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Evolution of sperm morphology in potamid freshwater crabs (Crustacea: Brachyura: Potamoidea)

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We investigated sperm cells and spermatophores of four species of Old World freshwater crabs belonging to three different genera of the subfamily Potaminae (family Potamidae). Characters previously believed to be apomorphic for the potamid subfamily Potamiscinae were also found to occur in the Potaminae. To infer the morphological ancestral character state combination of the Potamidae, ancestral character state analysis of four different sperm traits was performed, based on a 16S rDNA phylogeny of the investigated species. Comparing molecular phylogeny and character state distribution, several cases of convergent evolution could be identified. The densely packed, coenospermic spermatophores and the occurrence of a 'tongue-and-groove' connection between operculum and acrosomal zones are probably apomorphies for the whole Potamidae. The spermatozoa of *Socotrapotamon socotrense* show several unique characters. We also analysed the evolution of acrosome size. The sperm cells of the Potamidae and their sister-group Gecarcinucidae only slightly overlap in acrosome size. Within the investigated species, the 'East Asia' subclade (subfamily Potamiscinae) developed significantly larger acrosomes than the subfamily Potaminae. Our results suggest that the use of brachyuran acrosome morphology for phylogenetic inference at the family level is strongly affected by small sample size, and by convergent character evolution.

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ADDITIONAL KEYWORDS: ancestral character state reconstruction – Potamidae – spermatophores – spermatozoa.

INTRODUCTION

Brachyuran sperm morphology has been used for phylogenetic inference in the past (reviewed in Jamieson, 1994; Jamieson, Guinot & Richer de Forges, 1995; Jamieson & Tudge, 2000; Tudge, 2009), and has been claimed to reflect phylogenetic relationships. Within the Brachyura, spermatozoa of the freshwater crab family Potamidae have already been the subject of ultrastructural investigations (see Table 1). This family represents the most speciose group of the true freshwater crabs (Ng, Guinot & Davie, 2008). The current recognition of two subfamilies (Potaminae and Potamiscinae) within the Potamidae is supported by sternal morphology (Yeo & Ng, 2003) and molecular phylogenetics based on 16S rDNA (Shih, Yeo & Ng, 2009). The only difference between both approaches concerns the placement of the potamids inhabiting the island of Socotra between Africa and the Arabian Peninsula. Sternal morphology assigns these crabs to the subfamily Potaminae, whereas molecular phylogenetics places them in the subfamily Potamiscinae. As Yeo & Ng (2003) and Shih *et al.* (2009) already discussed, the systematic position of the Socotra potamids is perplexing.

Of the potamids so far investigated with respect to sperm morphology, only the closely related *Potamon ibericum* and *Potamon fluviatile* belong to the potamid subfamily Potaminae (Table 1). Several spermatozoal character states of the subfamily Potamiscinae were identified that separate them from the sperm cells of *Potamon*, and thus seem to support the

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taxonomic dichotomy of the family (Klaus, Schubart & Brandis, 2009a). In detail, these differences are: (1) the existence of a middle acrosomal zone in the Potamiscinae; (2) the potamiscine 'tongue-and-groove' connection of operculum and the acrosomal zones beneath; and (3) the occurrence of coenospermia (several spermatozoa in one spermatophore) with densely packed sperm cells in the Potamiscinae (except *Johora singaporensis* and *Thaiphusa sirikit* that show cleistospermia, i.e. only a single sperm cell per spermatophore). However, the number of investigated species seems to be a critical issue for the confirmation of hypotheses of homology, and for the identification of plesiomorphies and apomorphies of spermatozoal characters in the Brachyura.

Here, we investigate the sperm ultrastructure of four species of the subfamily Potaminae, from three different genera including *Socotrapotamon socotrense* from the island of Socotra. The aim of our study was to assess the morphological ground pattern of the spermatozoa in the different potamid lineages. We define the ground pattern of a lineage as the character state of its last common ancestor. This state can be either apomorphic (i.e. newly evolved) or plesiomorphic (inherited, and thus also possibly occurring in other lineages).

Ancestral character state analysis is able to reconstruct the morphological evolution along a molecular phylogeny, and in this way can be used to investigate the direction of character loss and convergent evolution. This is of special interest concerning the potamids of Socotra, where morphology-based taxonomy and molecular phylogeny are not congruent (Shih *et al.*, 2009).

Acrosome size was proposed as a tool to discriminate between the Old World freshwater crab families (the Potamoidea, see Klaus *et al.*, 2009b), as the potamids investigated so far have larger acrosomes than the Gecarcinucidae (Klaus *et al.*, 2009a). However, acrosome sizes of the subfamily Potaminae range at the lower end within the Potamidae. Here, we investigate the change of potamid acrosome size in a phylogenetic context, answering the questions of whether acrosome size of the subfamily Potaminae overlaps with that of the Gecarcinucidae, and whether different acrosome sizes of the Potamidae represent lineage-dependant evolutionary trends.

MATERIAL AND METHODS

Material

See Tables 1 and 2.

Ultrastructural and morphometric data

The vasa deferentia of alcohol-stored male specimens from the collection of the Natural History Museum and Research Institute Senckenberg, Frankfurt/Main (Table 2) were removed and fixed in cacodylate-buffered glutaraldehyde (pH 7.4). All specimens were initially fixed in 10% formalin. The vas deferens of *Potamon fluviatile* was taken from a specimen that had unfortunately been frozen after natural death.

The tissue was prepared for transmission electron microscopy (TEM) as described in Klaus *et al.* (2009a), including postfixation in 1% osmium tetroxide for 2 h and en-bloc staining in 1% uranyl-acetate overnight. The tissue was embedded in Spurr's resin. Thin sections (of 75-nm thickness) were post-stained with aqueous lead citrate for 1 min. Electron micrographs were taken on a Zeiss EM10 transmission electron microscope.

Acrosome measurements (acrosome width and length, operculum width and height) were taken with a sliding caliper from the negative. For ancestral reconstruction of acrosome size, the measured variables were reduced by principle component analysis to one factor (PCA1). Operculum height was obviously not lineage dependant and was highly influenced by

Table 1. Potamid species with published sperm morphology

Species	Subfamily	Published in
Potamon ibericum (Bieberstein, 1808)	Potaminae	Guinot, Jamieson & Tudge, 1997
Potamon fluviatile (Herbst, 1785)	Potaminae	Guinot, Jamieson & Tudge, 1997
Sinopotamon yangsekiense Bott, 1967	Potamiscinae	Du *et al.*, 1999; Wang, Du & Lai, 1999
Geothelphusa albogilva (Shy, Ng & Yu, 1994)	Potamiscinae	Klaus, Schubart & Brandis, 2009a
Johora singaporensis (Ng, 1986)	Potamiscinae	Klaus, Schubart & Brandis, 2009a
Larnaudia beusekomae (Bott, 1970b)	Potamiscinae	Klaus, Schubart & Brandis, 2009a
Malayopotamon brevimarginatum (DeMan, 1892)	Potamiscinae	Klaus, Schubart & Brandis, 2009a
Indochinamon beieri (Pretzmann, 1966)	Potamiscinae	Brandis, 2000
Pudaengon thatphanom Ng & Nayanetr, 1995	Potamiscinae	Klaus, Schubart & Brandis, 2009a
Thaiphusa sirikit (Nayanetr, 1992)	Potamiscinae	Klaus, Schubart & Brandis, 2009a

Table 2. Specimens used for preparation of the vas deferens, indicating taxon name, catalogue number in the collection of the Natural History Museