposed to excluding the outgroup (Table S2 in Suppl. material 3), suggesting that *H. convexus* is quite distantly related to *H. fuscipes* and *H. arcticus*. This is supported by Short and Liebherr (2007) and Short and Fikáček (2013) who suggested that *Hydrobius* is paraphyletic with respect to species of *Limnocyclus*, *Limnoxenus*, *Hydramara, Sperchopsis, Ametor* and *Hybogralius*. Short and Fikáček (2013) used molecular data and found evidence for *H. fuscipes* being more closely related to species of *Ametor* and *Sperchopsis* than to *Hydrobius melaenus*. However, it is not clear which genetic groups of *H. fuscipes* they had sampled and they did not include *H. convexus* in their study. While *H. convexus* may not be the ideal outgroup for phylogenetic studies of the *H. fuscipes* species complex, it was the only other species of *Hydrobius* available to us for this study.

The most likely general explanation for the conflicting phylogenetic patterns in the gene trees (Figs S1–S3 in Suppl. material 3) is limited variation in the nuclear gene segments and incomplete lineage sorting (Pamilo and Nei 1988). Lack of variation is the best explanation for members of Clade IV being identical to *H. arcticus* and *H. f. rottenbergii* in the H3 gene tree (Fig. S2 in Suppl. material 3) and it is a likely explanation for Clade I and Clade II not being divergent in the H3 and ITS2 trees. Incomplete lineage sorting is probably also the best explanation for *H. f. fuscipes* being paraphyletic in the nuclear trees. The *H. f. fuscipes* group is the most divergent group in the COI tree (Fig. S1 in Suppl. material 3) and it would be interesting to see if more variable nuclear markers would group the specimens together in nuclear gene trees.

The most interesting conflict between the gene trees was in the lack of reciprocal monophyly of *H. arcticus* and *H. f. rottenbergii* for COI and H3. Specimens belonging to these morphotypes grouped together with almost identical sequences in both the COI and H3 gene trees (Figs S1 and S2), but were placed in moderately supported separate monophyletic groups in the ITS2 gene tree and the tree based on the concatenated dataset (Fig. 5 and Fig. S3 in Suppl. material 3). The H3 data probably did not separate the morphotypes due to low variation in this marker. However, this explanation is unlikely for the more variable COI marker. A possible explanation is introgression due to rare hybridization events between the morphotypes after geographical separation. Selective sweeps, where mtDNA was affected more strongly than nDNA, could lead to the observed pattern if one of the ancestral morphotypes had parts of mtDNA that led to higher fitness in the hybrid (Ballard and Whitlock 2004). It could be interesting to test for selective sweeps through for example linkage equilibrium tests, but this would require genetic data with more variation appropriate for population genetic studies.

Despite having widely different habitats and not being known to occur in sympatry, the *H. arcticus* and *H. f. rottenbergii* morphotypes may have had a relatively recent

hybridization event resulting in very similar COI sequences. A possible explanation for when this event occurred, although speculative, is related to their habitats at the end of the last ice age (10–14 000 years ago). As the ice cover melted, what was then coastal areas close to the retracting ice had similar environmental conditions as alpine/arctic areas do today (Lokrantz and Sohlenius 2006). As a result, the two morphotypes could have been sympatric at the time and may have hybridized at low frequency resulting in mixing of mtDNA.

Genetic species delimitation

GMYC results based on COI data (Fig. 6 and Table 6) strongly support most of the genetically divergent clades as distinct species, the largest uncertainty being whether Clade I and Clade II are separate species and whether Clade VII is the same species as *H. arcticus* and *H. f. rottenbergii*. GMYC assumes complete lineage sorting, no hybridization and species monophyly in the gene tree (Pons et al. 2006), requirements that might be violated in the COI data. The specimens within Clade VII were from Hendrich et al. (2015) and identified as *Hydrobius fuscipes* var. indet, making it difficult to know which morphotype they morphologically resemble. These specimens' geographic localities are inland Germany (Bavaria), which does not fit either *H. f. rottenbergii* (coastal localities) or *H. arcticus* (alpine-arctic localities). Morphology and ITS2 gene sequences from these specimens might reveal if Clade VII is a valid species.

The GMYC analyses on the nuclear gene segments were less informative, most likely because of the low variation. This appears to be especially true for the H3 data which was best explained by the null models (Table 6). The ITS2 data had four models within 3 Δ AICc (Table 6), with both null models having the lowest Δ AICc, which means that all models are about equally good at explaining the data among the models compared (Burnham and Anderson 2002). The GMYC-support values (in Fig. 7) are more interesting for the ITS2 data, as they suggest that Clade IV, *H. f. rottenbergii* and *H. arcticus* should be delimited as separate species (GMYC-support 0.26–0.49). However, GMYC can be prone to overdelimitation (Carstens et al. 2013) which may also explain this pattern. The fact that our *H. arcticus* specimens have relatively high support (GMYC-support 0.36–0.54) for being split into two separate species despite having identical haplotypes illustrates this well. The *H. f. fuscipes* morphotype, which is paraphyletic in the ITS2 gene tree, is delimited as several species probably because the GMYC method assumes species monophyly in the gene tree (Pons et al. 2006).

The BPP results, both with version 2.2 and v3.0 (Table 7, Fig. 8, Table S4 and Fig. S6 in Suppl. material 3) strongly support most of the genetically divergent clades that were reliably delimited with GMYC as separate species, suggesting that the genetic differences found between the clades are significant enough to consider the groups different species. Clade I and Clade II were the clades with the lowest split probabilities overall and since the priors were shown to have an effect on the split probabilities (Table S3 and S5 in Suppl. material 3), these specimens may be of the same species.

However, Clade II is only one specimen, meaning that the statistical power of the BPP analyses is low when testing the delimitation of Clades I and II. More specimens from these groups would be required to arrive at a more reliable BPP result.

The BPP results delimited Clade VII, *H. arcticus* and *H. f. rottenbergii* as separate species in all analyses, strongly suggesting that they are different species (Table 7, Fig. 8 and Fig. S6C–E in Suppl. material 3). Interestingly, *a priori* assigning specimens of Clade VII as *H. arcticus* or *H. f. rottenbergii* did not affect the split probability of the two morphotypes (Table 7 and Fig. S6B in Suppl. material 3), showing the importance of the *a priori* assignment of samples. The fact that Clade VII consisted of only two specimens (with only COI data available) may explain why it did not affect the split probability of *H. arcticus* and *H. f. rottenbergii*.

Results from BPP v3.0 were very similar to the results from BPP v2.2, probably because the species trees with highest posterior probabilities in BPP v3.0 were very similar to the guide trees used in BPP v2.2 analyses (Figs S5 and S6 in Suppl. material 3). The guide tree and the number of terminals (i.e. potential species) can affect the results of the BPP analyses (Leache and Fujita 2010), but both BPP versions gave similar results, indicating that the guide trees used in BPP v2.2 likely did not affect the results. Conceptually, however, the new version of BPP represents a great step forward. It brings multi-locus Bayesian species delimitation under the multispecies coalescent (MSC) model into the realm of discovery methods at least for small datasets (although apart from computational limitations the presently implemented priors may be inappropriate, see Yang and Rannala 2014). Even when not fully a discovery method, minimum population level assignments may often be straightforward and BPP version 3 will jointly infer species delimitation and species phylogeny under the MSC while taking gene tree uncertainties (topology and branch lengths) into account.

Overall, both GMYC and BPP suggest that Clades III, IV, V, VI, VII, *H. arcticus*, *H. f. fuscipes*, *H. f. rottenbergii* and *H. f. subrotundus* are sufficiently genetically divergent to be considered separate species, whereas they do not agree upon whether or not Clades I and II are the same species. BPP uses multi-locus data, is not affected by incomplete lineage sorting and can handle small amounts of hybridization between species (Zhang et al. 2011). This may explain why it provides a clearer result in terms of species boundaries than the GMYC method for our data.

Male genital morphometrics

Several significant differences in genital characters were found between the morphotypes (Table S6 and S9 in Suppl. material 3). The effect size (i.e. how large the differences are) do vary among the characters, with three of the characters (length of parameres and width of parameres in both lateral and dorsal view) having a relatively low effect size of less than 7% difference at most. With such a relatively low effect size, it is difficult to observe the difference without doing measurements, whereas the robustness of parameres had an effect size of approximately 20% and this difference is observable

in Fig. 9. *Hydrobius arcticus* and *H. f. rottenbergii* clearly have more robust parameres than *H. f. fuscipes* and *H. f. subrotundus*. Similarly, the effect size in the character curvature of the paramere tip is approximately 25% and is also observable in Fig. 10. In this case the *H. f. subrotundus* morphotype (Fig. 10D) had a clearly more strongly curved paramere tip than the other morphotypes (Fig. 10A–C).

Some overlap was found between at least two of the morphotypes in all characters (Figs 11 and 12), which may suggest that some hybridization occur between the morphotypes. However, this is not concordant with the genetic data, as one would expect different morphotypes to group together to a larger degree in the phylogenetic trees. Any substantial hybridization would likely also have led to the morphotypes not being delimited in BPP. If hybridization does occur it is likely that other post-mating isolation mechanisms may be at work, for example infertile hybrids.

Several of the genital characters examined here are correlated to each other, meaning that the number of independent characters examined is low. For instance, the robustness of parameres is a ratio between the character length of parameres and the character width of paramere in dorsal view. These correlations also make it probable that an outlier in one character will also be an outlier in another character. The relatively low number of specimens measured (approximately 15 of each morphotype, limited by the number of sequenced specimens) make the results more prone to artifacts. However, several of the differences were highly statistically significant ($p < 0.001$), suggesting that coincidence is not a likely explanation.

Hydrobius f. subrotundus and *H. f. fuscipes* specimens were collected in sympatry, but grouped nevertheless with specimens of their respective morphotype in all phylogenetic trees (Fig. 5 and Figs S1–S3 in Suppl. material 3). The genital morphometric analyses also moderately support this observation and the sympatric specimens of different morphotypes have no overlap in width of parameres in dorsal view and very little overlap in curvature of paramere tip (Fig. 11A). The low number of morphologically compared sympatric specimens ($n = 5$) makes this comparison inconclusive. However, in concert with the genetic data, genital morphology does indicate that these belong to separate species.

Diagnostic body characters

Hydrobius arcticus is the morphotype easiest to identify, while *H. f. fuscipes* can be difficult to separate from *H. f. subrotundus*. The latter may have led to misidentifications of specimens, especially for specimens outside of northern Europe. Our genetic data indicate the presence of six or seven additional species outside of northern Europe. To enable reliable morphological identification of these, more specimens from a larger geographical range should be analyzed, especially if they are to be described as species new to science.

The relative position of trichobothria is one of the characters Hansen (1987) used to separate *H. f. rottenbergii* from the other morphotypes, but our results show that

the character is only useful under certain conditions (only consistent for trichobothria located on the upper part of the elytra posterior to the scutellum) and only works on Fennoscandian morphotypes. This is also evident from Fig. 5, where 2 specimens identified as *H. f. rottenbergii* from outside northern Europe belong to the genetically divergent Clade III and Clade V.

Body shape (EI) has been used to separate *H. f. fuscipes* from *H. f. subrotundus* (Hansen (1987), but is not ideal as a diagnostic character since body size affects the character and there is some overlap between the morphotypes (Fig. 16 and Table 9). The best use of the character is when comparing specimens of similar size. A combination of body shape, color of legs, and the male genital character curvature of the paramere tip may be the best way to separate *H. f. fuscipes* from *H. f. subrotundus*, preferably comparing them side by side. If the specimen has an extreme character value (e.g. entirely black or yellow femora or a very clearly short and convex body shape) it may not be necessary to look at all the characters.

The large number of listed synonyms for each species (especially *H. fuscipes*) makes certain association of morphotypes with nominal species challenging. Type specimens of senior synonyms were examined when available, but we were unable to borrow types of *H. arcticus*, and could not study the genitalia of the *H. f. fuscipes* type. The position of trichobothria, shape of mesoventral process and color of legs were as expected for type specimens, suggesting that the correct name have been applied to the different morphotypes analyzed. However, other quantitative measurements of the types were not necessarily concordant with measurements from other specimens of the respective morphotypes. The type of *H. f. fuscipes* generally grouped together with other *H. f. fuscipes* specimens, but the *H. f. subrotundus* type and some of the *H. f. rottenbergii* types had character values, both on body and genitalia, that were larger or smaller than most of their respective morphotypes (e.g. Fig. 12). These incongruities indicate that wrong names may have been applied or that the few type specimens examined represent outliers for certain measurements. Correlation between some of the characters, especially ratios that use elytra or paramere lengths, can also explain why a specimen is an outlier in more than one character.

The large number of synonyms must be considered when dealing with the genetically divergent clades (Table 5) as potential separate and valid new species, complicating the taxonomic work in *Hydrobius*. *Hydrobius arcticus* has been reported from northeastern Algeria (İncekara 2007), Iran (Ghahari and Jedryczkowski 2011) and Turkey (Mart et al. 2006). Our results suggest that these specimens may have been wrongly identified, as there are several potential cryptic species within *Hydrobius*. It is not unreasonable to think that *H. arcticus* specimens from the Mediterranean region and the Middle-East actually are something different from the northern European artic/alpine *H. arcticus*. Mart et al. (2006) provided a sketch of the male genitalia of their *H. arcticus*. Assuming that the sketch is accurate, the paramere robustness ratio is 13–15, too high to be *H. arcticus*.

COI barcodes could not distinguish *H. f. rottenbergii* from *H. arcticus*, making this an example of where DNA barcoding fails to identify different morphospecies.

Using ITS2 as an additional marker will separate these species, however. On the other hand, traditional DNA barcodes can be used to separate all other genetically divergent clades (Fig. S1 in Suppl. material 3), including potentially cryptic species within the *H. fuscipes* complex. Data in BOLD currently identified as *Hydrobius fuscipes* need to be revised to reflect this for the database to be efficient in the identification of closely related species in the *H. fuscipes* complex.

This study shows that using multiple methods, based on both morphology and molecular data, is important in species delimitation studies. This has also been shown in other integrative taxonomic studies, where using only one method to delimit species can and often will result in erroneous delimitations (e.g. Carstens et al. 2013; Padial et al. 2010; Schlick-Steiner et al. 2010).

Overall our results correspond well with the conclusion of Lindberg (1943) on the variants of *H. fuscipes* being different species. Compared to Lindberg (1943), this study expands the taxon sampling by including *H. arcticus*, a close relative to *H. f. rottenbergii* and by looking at genital morphometrics and genetic data in addition to traditional diagnostic characters. We also show that populations from central and southern Europe and North America might be additional species in the *Hydrobius fuscipes* species complex.

Conclusions, taxonomy and key

The four *Hydrobius* morphotypes examined in northern Europe should be regarded as separate species and elevated:

Hydrobius arcticus Kuwert, 1890
Hydrobius fuscipes (Linnaeus, 1758)
Hydrobius rottenbergii Gerhardt, 1872, **stat. n.**
Hydrobius subrotundus Stephens, 1829, **stat. n.**

The fact that *H. rottenbergii* is much more closely related to *H. arcticus* (based on both genetic data and similarity in male genitalia), the morphotype of which has been regarded as a separate and valid species for the longest time, than to the other *H. fuscipes* variants clearly indicates that it is a valid species. The consistent difference in the position of trichobothria in the elytral serial punctures rather than in the elytral intervals as in *H. fuscipes* and *H. subrotundus*, is further evidence of significant morphological divergence that cannot be disregarded as intraspecific variation since it covaries with 1) the male genitalia of short and broad *arcticus*-type, 2) the genetic evidence, and 3) the difference in ecological niche being coastal rock pools.

The strongest argument for *H. subrotundus* being a separate species is the fact that despite being sympatric with *H. fuscipes*, they are well differentiated clades genetically which covaries with significantly different, albeit overlapping, genitalic and body shape characters as well as partly subdivided ecological niches. This indicates little or no gene

flow between the species despite living in sympatry, which rules out treating them as subspecies according to the most commonly used concept (Mayr and Ashlock 1991). We are aware that the taxonomic level of subspecies is sometimes used in another sense, sometimes as a kind of compromise bin for complex or uncertain situations. However, we feel following a precise and predictive (hence testable) definition for subspecies is preferable for scientific progress. Even though our study is limited by focusing on a subset of the complete geographical range of the *Hydrobius fuscipes* complex, our data is clear enough to reject both a conspecific and subspecific status of the four examined taxa in northern Europe.

There is a chance that the names *H. subrotundus* and *H. rottenbergii* are inappropriately used for the clades here referred to (genetic data were not retrieved from type material). Type localities are in England for *H. subrotundus* and in Central Europe for *H. rottenbergii* and we have shown that specimens that could be associated with these names from Central Europe may represent additional species, genetically distinct. To solve the situation in central as well as southern Europe will require further taxonomic work for sure. However, we consider recognition of four clearly valid species in northern Europe under traditional names the best stimulus for further decrypting the *Hydrobius fuscipes* complex in the rest of Europe, east Palearctic and the Nearctic. In fact, since *Hydrobius fuscipes* has for a long time been suspected or even known to be a species complex by Hydrophilid-workers, yet still not solved or moved further to a solution, indicates that it is a multifaceted problem that may need to be solved step by step. Future studies benefit from the possibility of sequencing DNA fragments from old type material and in this way match type specimens with appropriate genetic groups and will show if alternative names should be applied. These comparisons in combination with conducting morphological analyses of the genetically divergent clades not present in northern Europe (this study; Hendrich et al. 2015) should yield conclusive results regarding the taxonomy of these potential additional species. One should keep in mind, however, that deep genetic divergence in itself does not necessarily prove heterospecific status of individuals. Deep divergences may result from the survival of two or more old and divergent copies at a genetic locus within a lineage with full panmixis. Covariation with differences in other characters is a prerequisite to reject this situation as panmixis is predicted to erase any population divergence in other traits.

Identification key to *Hydrobius* species of northern Europe

1 Mesoventral process blunt, angle >100° (Fig. 14B). Body size smaller (length of pronotum + elytra = 4.6–6.2 mm). Male parameres robust (Fig. 9D). Alpine-arctic environment. ..... ***H. arcticus***

– Mesoventral process acute and dentiform, angle <100° (Fig. 14A). Body size larger (length of pronotum + elytra = 5.1–7.4 mm). Male parameres more elongate and thin (Fig. 9A–B), or if robust (Fig. 9C) then trichobothria on anterior half of elytra situated in, or very close to, the 3th and 5th row of elytral serial punctures (Fig. 15B)..... **2**

2 Trichobothria on anterior half of elytra situated in, or very close to, the 3rd and 5th row of elytral serial punctures (Fig. 15B). Male parameres robust (Fig. 9C). Coastal rock pools. ..... ***H. rottenbergii***

– Trichobothria on anterior half of elytra situated in the intervals between the 2nd and 3rd, and between the 4th and 5th row of serial punctures (Fig. 15A). Male parameres elongate and thin (Figs 9A–B). **3**

3 Body shape generally compact and shorter (Elytral length/width = 1.14–1.33, Fig. 17D). Male parameres in lateral view significantly curved towards apex (Fig. 10D). Legs dark brown to black. More shaded or colder waters and at vegetation rich edges of slow flowing waters..... ***H. subrotundus***

– Body shape generally more elongate (Elytral length/width = 1.25–1.40, Fig. 17C). Male parameres in lateral view weakly curved towards apex (Fig. 10C). Legs yellow to dark brown. Characteristic species in open sun exposed, temporary or eutrophic, stagnant pools and ponds. ..... ***H. fuscipes***

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Supplementary material 1

Specimen information

Authors: Erlend I. Fossen, Torbjørn Ekrem, Anders N. Nilsson, Johannes Bergsten

Data type: specimen data

Explanation note: Excel file containing information (locality data, voucher info, gender, BOLD ID) about all specimens that where measured and/or sequenced.

Supplementary material 2

Additional morphological characters

Authors: Erlend I. Fossen, Torbjørn Ekrem, Anders N. Nilsson, Johannes Bergsten

Data type: Word file

Explanation note: Word file containing a list and descriptions of additional morphological characters that were measured. Contains 11 external body and 8 male genital characters.

Supplementary material 3

Supplementary tables and figures

Authors: Erlend I. Fossen, Torbjørn Ekrem, Anders N. Nilsson, Johannes Bergsten

Data type: Tables and figures

Explanation note: Additional tables (S1-S10) and figures (S1-S6). Phylogenetic trees, BPP results, post-hoc comparisons of morphological characters etc.

Supplementary material 4

Morphological dataset

Authors: Erlend I. Fossen, Torbjørn Ekrem, Anders N. Nilsson, Johannes Bergsten

Data type: Morphological measurements

Explanation note: Excel file containing the complete morphological measurements. Includes a second data sheet with non-abbreviated variables and units for the measurements.

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RESEARCH ARTICLE

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Contributions to the knowledge of subterranean trechine beetles in southern China's karsts: five new genera (Insecta, Coleoptera, Carabidae, Trechinae)

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Abstract

Recent discoveries reveal that southern China's karsts hold the most diverse and morphologically modified subterranean trechine beetles in the world, albeit the first troglobitic blind beetle was only reported in the early 1990's. In total, 110 species belonging to 43 genera of cavernicolous trechines have hitherto been recorded from the karsts of southern China, including the following five new genera proposed below: *Shiqianaphaenops* Tian, **gen. n.**, to contain two species: *Shiqianaphaenops majusculus* (Uéno, 1999) (= *Shenaphaenops majusculus* Uéno, 1999, **comb. n.**), the type species from Cave Feng Dong, Shiqian, Guizhou, and *Shiqianaphaenops cursor* (Uéno, 1999) (= *Shenaphaenops cursor* Uéno, 1999, **comb. n.**), from Cave Shenxian Dong, Shiqian, Guizhou; and the monotypic *Dianotrechus* Tian, **gen. n.** (the type species: *D. gueorguievi* Tian, **sp. n.**, from Cave Dashi Dong, Kunming, Yunnan), *Tianeotrechus* Tian & Tang, **gen. n.** (the type species: *T. trisetosus* Tian & Tang, **sp. n.**, from Cave Bahao Dong, Tian'e County, Guangxi), *Huoyanodytes* Tian & Huang, **gen. n.** (the type species: *H. tujiaphilus* Tian & Huang, **sp. n.**, from Longshan, Hunan) and *Wanhuaphaenops* Tian & Wang, **gen. n.** (the type species: *W. zhangi* Tian & Wang, **sp. n.**, from Cave Songjia Dong, Chenzhou, Hunan).

Keywords

New genus, new species, new combination, cavernicolous, ground beetle, Guizhou, Yunnan, Guangxi, Hunan

Introduction

China is long known to support with the largest karst landscapes and ecosystems in the world (Waltham 2009). Like in many other fields of natural sciences, however, modern speleological activities in China started much later than in other countries of Europe, North America or Japan, although the well-known Ming Dynasty traveler Xu Xiake made important contributions to cave surveys already during the first half of the 17th century. While in many countries caves are described and registered (e.g. more than 34,000 caves in Italy, see Stoch 2002), there is still no available cave database in China, where only rather few caves are well explored and surveyed. The situation is the same for cave biology. In China, cave-dwelling animals have been continuously reported since the 1980's, mostly by foreign researchers, but there is still a long way to go. With the exception of several vertebrates such as fishes, bats and frogs, almost nothing is known about troglobitic invertebrates presented in the Red Data book of China or on the list of endangered species which are strictly protected by law in China (Wang and Xie 2005).

However, this situation is changing. Discoveries during last two decades reveal that the karsts of southern China host the globe's most diverse fauna of subterranean trechine beetles, including that at the generic level. Up to date, 38 genera and 106 species of cave trechines have been recorded from southern China. Below is a checklist of cavernicolous trechine genera known from southern China's karsts, coupled with data on their species diversity and geographical ranges.

Agonotrechus Jeannel, 1923: only a single troglophilous species recorded from a cave in western Hubei (Deuve 1999);

Aspidaphaenops Uéno, 2006: three species, all troglobitic and all known from Qianxinan, Guizhou (Uéno 2006b);

Bathytrechus Uéno, 2005: one species, troglobitic, in Leye, northwestern Guangxi (Uéno 2005a);

Boreaphaenops Uéno, 2002: a single species, troglobitic, in Shennongjia, western Hubei (Uéno 2002, 2010);

Cathaiaphaenops Deuve, 1996: six species, troglobitic, known from Hunan, Hubei and Chongqing (Deuve 1996, 1999, Uéno 2000b);

Cimmeritodes Deuve, 1996: two species, troglobitic, one in Longshan, northwestern Hunan, the other in Quzhou, eastern Zhejiang (Deuve 1996, Uéno 2006b, Deuve and Tian 2015);

Dongodytes Deuve, 1993: 12 species, highly modified, troglobitic, in several counties of northwestern Guangxi (Deuve 1993, Uéno 1998, 2005c, Tian 2011, Tian et al. 2014);

Giraffaphaenops Deuve, 2002: two species, extremely modified, troglobitic, in several caves in Leye and a cave in Tianlin, northwestern Guangxi respectively (Deuve 2002, Uéno 2003b, Tian and Luo 2015);

Guiaphaenops Deuve, 2002: one species, troglobitic, in Lingyun, northwestern Guangxi (Deuve 2002; Uéno 2006a);

Guizhaphaenops Vigna Taglianti, 1997: 11 species in two subgenera, troglobitic, in Guizhou and Yunnan (Vigna Taglianti 1997, Deuve 1999, 2001, Uéno 1998, 2000a, Uéno and Ran 2004, Deuve and Quéinnec 2014);

Jiangxiaphaenops Uéno & Clarke, 2007: a single species, troglobitic, known from Wannian, Jiangxi (Uéno and Clarke 2007);

Jiulongotrechus Tian, Huang & Wang, 2015: a single species, troglobitic, known from Tongren, eastern Guizhou (Tian et al. 2015);

Junaphaenops Uéno, 1997: a unique species, troglobitic, known from Kunming, the capital City of Yunnan Province (Uéno 1997);

Libotrechus Uéno, 1998: two species, troglobitic, in southernmost Guizhou and northern Guangxi (Uéno 1998, Lin and Tian 2014);

Luoxiaotrechus Tian & Yin, 2013: two species, troglobitic, from eastern Hunan (Tian and Yin 2013, Tian and Huang 2015);

Microblemus Uéno, 2007: a single species, troglobitic, in Jinhua, eastern Zhejiang (Uéno 2007);

Minimaphaenops Deuve, 1999: a single species, troglobitic, in Fengjie, Chongqing (Deuve 1999);

Oodinotrechus Uéno, 1998: Three species, troglobitic, two in southernmost Guizhou and northernmost Guangxi (Uéno 1998, Tian 2014), one in Pingle, northeastern Guangxi (Sun and Tian 2015);

Pilosaphaenops Deuve & Tian, 2008: 2-3 species, highly modified, troglobitic, in southernmost Guizhou and northernmost Guangxi (Uéno 2002, Deuve and Tian 2008, Tian 2010);

Plesioaphaenops Deuve & Tian, 2011: a single species, troglobitic, in Longlin, western Guangxi (Deuve and Tian 2011);

Qianaphaenops Uéno, 2000: six species, troglobitic, from northeastern Guizhou (Uéno 2000c, Tian and Clarke 2012, Tian et al. 2015);

Qianotrechus Uéno, 2000: five species, troglobitic, in northern Guizhou and southeastern Sichuan (Uéno 2000c, 2003a);

Satotrechus Uéno, 2006: two troglobitic species, one in southwestern Guizhou, the other in northwestern Guangxi (Uéno 2006b, Deuve and Tian 2011);

Shenaphaenops Uéno, 1999: one species, troglobitic, in northwestern Guizhou (Uéno 1999a). The other two species originally assigned to this genus (Uéno 1999b) are transferred into a new genus described below;

Shuaphaenops Uéno, 1999: one species, troglobitic, in southern Chongqing (Uéno 1999c);

Shilinotrechus Uéno, 2003: two species, troglobitic, from eastern Yunnan (Uéno 2003a, Huang and Tian 2015);

Sichuanotrechus Deuve, 2005: five species, troglobitic, in northern Sichuan (Deuve 2005, Uéno 2006a, 2008, Huang and Tian 2015);

Sidublemus Tian & Yin, 2013: one species, troglobitic, from southeastern Hunan (Tian and Yin 2013);

Sinaphaenops Uéno & Wang, 1991: nine species in three subgenera, highly modified, troglobitic, from western to southern Guizhou, and northernmost Guangxi (Uéno

and Wang 1991, Magrini et al. 1997, Uéno and Ran 1998, Uéno 2002, Deuve and Tian 2014, Tian and Huang 2015);

Sinotroglodytes Deuve, 1996: two species, troglobitic, in northwestern most Hunan (Deuve 1996, Uéno 2009);

Superbotrechus Deuve & Tian, 2009: a single species, troglobitic, in western Hubei (Deuve and Tian 2009);

Toshiaphaenops Uéno, 1999: two species, troglobitic, from northwestern Hunan and western Hubei (Uéno 1999a);

Trechiotes Jeannel, 1954: three species, troglophilous, in Guizhou and Guangxi (Deuve et al. 1999, Deuve 1995, Uéno 2007);

Uenotrechus Deuve & Tian, 1999: one species, highly modified, troglobitic, in southern Guizhou and northern Guangxi (Deuve et al. 1999, Deuve and Tian 2010);

Wulongoblemus Uéno, 2007: one species, troglobitic, in western Zhejiang (Uéno 2007);

Yanzaphaenops Uéno, 2010: a single species, highly modified, troglobitic, in Shennongjia, western Hubei (Uéno 2005b, 2010);

Yunotrechus Tian & Huang, 2014: a single troglobitic species from southernmost Yunnan (Tian and Huang 2014);

Zhijinaphaenops Uéno & Ran, 2002: five species, troglobitic, in western and central Guizhou (Uéno and Ran 2002; Deuve and Tian 2015).

Uéno (1999a) set up the genus *Shenaphaenops* based on a single female collected from a limestone cave called Shen Dong (actually, the complete name of the cave is Shendong Migong) in Shuicheng County, northwestern Guizhou Province. Later, he found two new species from caves in Shiqian County, northeastern Guizhou and described them in *Shenaphaenops*, even though he realized that both two counties lay very far from each other, and the beetles found in Shiqian showed some peculiar features, in particular, a dilated protarsomere 1 in both sexes, a character never seen in other trechines (Uéno 1999b). We visited the type localities in both counties twice in 2014 and 2015, respectively, and successfully collected a male of *Shenaphaenops humeralis* Uéno, 1999, the type species, as well as an abundant material of *S. majusculus* Uéno, 1999. The newly collected samples provided enough evidence to show that both of these species are sufficiently different in many characters of generic importance to warrant the placement of the two known species from Shiqian into a new genus, not *Shenaphaenops*.

Thanks to Dr. Borislav V. Gueorguiev (National Museum of Natural History, Sofia, Bulgaria), we received a very peculiar trechine for study and as a gift. This female specimen was collected in a limestone cave called Dashi Dong in a suburb of Kunming, the capital city of Yunnan Province in autumn 2011 during a China-Bulgaria joint cave exploration in Yunnan organized by Prof. Fan Zhang (Yunnan Institute of Geography, Yunnan University). This interesting anophthalmic beetle appears to be new both at the specific and generic levels.

Among the new findings of cave-dwelling trechines during our cave surveys in Guangxi in July and August 2015, perhaps the most interesting was an unexpected

species collected in Cave Bahao Dong, Tian'e County. It is sympatric with *Dongodytes giraffa* Uéno, 2005, an extremely troglomorphic beetle, but shows very unusual morphological characters not seen in any other cavernicolous trechines known in China, such as the right mandible being quadridentate, the pronotal lateral borders invisible from above and the elytra with three dorsal pores.

In July 2014, a single female trechine was discovered in the famous Huoyan Karst, Longshan County, northwesternmost Hunan Province. It lives there together with *Cathaiaphaenops*, *Sinotroglodytes*, *Cimmeritodes* and *Toshiaphaenops* species. However, this semi-aphaenopsian beetle is very different from the other sympatric Trechini, with several peculiar characters such as a very long and tube-like head, strongly dilated femora, very convex pronotum and elytra, and a strange elytral chaetotaxy, in which the humeral group of marginal umbilicate series is composed of five, not four, pores.

In late August 2015, we were invited to undertake a cave biodiversity survey in the Wanhuayan cave system, southern Hunan, as part of the cave exploration activities headed by Prof. Yuanhai Zhang (Institute of Karst Geology, Chinese Academy of Geological Sciences, Guilin). Several specimens of a blind trechine species were collected. This peculiar troglobitic species is probably related to *Shenaphaenops* or even the *Sinaphaenops* complex, but with many different characteristics of generic importance.

In order to properly assess the above trechine species, five new genera are established below, including the proposal of two new combinations for both Shiqian species formerly treated in *Shenaphaenops*, and the description of four new species forming four monotypic genera from limestone caves in Yunnan, Guangxi and Hunan, respectively.

Material and methods

The beetle specimens were collected by using an aspirator inside the cave, and kept in 55% ethanol before study. Dissections and observations were made under a Leica S8AP0 microscope. Dissected genital pieces, including the median lobe and parameres of the aedeagus, were glued onto small transparent plastic plates and pinned under the specimen they belonged to. Habitus pictures were taken by means of a Keyence VHX-5000 digital microscope. Genital pictures were taken using a Canon EOS 40D camera connected to a Zeiss AX10 microscope, and then stacked and processed by means of Adobe Photoshop CS5 software. Distribution maps created using Mapinfo software.

The length of the body was measured from the apex of the right mandible (in open position) to the elytral apex; the width of the body was taken as the maximum width of the elytra.

Abbreviations of other measurements used in the text are as follows:

HLm length of head including mandibles, from apex of right mandible to occipital suture;
HLl length of head excluding mandibles, from front of labrum to occipital suture;
HW maximum width of head;

PrL length of prothorax, along the median line;
PnL length of pronotum, as above;
PrW maximum width of prothorax;
PnW maximum width of pronotum;
PfW width of pronotum at front;
PbW width of pronotum at base;
EL length of elytra, from base of scutellum to elytral apex;
EW maximum width of combined elytra.

Taxonomy

Shiqianaphaenops **Tian, gen. n.**
http://zoobank.org/D7A2BFB8-59A2-4BA4-A26C-630F9E5B7488

Type species. *Shenaphaenops majusculus* Uéno, 1999 (Cave Feng Dong, Shiqian, Guizhou).

Diagnosis. Medium-sized aphaenopsian trechine beetles, with sparsely pubescent body, elongated head, reduced frontal furrows, tridentate right mandible, 4-setose mentum, tumid propleura, widened 1st protarsomere and bisetose on each of abdominal ventrites.

Generic characteristics. Medium-sized aphaenopsian trechines, yet not too highly modified morphologically; eyeless, unpigmented and apterous; body slender and elongate, with slender and long appendages; covered with sparse pubescence, hairs being much longer on head and pronotum than on elytra; head elongate, longer than prothorax, much longer than wide; frontal furrows short, with two pairs of supraorbital pores; right mandible tridentate; labial suture clear; mentum 4-setose, tooth simple and short, blunt at apex; submentum 9-setose; antennae long, nearly extending to elytral apex; prothorax longer than wide, propleura visible from above; pronotum elongate, longer than wide, widest near front; only anterior pairs of lateromarginal setae; elytra elongate-ovate, strongly convex, shoulders distinct and rounded, lateral margins ciliated throughout; striae faintly impressed; two dorsal and the pre-apical pores present on each elytron; humeral group of marginal umbilicate pores not aggregated; protibia without longitudinal groove externally; 1st protarsomere in both sexes widened and angularly produced externally, with a row of comb-like setae on ventral side; 1st and 2nd protarsomeres modified in male; ventrite VII with two pairs of setae in both sexes.

Remarks. Uéno (1999) hesitantly treated the two trechine species found in Shiqian County, eastern Guizhou as members of the genus *Shenaphaenops* Uéno, 1999, which had been set up based on a single female collected in Shuicheng County, northwestern Guizhou. However, he realized some striking differences and the geographical gap between the Shiqian species and the type species *S. humeralis* Uéno, 1999. Apart from the somewhat similar general body configurations in both *Shiqianaphaenops* gen. n. (Fig. 1) and *Shenaphaenops* (Fig. 2), many character states of generic importance are

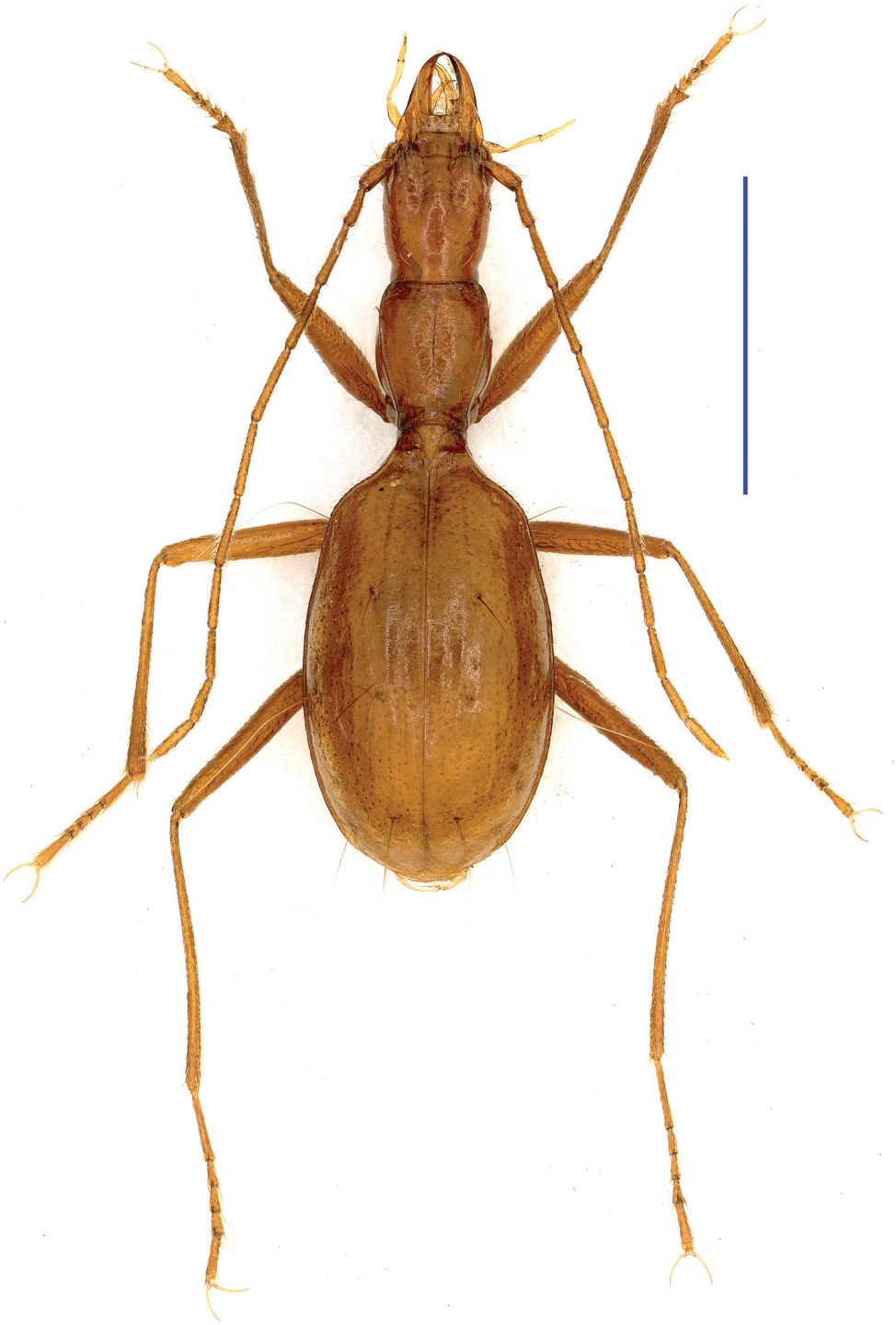

Figure 1. Habitus of *Shiqianaphaenops majusculus* (Uéno, 1999), comb. n., male Scale bar: 2.0 mm.

Figure 2. Habitus of *Shenaphaenops humeralis* Uéno, 1999, male Scale bar: 2.0 mm.

different, such as: (1) 1st protarsomere in both sexes widened apically and distinctly produced externally, covered with a ctenidium structure ventrally in *Shiqianaphaenops* which, amongst trechines, is only found in this genus; (2) right mandible tridentate in *Shiqianaphaenops*, versus bidentate in *Shenaphaenops*; (3) labial suture clear in *Shiqianaphaenops*, but completely missing in *Shenaphaenops*; (4) ventrite VII in male bisetose in *Shiqianaphaenops*, versus 4-setose in *Shenaphaenops*; (5) in male, both 1st and 2nd protarsomeres modified in *Shiqianaphaenops*, versus only 1st protarsomere being modified in *Shenaphaenops*; (6) pubescence weaker and sparser, hairs much longer on head and pronotum than on elytra in *Shiqianaphaenops*, versus generally denser and hairs being of same length in *Shenaphaenops*; (7) base of pronotum much narrower than front in *Shiqianaphaenops*, versus only slightly narrower in *Shenaphaenops*; (8) elytra broader, with humeral angles rounded in *Shiqianaphaenops* (Fig. 3A), versus more slender, with humeral angles distinctly angular in *Shenaphaenops* (Fig. 3B); (9) marginal sides of elytra ciliate throughout, versus smooth in *Shenaphaenops*; (10) in *Shiqianaphaenops*, median lobe of aedeagus stout and thick, broadly rounded dorsally, basal part comparatively small, with an indistinct sagittal aileron, inner sac armed with a very large copulatory piece; apical lobe contracted at apex, roundly blunt in lateral view, but pointed in dorsal view; parameres thin, much shorter than median lobe, each bearing three long setae at apex (Fig. 4A, B). In contrast, median lobe of aedeagus in *Shenaphaenops* slender and thin, gently expanded dorsally, basal part comparatively large, with a small but distinct sagittal aileron; apical lobe indistinctly contracted at apex, obtuse in lateral view, broad in dorsal view; inner sac armed with a large copulatory piece; parameres shorter than median lobe, left one slightly longer than right one, each bearing two long setae at apex (Fig. 4C, D).

Etymology. Shiqian+Aphaenops, to indicate that both known members of this genus occur in Shiqian County, eastern Guizhou Province. Gender masculine.

Range. China (eastern Guizhou) (Fig. 5c).

Known so far by two very similar species from two caves in Shiqian County: Shenxian Dong and Feng Dong. Since Shenxian Dong lies very close to Feng Dong, only about one kilometre in distance across a shallow valley, with still another cave just between them, all three caves may prove belong to a same cave system. Perhaps this is why both Uéno's species from Shiqian County are not too different from each other. Feng Dong is a large and beautiful cave (Fig. 6A, B). The beetle (Fig. 6C) is sympatric with millipedes, crickets and frogs.

Material examined. *Shenaphaenops humeralis* Uéno, 1999: a male, Cave Shendong Migong (Shendong in the original description), Muqiao, Laoyingshan, Shuicheng County, northwestern Guizhou, 26°35'15"N/ 104°59'47"E, 1910 m in altitude, VIII-22-2014, Mingyi Tian leg., deposited in the insect collections of South China Agricultural University (SCAU).

Shiqianaphaenops majusculus (Uéno, 1999), comb. n.: 3 males, V-1-2015, Cave Feng Dong, Tangshan, Shiqian County, 27°29'10"N/108°15'23"E, 700 m in altitude, Mingyi Tian & Jingli Cheng leg., in SCAU; 6 males & 4 females, ibid., VIII-1-2015, same collectors, in SCAU.

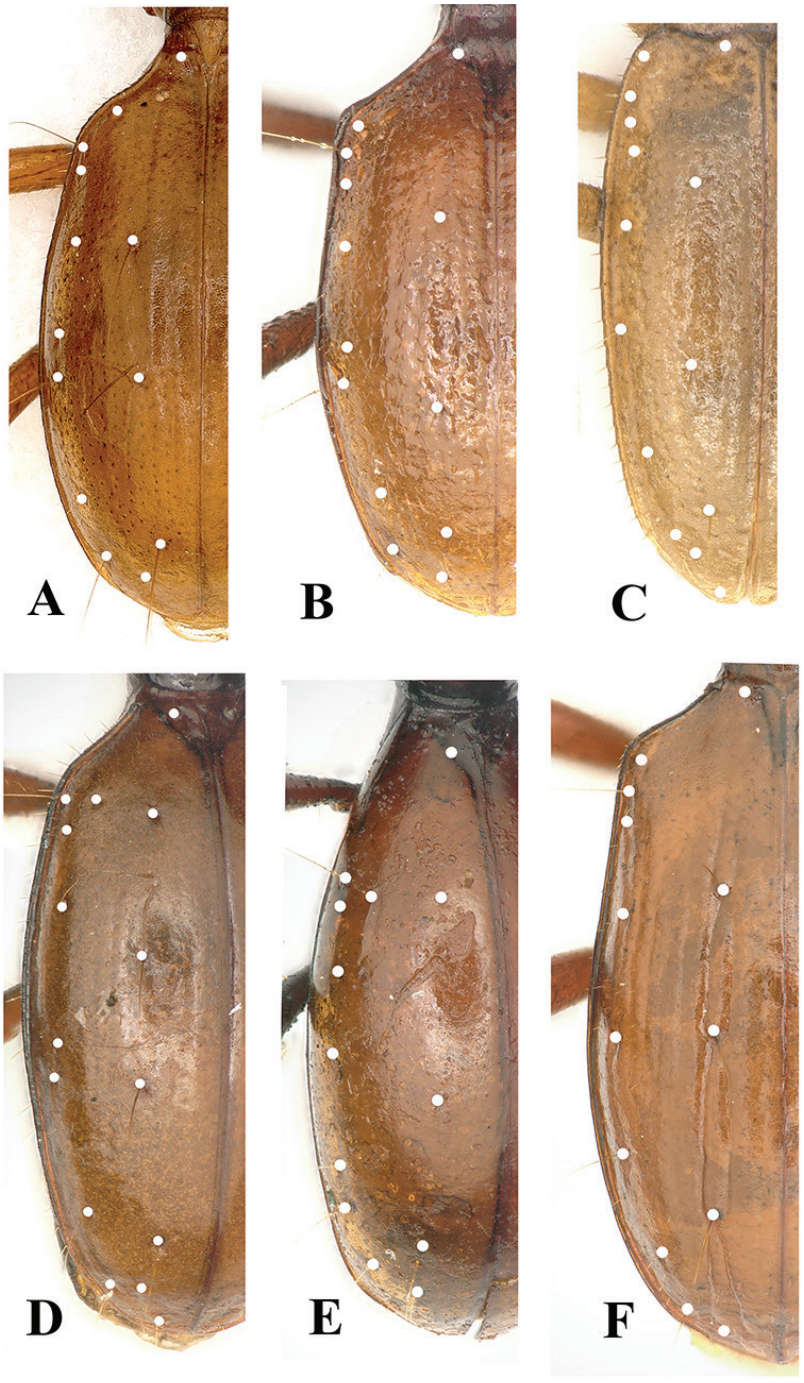

Figure 3. Elytral chaetotaxy **A** *Shiqianaphaenops majusculus* (Uéno, 1999), comb. n. **B** *Shenaphaenops humeralis* Uéno, 1999 **C** *Dianotrechus gueorguievi* Tian, gen. n., sp. n. **D** *Tianeotrechus trisetosus* Tian & Tang, gen. n., sp. n. **E** *Huoyanodytes tujiaphilus* Tian & Huang, gen. n., n. sp. **F** *Huoyanodytes tujiaphilus* Tian & Huang, gen. n., sp. n. **G** *Wanhuaphaenops zhangi* Tian & Wang, sp. n.

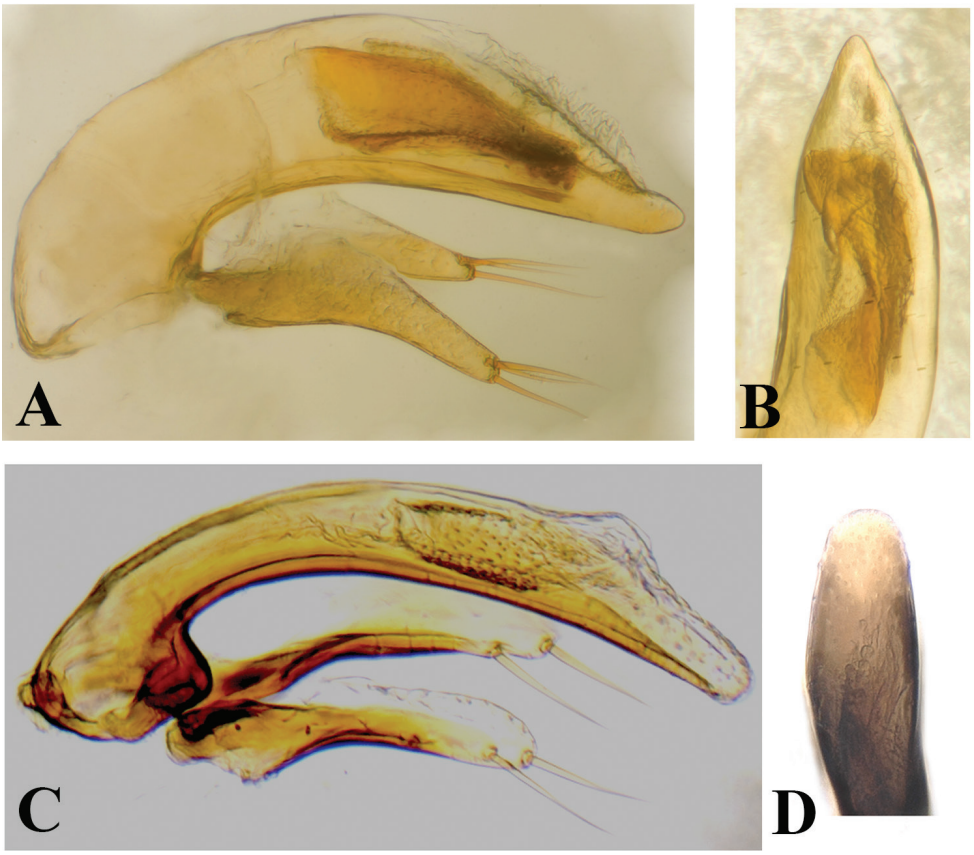

Figure 4. Male genitalia **A** and **C** median lobe, lateral view **B** and **D** apical lobe, dorsal view **A** and **B** *Shiqianaphaenops majusculus* (Uéno, 1999), comb. n. **C** and **D** *Shenaphaenops humeralis* Uéno, 1999.

Shiqianaphaenops cursor (Uéno, 1999), comb. n.: We have not seen any material. We visited Cave Shenxian Dong, the type locality, in August 2015, but failed to catch anything. The cave is badly disturbed by human activities.

***Dianotrechus* Tian, gen. n.**
http://zoobank.org/844915FD-7B9B-421B-8143-0ACA207CEF54

Type species. *Dianotrechus gueorguievi* Tian, sp. n. (Cave Dashi Dong, Kunming, Yunnan).

Diagnosis. Small-sized and anophthalmic trechine beetles, with robust head, complete frontal furrows, bidentate right mandible, 4-setose mentum, fused mentum and submentum, short appendages, quadrate pronotum, widely spaced middle pores of the marginal umbilicate setiferious series and 6-setose ventrite VII in female.

Generic characteristics. Small-sized, anophthalmic trechine; unpigmented and apterous; head robust, genae strongly convex laterally; frontal furrows entire, two

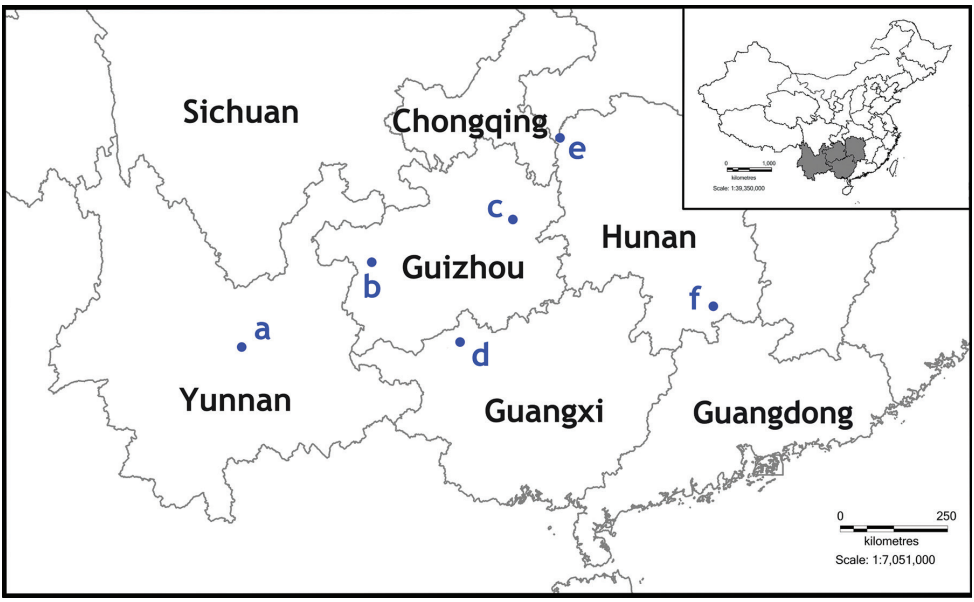

Figure 5. Distribution of the trechine beetles **a** *Dianotrechus gueorguievi* Tian, gen. n., sp. n. **b** *Shenaphaenops humeralis* Uéno, 1999 **c** *Shiqianaphaenops majusculus* (Uéno, 1999), comb. n. **d** *Tianeotrechus trisetosus* Tian & Tang, gen. n., sp. n. **e** *Huoyanodytes tujiaphilus* Tian & Huang, sp. n. **f** *Wanhuaphaenops zhangi* Tian & Wang, gen. n., sp. n.

pairs of supra-orbital pores; right mandible bidentate; mentum and submentum completely fused; submentum 7-setose, a shorter seta present in the middle; mentum 4-setose; antennae short and stout, extending to basal third of elytra; prothorax with propleura invisible from above; pronotum quadrate, two pairs of laterodorsal setae present; elytra elongate-ovate, moderately convex, without prehumeral angles, lateral margins gently expanded, ciliated throughout; punctate-striate, two dorsal and the pre-apical pores present, apical stria present, humeral group of marginal umbilicate pores not aggregated, 5th and 6th pores of middle group widely separated, 5th pore strikingly shifted forward, much closer to 4th than to 6th; scutellum small; legs short and stout; protibia without longitudinal groove externally; ventrite VII in female with three pairs of setae.

Remarks. The most striking peculiarities of this new genus lie in the conformation of the pronotum and the chaetotaxal pattern on the elytra, especially the middle group of umbilicate pores, in which the 5th pore is much closer to the 4th than to the 6th. *Dianotrechus* gen. n. seems to be particularly close to *Shilinotrechus* Uéno, 2003, an anophthalmic trechine genus also recorded from eastern Yunnan, but the former genus differs from the latter by the following character states: small-sized (versus medium-sized in *Shilinotrechus*); right mandible bidentate (versus tridentate in *Shilinotrechus*); body shape nearly parallel-sided (versus fusiform in *Shilinotrechus*); head of anophthalmic type (versus aphaenopsian type in *Shilinotrechus*); ventrite VII with three pairs

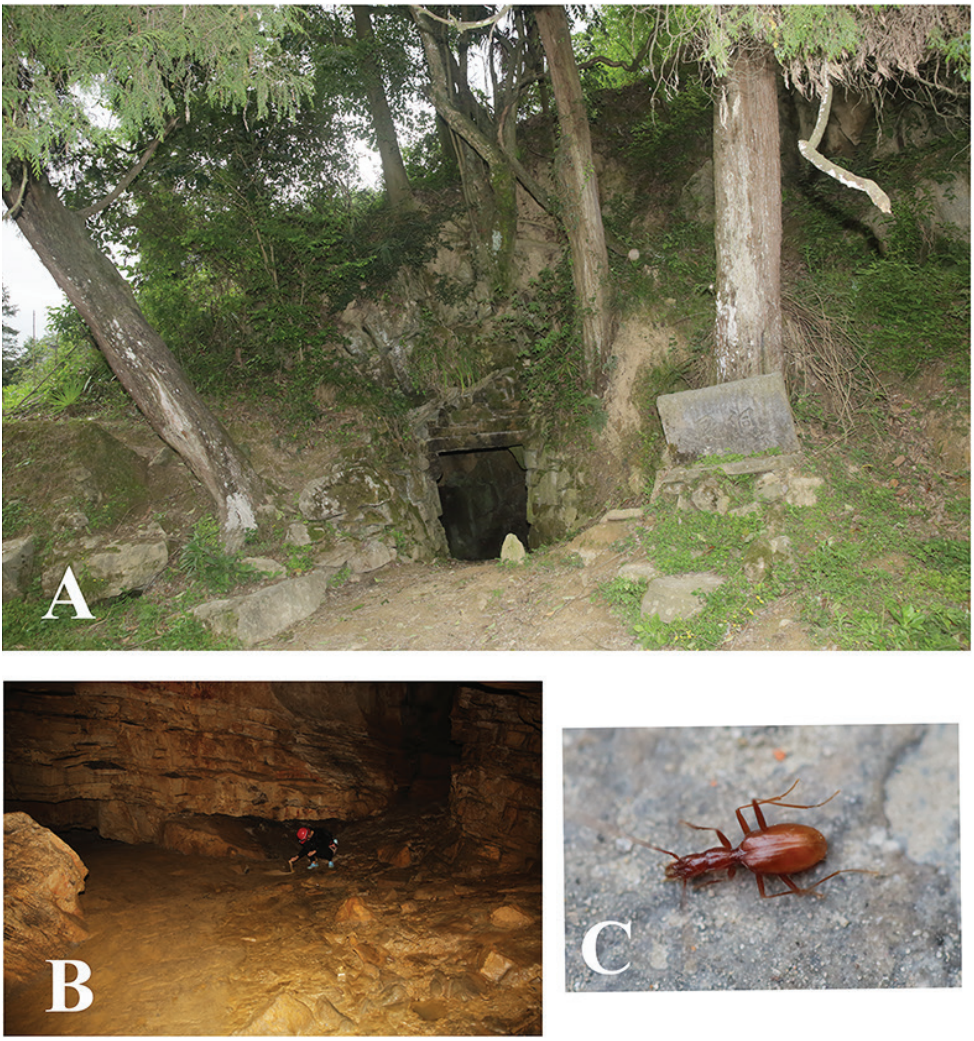

Figure 6. Cave Feng Dong, type locality of *Shiqianaphaenops majusculus* (Uéno, 1999), comb. n. **A** entrance **B** cave chamber, to show where the beetles were collected **C** a wandering individual in cave.

of setae in female (versus two pairs in *Shilinotrechus*). Some more differences are also evident in the conformation of the pronotum and elytra, as well as the chaetotaxy pattern of the marginal umbilicate series.

Compared to *Cimmeritodes* Deuve, 1996, a small-sized trechine genus originally reported from the Huoyan Karst of Longshan County, northwestern Hunan Province, but also occurring in Zhejiang (Deuve and Tian 2015), *Dianotrechus* gen. n. is easily distinguished by the bidentate right mandible (versus tridentate in *Cimmeritodes*), the quadrate pronotum (versus cordate in *Cimmeritodes*), the chaetotaxy pattern of the marginal umbilicate series, in which the 5th is more distant from the 6th than from the

4th (reverse in *Cimmeritodes*), and ventrite VII has three pairs of setae in the female (two pairs in *Cimmeritodes*).

Etymology. Dian + Trechus, "Dian" is a short name for Yunnan Province in Chinese. The name of the new genus reflects the occurrence of this cavernicolous trechine in Yunnan. Gender masculine.

Range. China (eastern Yunnan) (Fig. 5a).

***Dianotrechus gueorguievi* Tian, sp. n.**
http://zoobank.org/1618C05C-2922-4288-94C0-AF26A23054B2

Holotype. Female, labeled "China, Yunnan Province, Ermu Vill., Kunming District, Dashi Dong (Big Rock Cave), 24°49'13"N/102°27'56"E, 1940 m in altitude, XI-8-2011, B. Petrov leg.", in SCAU.

Diagnosis. A small, stout, yellowish brown beetle which is densely pubescent, with short fore body and appendages, convex head, not tumid propleura which invisible from above, rather flat elytra and coarsely punctate elytral striae.

Description. Length: 3.1 mm (including mandibles), width: 0.9 mm. Habitus as in Fig. 7

Whole body yellowish brown, with palps pale; head and pronotum shiny, elytra dim; frons and vertex glabrous, genae with several short hairs, pronotum with a few fine setae, whole elytra covered with erect setae, these being as long as those on genae; underside generally glabrous, smooth and polished, but a few short hairs present on ventrites II and IV, and in lateral areas of prosternum; microsculptural meshes vanishing on head and pronotum, densely and moderately engraved on elytra. Fore part of body much shorter than elytra, EL/(HLm+PnL) = 1.39.

Head short and stout, much longer than wide (including mandibles), HLm/HW = 1.44, or as long as wide (excluding mandibles), genae broadly convex, frons and vertex moderately convex, frontal furrows entire, strongly divergent backwards; both supra-orbital pores closely located, posterior ones almost on frontal furrows, distance between anterior and posterior pores shorter than that between supra-orbital furrows at the closest point, neck short and broad; clypeus 4-setose, labrum transverse, nearly straight at frontal margin, 6-setose; mandibles rather short; labial suture missing; mentum 4-setose (two laterally and two at base of mental tooth); mentum tooth simple, very short, broad at apex; basal emargination wide and rather deep; ligula small and short, adnated to paraglossae, widened at apex, 6-setose; palps stout and short, penultimate joints much stouter than apical ones; 3rd maxillary palpomere slightly longer than 4th, labial palpomere 2nd distinctly longer than 3rd, bisetose at inner margin, with two additional setae in outer apical parts; 3rd maxillary palpomere with two tiny setae near apex; suborbital pores present, located in median portion of ventral genae, lying far from base of head; antennae short and stout, wholly pubescent, 1st antennomere stouter than others, 1st, 2nd and 4th-10th subequal in length, 3rd slightly longer than 2nd, but slightly shorter than 11th.

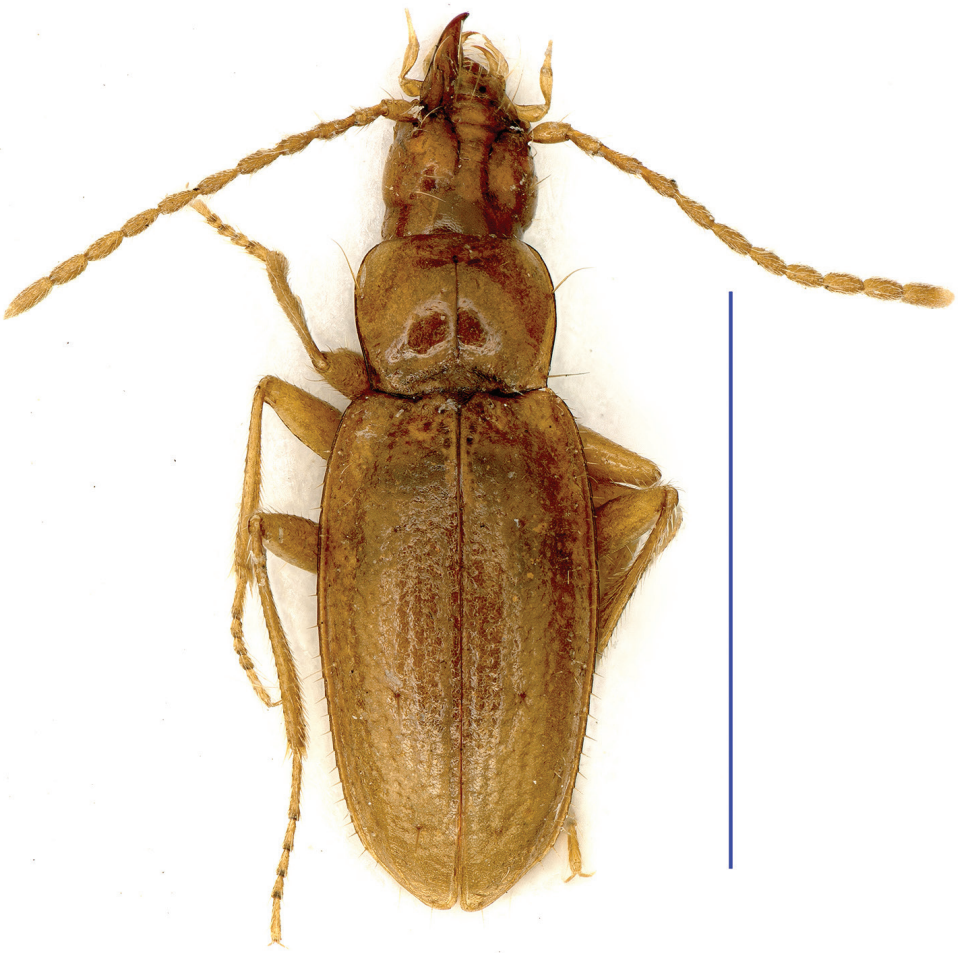

Figure 7. Habitus of *Dianotrechus gueorguievi* Tian, gen. n., sp. n., holotype, female Scale bar: 2.0 mm.

Prothorax: propleura not tumid, invisible from above; pronotum transverse, PnL/PnW = 0.84, wider than head, PnW/HW = 1.27, much shorter than head (including mandibles), HLm/PnL = 1.44, or as long as head (excluding mandibles); disc moderately convex; widest at about middle where lateral sides slightly expanded but remaining nearly parallel-sided, reflexed near hind angles; fore lateromarginal seta located at a little before middle, basal one a little before hind angle; fore angles rounded, basal ones rectangular and pointed; base as wide as front; front almost straight, base nearly straight medially, obtusely sinuate near hind angles; median line fine and well-defined, reaching front margin, but ending before basal transverse impression, the latter being distinctly marked and connected to basal foveae; front transverse impression unclear. Scutellum small and short.

Elytra elongate, slender, moderately convex, wider than pronotum, almost twice as long as wide, EL/EW =1.93, widest at about middle of elytra, gently narrowed towards

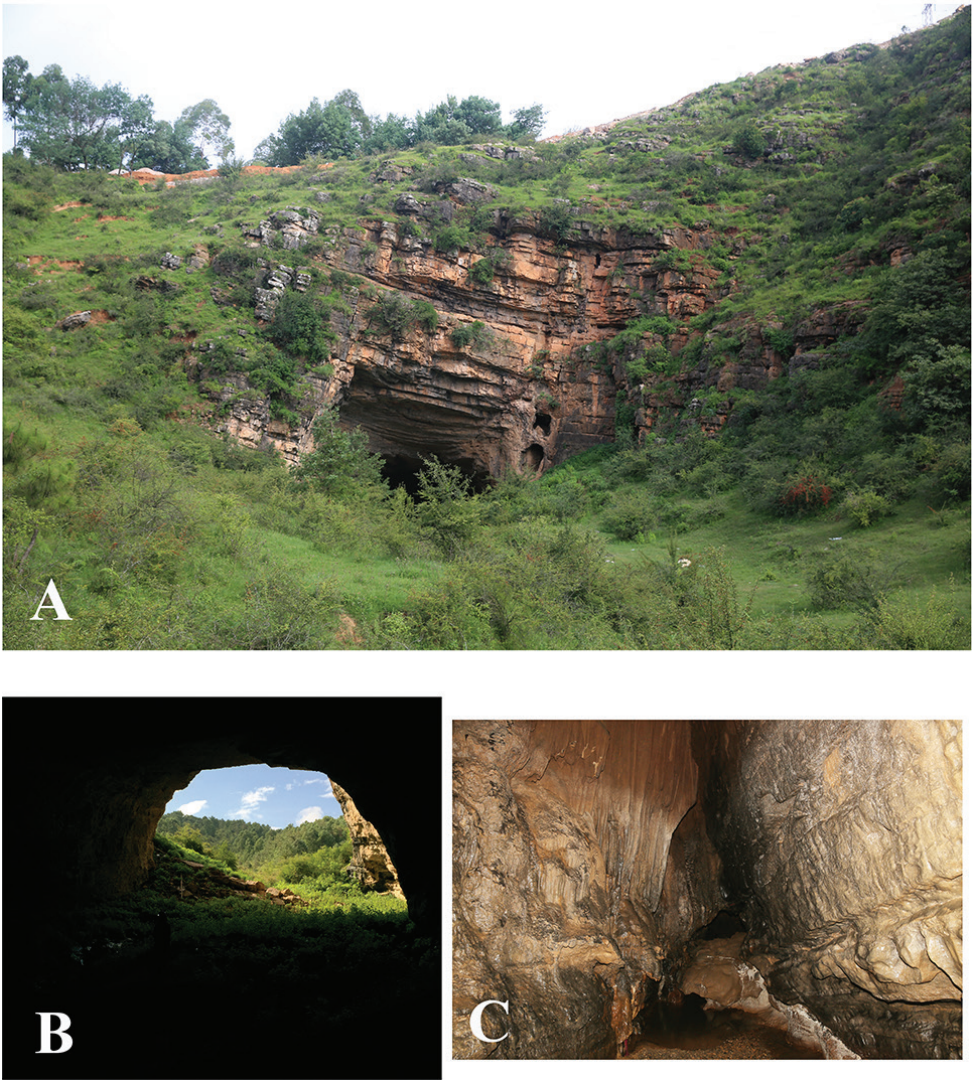

Figure 8. Cave Dashi Dong, type locality of *Dianotrechus gueorguievi* Tian, gen. n., sp. n. **A** entrance, outside view **B** entrance, inside view **C** main passage.

base and subapex; base wide, shoulders rounded, prehumeral angles missing; apex of each elytron rounded; disc moderately convex, striae coarsely punctate, intervals slightly convex; 1st-4th striae and apical striae well-marked, 1st-3rd striae complete, 4th finished at level before median dorsal pore; other striae wanting; basal pore present, lying near basal margin and on side of scutellum; both dorsal pores located on 4th intervals, at about basal third and a little behind the middle of elytra, respectively, pre-apical pore located at apical fusion of 2nd and 3rd striae, level to ending point of apical stria, about twice as far from apex as from suture; marginal umbilicate series with 1st, 2nd, 6th and

apical pores close to marginal gutter, 2nd-4th pores equidistant, but 1st more isolated; 5th pore widely removed away from 6th and closer to 4th pore.

Legs moderately long, covered with dense and short hairs; protarsi short, 1st tarsomere not distinctly wider than others, longer than 2nd and 3rd combined, but shorter than 2nd-4th combined; meso- and metatarsi longer, 1st tarsomere as long as 2nd-4th combined, respectively. Ventrites IV-VI each with a pair of paramedian setae, ventrite VII in female with three pairs of setae.

Male: Unknown.

Etymology. In honour of Dr. Borislav V. Guéorguiev (National Museum of Natural History, Sofia, Bulgaria), an expert in Carabidae.

Distribution. China (Yunnan) (Fig. 5a). Known only from the limestone Cave Dashi Dong in a western suburb of Kunming City (Fig. 8A–C).

Dashi Dong is located more than 1 km away from Ermu Village, Xianjie Zhen, Anning, Kunming. The opening of this cave is 27 m wide and 17 m high. Its total length is 1394 m and the total depth is 39.30 m. The temperature in the dark parts is 21 °C. The unique beetle was collected in the dark area. In order to find more specimens of this interesting beetle, we visited this cave three times in July 2014, July 2015 and August 2015, but of no avail.

Tianeotrechus Tian & Tang, gen. n.

http://zoobank.org/49A4C222-27E7-4A48-8A0D-42C391FF432C

Type species. *Tianeotrechus trisetosus* Tian & Tang, sp. n. (Bahao Dong, Tian'e County, Guangxi).

Diagnosis. Medium-sized cave beetles, with typical aphaenopsian head, reduced frontal furrows, quadridentate right mandible, evident labial suture, bisetose mentum, robust pronotum, invisible propleura from above though which is tumid, and strongly covex elytra which have three pairs of dorsal setiferious pores.

Generic characteristics. Medium-sized and semi-aphaenopsian trechines, eyeless, unpigmented and apterous; head evidently aphaenopsian, with incomplete frontal furrows and a somewhat elongated head, with two pairs of supra-orbital pores; mandibles developed, right mandible quadridentate, molar and retinacular teeth more developed than premolar tooth which is bifid (Fig. 9A); mentum and submentum well separated by labial suture; mentum bisetose, distinctly concave, mental tooth short and thick, bifid apically; submentum provided with a row of seven setae, median one minute and much shorter than others; antennae fairly short, reaching a little beyond middle of elytra; pronotum robust, longer than wide, sides expanded at apical third, making lateral suture invisible from above; posterior lateromarginal setae absent; elytra strongly convex, nearly as long as fore body (including mandibles), humeral shoulders roundly angulate, lateral sides smooth; striae reduced but more or less traceable; three dorsal and the pre-apical setae present; marginal umbilicate series not aggregate, only 2nd pore close to marginal gutter; 1st pore of humeral group shifted backward and about level

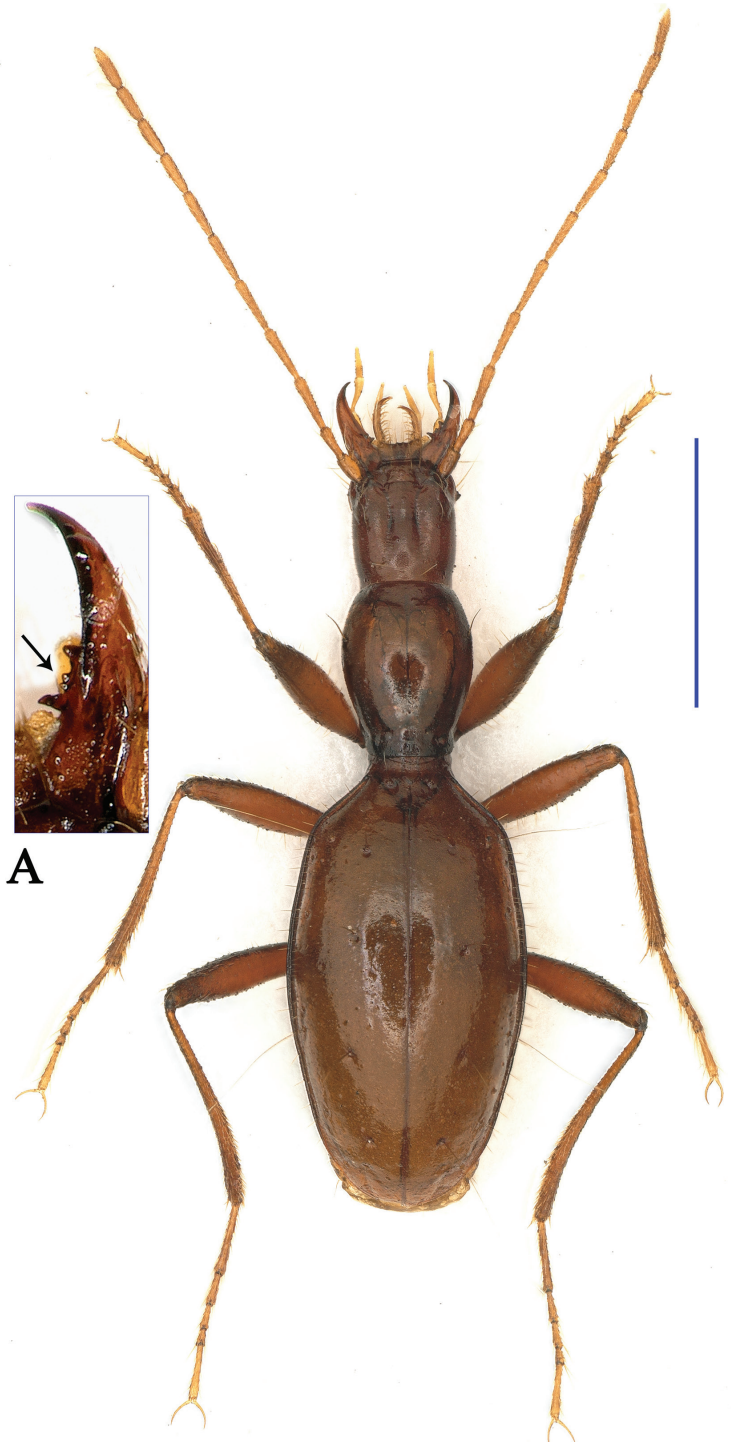

Figure 9. Habitus of *Tianeotrechus trisetosus* Tian & Tang, gen. n., sp. n., holotype, male Scale bar: 2.0 mm, **A** enlarged right mandible to show the quadrisetose teeth

to 5th stria, a little behind 2nd; 4th distant from 3rd; both pores of middle group lying close to each other; legs moderate for cave trechines, tibiae without longitudinal furrows externally; protarsi in male not modified; ventrite VII bisetose in male, 4-setose in female; male genitalia minute, well-sclerotized, moderately curved in middle part, apex slightly raised and pointed in lateral view; basal part quite large, sagittal aileron present; inner sac armed with a thin and scale-covered copulatory piece; parameres large but short, each bearing three long setae at apex.

Remarks. It is not easy to determine the taxonomic position of this new genus. Several generically important characters of *Tianeotrechus* gen. n. do not exist in the other genera of Chinese Trechini: a quadridentate right mandible, invisible pronotal lateral borders and the presence of three dorsal pores on each elytron. We hope more discoveries in the near future will be able to shed additional light to clarify this problem.

Etymology. Tian'e + Trechus, in reference to the provenance of the type species from Tian'e County. Gender masculine.

Range. China (northern Guangxi) (Fig. 5d).

Tianeotrechus trisetosus Tian & Tang, sp. n.

http://zoobank.org/CDEDE83E-8CC2-4BAE-A706-45020D2D6265

Holotype. Male, Cave Bahao Dong, Gandong Village, Bala Xiang, Tian'e County, northern Guangxi, 24°55'57.10"N/107°02'40.80"E, 686 m in altitude, VIII-7-2015, Mingruo Tang leg., in SCAU; paratypes: 4 males and 1 female, ibid., in SCAU.

Diagnosis. A medium-sized trechine, with shiny and robust body, moderated appendages, convex pronotum and elytra, and elongated elytra which have round shoulders and reduced striae.

Description. Length: 5.6–5.7 mm (mean 5.66), width: 1.6 mm. Habitus as in Fig. 9.

Body brownish red, with palps, antennae and tarsi pale; frons, vertex and underside of head, pronotum, inner part of elytra glabrous, propleura and prosternum glabrous; genae, lateral parts of elytra, meso- and metasterna, and visible ventrites clothed with short pubescence; legs densely pubescent, covered with longer setae; microsculptural engraved meshes moderately transverse on head, strongly striate on pronotum and elytra.

Head elongate, much longer than wide, HLm/HW = 1.78–1.80 (mean 1.79), HLl/HW = 1.29–1.34 (mean 1.32); frons and vertex moderately convex; frontal furrows fairly long but incomplete, nearly parallel-sided, albeit slightly divergent posteriad, ending near neck constriction; genae barely expanded laterally, both sides held almost parallel; anterior and posterior supra-orbital pores located in the middle of genae and near neck constriction, respectively, distance between anterior and posterior pores much less than that between anterior pores; clypeus 4-setose, labrum transverse, widely but shallowly emarginated at front margin, 6-setose; mandibles distinctly curved at apex; palps thin and moderately long, 3rd and 4th maxillary pal-

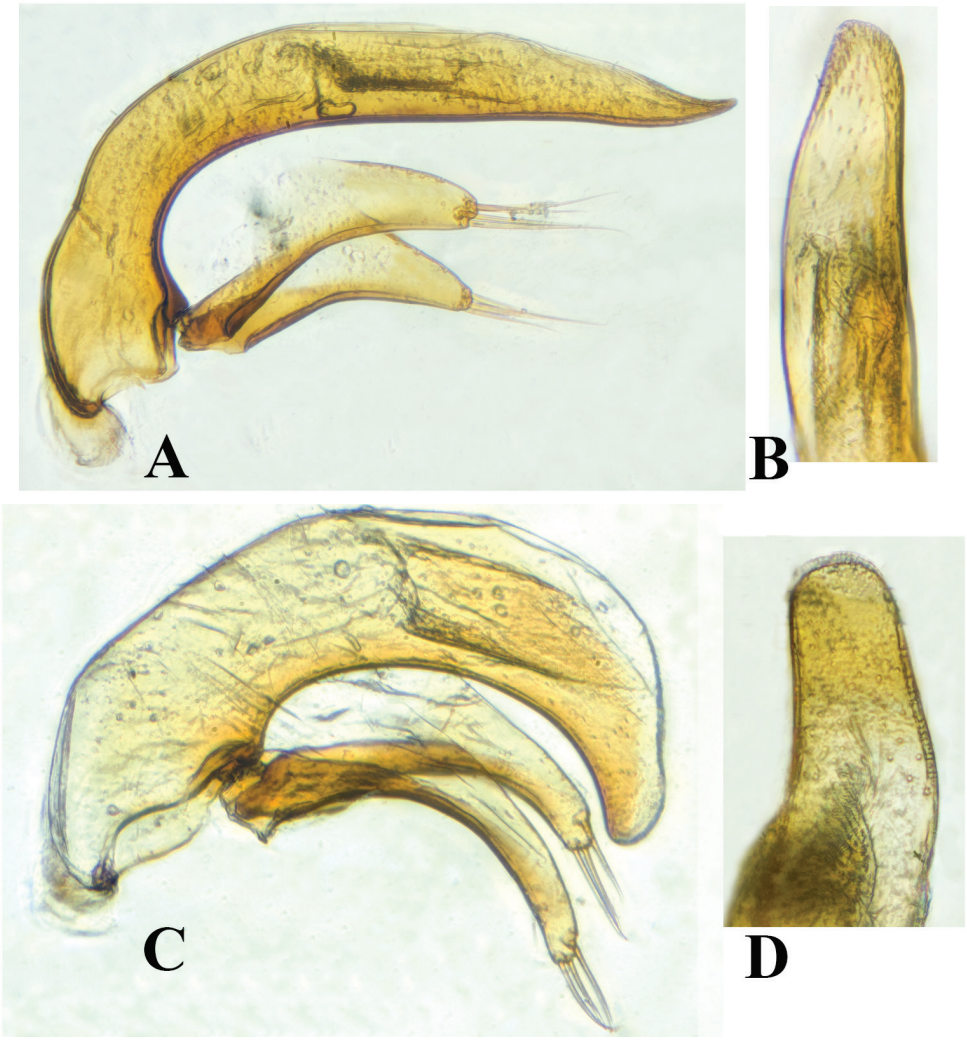

Figure 10. Male genitalia **A** and **C** median lobe, lateral view **B** and **D** apical lobe, dorsal view **A** and **B** *Tianeotrechus trisetosus* Tian & Tang, gen. n., sp. n. **C** and **D** *Wanhuaphaenops zhangi* Tian & Wang, gen. n., sp. n.

pomeres, and 3rd labial palpomere glabrous, 2nd labial palpomere 4-setose; penultimate palpomere evidently longer than apical one of labium, slightly longer in maxilla; suborbital pores on ventral side of genae, located closer to submentum than to base of head; antennae extending to about apical 3/4 of elytra, pubescent from 2nd in apical half; 1st antennomere stout and bearing several setae, slightly shorter than 2nd; 3rd 1.6 times longer than 2nd; 3rd, 4th and 5th subequal in length, then gradually shortened from 6th to 10th; 11th as long as 9th.

Prothorax expanded due to propleura, but concealed dorsally by pronotum, the latter being more strongly tumid laterally; pronotum longer than wide, PnL/PnW = 1.22-1.32 (mean 1.27); shorter than head with mandibles, PnL/HLm = 0.89-0.94 (mean 0.91); wider than head, PnW/HW = 1.24-1.32 (mean 1.28); base narrower than front, PbW/PfW = 0.885-0.894 (mean 0.889), both nearly straight and unbeaded; widest before middle, lateral margins of pronotum invisible from above; anterior lateromarginal pores present, located at about apical third; middle line fine; frontal impression faint, depressed medially, basal transverse sulcus well-marked; disc strongly convex. Scutellum small and short.

Elytra elongate ovate, much longer than wide, EL/EW = 1.72-1.75 (mean 1.74); as long as head (including mandibles) plus pronotum; much wider than pronotum, EW/PnW = 1.80-1.88 (mean 1.83); widest at about 3/7^{ths} from base; prehumeral part short, humeral angles rounded; lateral sides smooth and well-beaded, ciliated throughout; disc strongly convex except for a small area near base just behind scutellum, the latter being somewhat depressed; striae more or less obliterated but traceable, intervals slightly convex; base not bordered; basal pores on either side of scutellum, close to basal margin; three dorsal pores present on 3rd stria, located at about 1/6th, 2/5^{ths}, 2/3^{rds} and 5/6^{ths} of elytra from base, respectively; pre-apical pore lying at about 5/6^{ths} of elytra, at site of junction of 2nd, 3rd and 4th striae, much closer to elytral suture than to apical margin; humeral group of marginal umbilicate pores not aggregated, 1st, 2nd and 3rd pores forming an equilateral triangle, 4th widely distant from other three, 2nd close to, 4th far from, marginal gutter; middle group located behind middle of elytra, close to each other, distant from marginal gutter; apical pore minute, placed near elytral apex.

Legs moderately long, tibiae not longitudinally furrowed, hind tibia as long as elytral width; protarsi short; 1st tarsomere shorter than, or subequal to, or longer than 2nd-4th tarsomeres combined in pro-, meso- and metatarsi, respectively; 4th tarsomere wider than long in fore leg, as long as wide in middle leg, and evidently longer than wide in hind leg. Ventrites IV-VII each with one pair of setae.

Male genitalia (Fig. 10A, B): The median lobe of aedeagus well-sclerotized, small and slender, moderately curved ventrally in middle part, pointed at apex in lateral view; in dorsal view, apical lobe roundly broad at apex, nearly parallel-sided; base widely opening, with a large and thick sagittal aileron; parameres broad, much shorter than median lobe.

Etymology. To refer to the presence of three dorsal pores on elytron.

Distribution. China (Guangxi) (Fig. 5d). Known so far from the limestone Cave Bahao Dong, southern Tian'e County (Fig. 11A).

The cave opens below a hill, surrounded by trees and bushes and is invisible from outside. The entrance is large, but the length remains unknown. It is deep and hardly accessible, accumulated by random ripraps; it takes the cavers about an hour to reach the underground river which runs through the deepest part of the cave. All of the type series were collected under stone in twilight and transition zones, thirty to fifty meters from the entrance. It is sympatric with *Dongodytes giraffa* Uéno, 2005 (Fig. 11B, C).

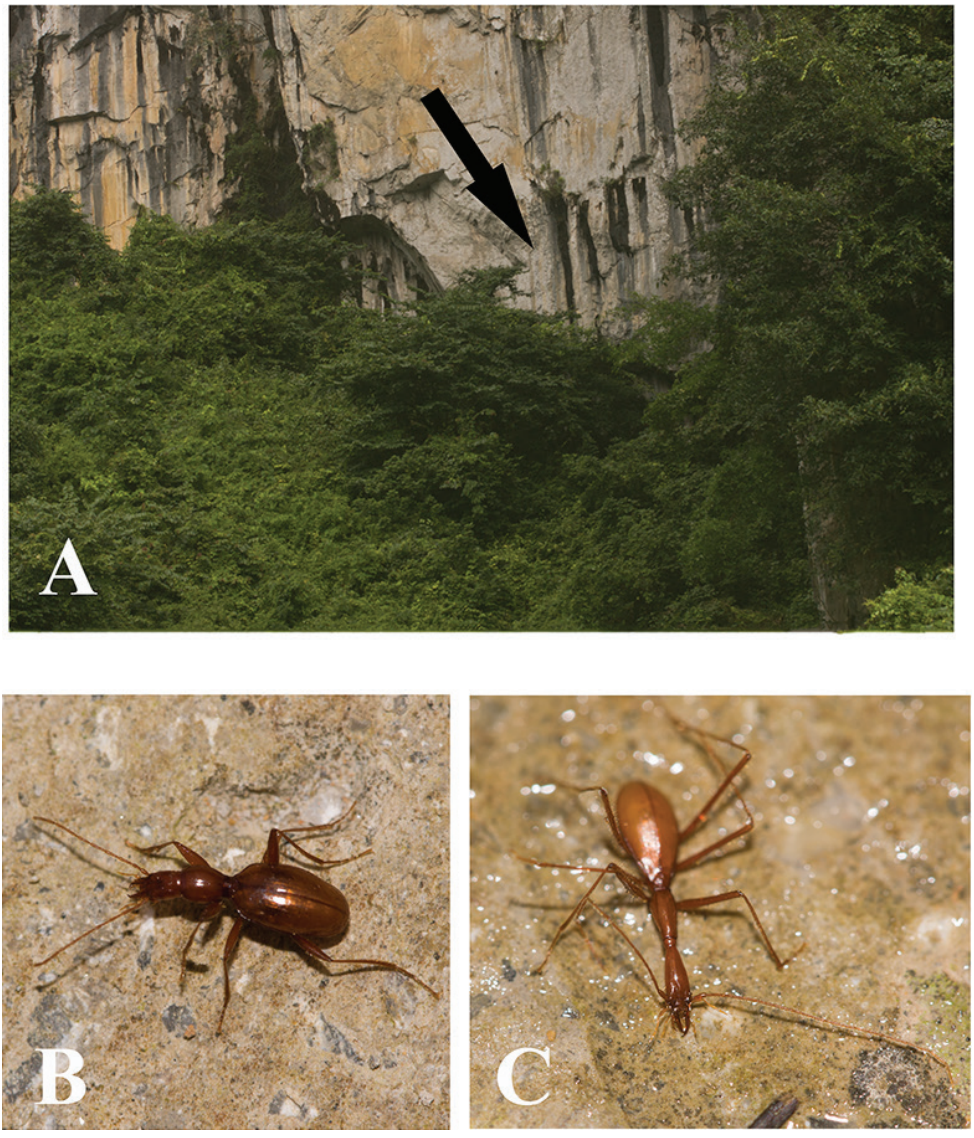

Figure 11. Cave Bahao Dong, type locality of *Tianeotrechus trisetosus* Tian & Tang, gen. n., sp. n. **A** outside cave, arrowhead shows the entrance **B** a wandering individual of *Tianeotrechus trisetosus* in cave **C** a wandering individual of *Dongodytes giraffa* Uéno, 2005, in cave.

Huoyanodytes Tian & Huang, gen. n.

http://zoobank.org/1A50BCBA-6A05-4D77-8E78-3EC6C157CAC6

Type species. *Huoyanodytes tujiaphilus* Tian & Huang, sp. n. (Cave Tujiamei Dong, Longshan, Hunan)

Diagnosis. Large-sized, semi-aphaenopsian beetles, with elongated and tube-like head, long fore body, bidentate right mandible, bisetose mentum, well defined labial suture, tubiform and tumid prothorax, five pores in the humeral group of the marginal umbilicate series and disappeared elytral striae.

Generic characteristics. Large-sized, semi-aphaenopsian trechine, eyeless, unpigmented and apterous; fore body longer than elytra; head tube-like, parallel-sided, without neck constriction; much longer than wide, head (including mandible) as long as prothorax; mandible elongate, right mandible bidentate; ligula multisetose; submentum 10-setose, mentum bisetose, each of abdominal ventrites IV-VII 4-setose; labial suture clear, well separating mentum and submentum; frontal furrows short, subparallel-sided, two pairs of supra-orbital pores present; antennae long, extending to a little before elytral apex; prothorax elongate, somewhat tubiform, propleura distinctly tumid and thus visible from above; both fore and hind pronotal angles obtusely rounded; elytra ovate, strongly convex, making marginal side partly concealed and invisible from above; humeral angles rounded, widest at about middle, striae completely missing; apex broadly rounded; two dorsal and the pre-apical pores present on each elytron, humeral group of umbilicate marginal pores composed of five pores, middle group backwardly located, at about apical third of elytra; femora more dilated near subapex; tibiae long and slender, without longitudinal grooves externally.

Remarks. Again, the affinities of *Huoyanodytes* gen. n. are bound to remain obscure. Its tube-like head, the more dilated subapically femora, the very convex pronotum and elytra, and the peculiar elytral chaetotaxy are the apomorphies that make it unrelated to any other genera so far known in China. It must be pointed out that it is the first example in a trechine beetle which humeral group of umbilicate marginal pores as composed of five pores, instead of four.

Etymology. Huoyan+dytes, to refer to this genus occurring in Huoyan Karst. Gender masculine.

Range. China (northwestern Hunan) (Fig. 5e).

***Huoyanodytes tujiaphilus* Tian & Huang, sp. n.**
http://zoobank.org/C0AA3DF1-1854-4934-988E-B0A89C0E3B7C

Holotype. female, Cave Tujiamei Dong, Huoyan Karst, Huoyan Xiang, Wulongshan Geopark, Longshan County, NW Hunan Province, China, 29°12'20.11"N/109°18'37.21"E, 427 m in altitude, VII-3-2014, leg. Mingyi Tian, Weixin Liu, Haomin Yin, Sunbin Huang & Xinhui Wang, deposited in SCAU.

Diagnosis. A large cavernicolous beetle, with light dark brown fore body, light brown elytra, tubiform head and prothorax, strongly convex elytra and 4-setose on each of visible abdominal ventrites.

Description. Length: 7.0 mm including mandibles, width: 2.0 mm. Habitus as in Fig. 12.

Figure 12. Habitus of *Huoyanodytes tujiaphilus* Tian & Huang, gen. n., sp. n., holotype, female. Scale bar: 2.0 mm.

Head, pronotum legs excluding tarsi, antennomeres 1-2 light dark brown, elytra, antennomeres 3-11 light brown, palps pale; upper- and underside of head, pro-, meso- and metasterna sparsely covered with rather long setae; elytra glabrous; pronotum with two

short hairs in middle portion along mid suture; microsculptural engraved meshes moderately transverse on head, vanishing on pronotum, and strongly transverse on elytra.

Body quite large sized, rather stout, head (including mandibles) plus pronotum slightly longer than elytra, (HLm+PnL)/EL = 1.03.

Head evenly slender, much longer than wide, HLm/HW = 2.90, or HLl/HW = 2.08, genae well-developed and elongated, making head tube-like, nearly parallel-sided; frons, vertex and genae moderately convex; frontal furrows wide and deep, but short, ending at about middle of head from labrum, almost parallel to each other; anterior supra-orbital pores located at about basal 4/7th of head, lateral to frontal furrow and a little before its ending points, posterior ones located at about basal 1/5th of head excluding mandibles; distance between anterior pores as great as that between anterior and posterior pores of each side; clypeus 8-setose; labrum strongly transverse, straight at frontal margin, 6-setose; mandibles long and thin, gently incurved in apical half and distinctly unciform at apex; labial suture clear; mentum widely and deeply concave at base, bisetose, mental tooth simple, blunt at apex; submentum 10-setose; ligula 10-setose, setae being short; palps elongated, slender and subcylindrical, 3rd maxillary palpomere longer than 4th, both glabrous; 2nd labial palpomere longer than 3rd, bisetose at inner margin, and with two additional setae in subapical and apical parts, respectively; antennae long and pubescent, 1st antennomere stouter, about 2/3^{rds} as long as 2nd, which is about 3/4^{ths} as long as 3rd, 4th slightly longer than 3rd, 5th longest, slightly longer than 4th, 6th-11th as long as 4th; head (including mandibles) plus pronotum slightly longer than elytra.

Prothorax barrel-shaped, longer than wide, PrL/PrW = 1.53, widest at about third from base; longer or shorter than head excluding or including mandibles, PrL/HL = 0.76 or 1.09; much wider than head, PrW/HW = 1.45; propleura distinctly tumid, wholly visible from above; wider than pronotum, PrW/PnW = 1.17; pronotum much longer than wide, PnL/PnW = 1.79, wider than head, PnW/HW = 1.24; subparallel-sided, but narrowly and broadly contracted at both ends, making front and hind angles round off, albeit front ones fairly angulate; lateral margins not beaded; PrW/PnW = 1.17; base nearly as wide as front, frontal margin not beaded, finely emarginated in the middle, basal margin widely beaded and nearly straight; both fore and hind lateromarginal setae placed a little mesal to dorsolateral suture, at about basal fourth and apical fifth of pronotum, respectively; disc slightly convex; median line clear, reaching both ends; both transverse impressions not well-marked. Scutellum small and short.

Elytra ovate-oblong, strongly convex; twice as wide as prothorax, much longer than wide, EL/EW=1.89; widest a little behind middle, lateral margins smooth throughout, neither ciliated nor dentate; without prehumeral angles; apex broadly rounded; striae completely disappeared; two dorsal pores present on the location of 3rd stria, at about basal 2/7^{ths} and 3/5^{ths} of elytra, respectively; pre-apical pores located at about apical 2/11^{ths} of elytra; basal pore present, a little distant from scutellum; humeral group of marginal umbilicate pores not aggregated, composed of five pores, 1st pore transversally removed mesad and backward, at a little behind level to 2nd, but a little before the

anterior dorsal pore; 3rd pore close to 2nd; 3rd, 4th and 5th pores widely and equidistantly located; 6th and 7th pores of middle group shifted behind, lying at about apical fourth of elytra; apical group composed of three pores, apical pore located closer to suture than to elytral margin; only 2nd and 9th pores close to marginal gutter, others widely distant from the gutter.

Legs moderately long, femora gradually dilated from base towards subapical portions, then suddenly narrowed towards apices, covered with sparse, long and erect setae; tibiae and tarsi covered with dense and short hairs; tibiae thin, without longitudinal grooves; protarsi short, 1st tarsomere wider than others, longer than 2ndand 3rd combined, but shorter than 2nd-4th combined; meso- and metatarsi longer, 1st tarsomere as long as 2nd-4th combined.

Male: Unknown.

Etymology. tujia + philus, to refer to the fact that the new species is occurring in the country of Tujia people.

Distribution. China (Hunan)(Fig. 5e). Known only from the limestone Cave Tujiamei Dong, Wulongshan Geopark, Longshan County, northwesternmost Hunan Province.

This cave (Fig. 13A, B) lies very close to Feihu Dong, the longest cave in Huoyan Karst, along the main road, and opposite Tujiamei Restaurant. This is a water source cave, with a small underground stream running throughout, the length still being unknown. It is highly moist and muddy. We surveyed as long as about 400 m in the cave, and collected the unique specimen in the dark zone when it was wandering on the wall. The other three trechine species found in the cave are *Cathaiaphaenops delprati* Deuve, 1996 (Fig. 13C), *Sinotroglodytes bedosae* Deuve, 1996, and *Toshiaphaenops ovicollis* Uéno, 1999. We visited this and adjacent caves in July, 2015 in order to find more specimens of this interesting beetle, but failed to catch anything.

Wanhuaphaenops Tian & Wang, gen. n.

http://zoobank.org/C04A6404-CAD3-421C-97C8-5848046875BB

Type species. *Wanhuaphaenops zhangi* Tian & Wang, sp. n. (Cave Songjia Dong, Chenzhou, Hunan).

Diagnosis. Medium-sized, aphaenopsian beetles, body elongate, with short antennae and quite long legs, slender head, reduced frontal furrows, bisetose mentum, clear labial suture, short and tumid prothorax, elongated elytra and bisetose on each of abdominal ventrites.

Generic characteristics. Medium-sized, aphaenopsian type trechine, eyeless, unpigmented and apterous; body very strongly elongate, highly modified morphologically, albeit antennae rather short; head typically aphaenopsoid, extremely elongated as in *Dongodytes* Deuve, 1993 or some members of *Sinaphaenops* Uéno & Wang, 1991, much longer than wide, with short and incomplete frontal furrows ending at about middle of head from clypeus, two pairs of supra-orbital pores present, both anterior

Figure 13. Cave Tujiamei Dong, type locality of *Huoyanodytes tujiaphilus* Tian & Huang, sp. n. **A** environ outside cave, arrowhead showing the site of entrance **B** entrance **C** a wandering individual of *Cathaiaphaenops delprati* Deuve, 1996, a sympatric trechine beetle of *Huoyanodytes tujiaphilus* Tian & Huang, sp. n.

and posterior pores widely spaced; mandibles moderately long, well-developed, right mandible tridentate; labial suture clear; mentum bisetose, distinctly concave, tooth moderately long, thick and blunt at apex; submentum provided with a row of seven (or eight in a male individual) setae, median one much shorter than others; antennae quite short, extending to about middle of elytra; prothorax distinctly shorter than head, longer than wide, propleura strongly tumid, visible from above; pronotum subquadrate, base nearly as wide as front, both anterior and posterior lateromarginal setae present; elytra strongly elongate, slightly longer than head (including mandibles) plus prothorax; widest behind middle, marginal sides smooth throughout, but ciliate in humeral angle area; humera distinctly angulate; disc moderately convex, rather flat

near base, striae well-defined or obliterated, two dorsal and the pre-apical pore present; humeral pores of marginal umbilicate series not aggregated, middle group not close to each other; legs fairly long, 1st protarsomere in male modified, with a tiny apical denticle inward; tibiae without longitudinal furrow externally; ventrite VII with two pairs of setae in both sexes; aedeagus minute, well-sclerotized, short and broad, strongly arcuate, apex blunt, basal part large, with a small sagittal aileron, inner sac with a fairly large copulatory piece, parameres long, right one longer than left one, broad at apex, each bearing three long apical setae.

Remarks. The true affinities of *Wanhuaphaenops* gen. n. likewise remain uncertain. Probably the closest match is *Shenaphaenops* Uéno, 1999 (from northwestern Guizhou Province) because both share several important characters: a wholly pubescent body, humera strongly angulate, right mandibles tridentate, only 1st protarsomere modified in male, two pairs of supra-orbital pores present on head, two dorsal and the pre-apical pores present on elytron, and ventrite VII 4-setose. However, *Wanhuaphaenops* gen. n. is easily distinguished from *Shenaphaenops* by the following characters: (1) head much more elongated, with anterior supra-orbital pore widely distant from posterior one, and labial suture clear (reverse in *Shenaphaenops*); (2) antennae much shorter than in *Shenaphaenops*, in which these extending to nearly elytral apex; (3) pronotal posterior lateromarginal setae present in *Wanhuaphaenops* gen. n., but absent in *Shenaphaenops*; (4) aedeagus stouter and strongly arcuate in *Wanhuaphaenops* gen. n., with each paramere bearing three apical setae(Fig. 10C, D), versus aedeagus being slender and slightly arcuate, with each paramere bearing two apical setae in *Shenaphaenops* (Fig. 4C, D).

Wanhuaphaenops gen. n. might also be found related to the genus *Sinaphaenops* Uéno & Wang, 1991, one of the most highly modified genera among the Chinese cave-dwelling trechines which ranges from west, southern Guizhou and northernmost Guangxi. Both share a somewhat similar body configuration, but *Wanhuaphaenops* gen. n. is much smaller and less troglomorphic than *Sinaphaenops*, the appendages being much shorter, and only one joint of protarsi (1st protarsomere) is modified in the male, versus two, and a different elytral chaetotaxy.

Etymology. As Cave Songjia Dong represents one branch of the Wanhuayan cave system, the name of this new genus refers to the occurrence of this aphaenopsian beetle in Wanhuayan caves. Gender masculine.

Range. China (southern Hunan) (Fig. 5f).

Wanhuaphaenops zhangi Tian & Wang, sp. n.

http://zoobank.org/C164C788-0F9A-439E-A31A-B774DC7BB2DD

Holotype. male, Cave Songjia Dong, Beihu Qu, Chenzhou, southern Hunan Province, 25°40'08.05"N/112°53'59"E, 493 m in altitude, VIII-25-2015, Xinhui Wang, Sunbin Huang, Mingruo Tang & Pingjing Yang leg., in SCAU; paratypes: 9 females & 9 males, ibid., in SCAU.

Diagnosis. A slender and brown cave beetle, with a collar-like neck constriction on head, fairly long fore body which is slightly shorter than elytra, long head which is distinctly longer than prothorax, and distinct humeral angles of elytra.

Description. Length: 5.4–5.8 mm (mean 5.6); width: 1.4–1.6 mm (mean 1.5). Habitus as in Fig. 14.

Body wholly brown, upper surface covered with sparse and minute pubescence, genae and underside of head with some longer setae, abdominal ventrites covered with denser minute pubescence, prosternum, propleura and meso- and metasterna glabrous; legs densely pubescent; microsculpture composed of finely, densely and strongly transverse meshes on upper and underside surfaces. Body elongated, fore body, including mandibles slightly shorter than elytra.

Head strongly elongated, HLm/HW = 2.37–2.5 (mean 2.44), HLl/HW = 1.89–1.94 (mean 1.91), widest at about third of head from labrum, then gently narrowed towards a collar-like constriction of the neck; anterior supra-orbital pore level to the widest point, posterior one at about 1/5th of head from base, strongly behind end of frontal furrows; distance between anterior and posterior pores greater than that between both anterior pores; frontal furrows fine but well-defined, short, nearly parallel-sided in the middle, divergent posteriad, but then convergent before ending points; anterior supra-orbital pores located at the level of mid frontal furrows, posterior ones near collar-like constriction, distance between both posterior pores about half as that between anterior and posterior pores of either side; frons and vertex moderately convex; clypeus quadrate, 4-setose; labrum transverse, widely but shallowly emarginated at front margin; mandibles gently unciform at apex; palps fairly slender, 3rd and 4th maxillary palps glabrous, subequal in length; 2nd labial palp distinctly longer than 3rd, with two setae at inner margin, and 2–3 additional ones in subapical part, 3rd glabrous; suborbital pores on ventral side, near a collar-shaped beaded neck; 1st antennomere thick, as long as 2nd; 3rd antennomere longest, 2.5 times as long as 1st; 4th–7th and 11th slightly longer than 8th–10th.

Prothorax shorter than head, PrL/HLm = 0.60–0.67 (mean 0.63), PrL/HLl = 0.75–0.86 (mean 0.80); but much wider, PrW/HW = 1.11–1.17 (mean 1.14), longer than wide, PrL/PrW = 1.29–1.43 (mean 1.34), widest at about 3/7^{ths} from base; pronotum much longer than wide, PnL/PnW = 1.35–1.58 (mean 1.45), slightly wider than head, PnW/HW = 1.05–1.06 (mean 1.05); widest behind middle, sides beaded, gently narrowed both distad and basad, distinctly sinuate before hind angles, both front and hind angles obtuse, albeit hind ones more angulate and distinctly reflexed; anterior lateromarginal setae at about apical 2/5^{ths}, posterior ones close but a little before hind angles, distinctly shorter than the formers; base slightly wider than front, PbW/PfW = 1.05–1.07 (mean 1.06), both nearly straight, front thickly and widely bordered, base unbordered; disc convex; middle line deep, connected to both front and basal impressions. Scutellum short and small.

Elytra fairly long and elongate ovate, much longer than wide, EL/EW = 1.78–1.82 (mean 1.80), slightly longer than fore body; widest at about 4/9th from apex, lateral margins finely beaded from base to finish just before apex, finely ciliate throughout,

Figure 14. Habitus of *Wanhuaphaenops zhangi* Tian & Wang, gen. n., sp. n., holotype, male. Scale bar: 2.0 mm.

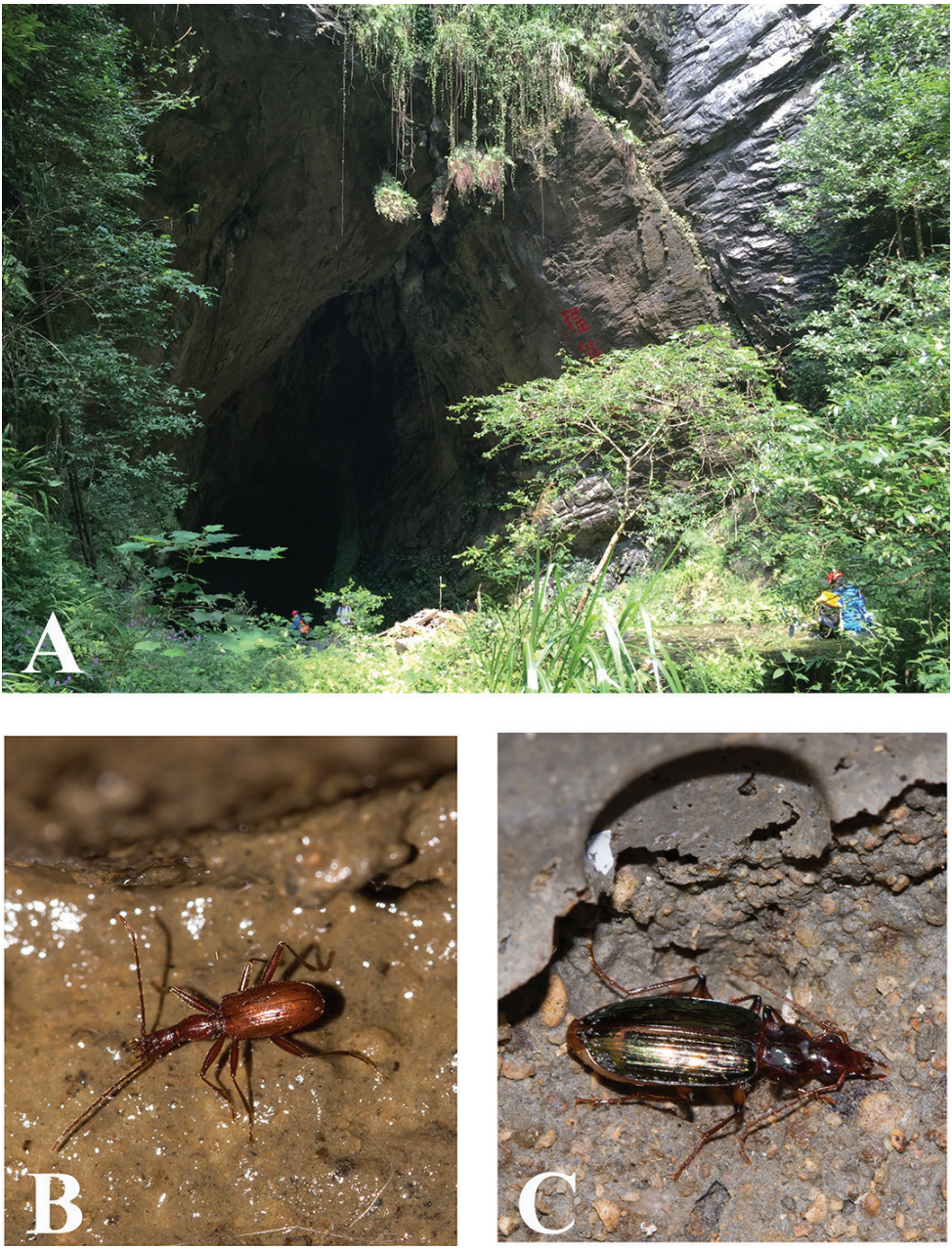

Figure 15. Cave Songjia Dong, type locality of *Wanhuaphaenops zhangi* Tian & Wang, gen. n., sp. n. **A** entrance **B** a wandering individual of *Wanhuaphaenops zhangi* **C** a platynine *Colpodes* beetle in the cave.

but remarkably distinct in angular area, nearly straight before and behind humeral angles; base not bordered; disc convex, but basal or humeral area distinctly depressed and almost flat; 2nd and 3rd striae well-marked and complete, others more or less oblit-

erated; all dorsal and pre-apical setiferous pores located exactly on interrupted and junction points of 2nd and 3rd striae, making 3rd interval with three regular longitudinal meshes between the pores; basal pores located near base, along both sides of scutellum; anterior and posterior dorsal pores at about basal third and middle of elytra, respectively, pre-apical pore at apical fourth of elytra, much closer to suture than to apex of elytra; humeral set of marginal umbilicate pores not aggregated, 1st-3rd pores equidistantly located, quite near the marginal gutter, 4th distant from 3rd; 5th and 6th isolated from each other, though 5th closer 6th than to 4th.

Legs thin and fairly long, femora moderate, tibiae not longitudinally furrowed, hind tibia slightly longer than elytral wide; protarsi short; 1st tarsomere shorter than, or subequal to, or longer than 2nd-4th tarsomeres combined in pro-, meso- and metatarsi, respectively.

Male genitalia (Fig. 10C, D): The median lobe of aedeagus very small, but well-sclerotized, with a small but distinct sagittal aileron and a fairly large copulatory piece; parameres well-developed.

Etymology. This species is named in honour of Prof. Yuanhai Zhang (Institute of Karst Geology, Chinese Academy of Geological Sciences, Guilin), who was leading the cave exploration project at Wanhuayan in late August 2015, one of the results being the discovery of this interesting species.

Distribution. China (Hunan) (Fig. 5f). Known only from the limestone Cave Songjia Dong, in the Wanhuayan cave system.

Songjia Dong is the upper part of the Wanhuayan cave system, about 10 km away from the main entrance of the Cave Wanhuayan. It is a water cave, with the entrance being as big as that in Wanhuayan (Fig. 15A). The beetles were collected in a dark area about 80 m deep from the entrance (Fig. 15B). A *Colpodes* species, a trogloxene, was also found in this cave (Fig. 15C).

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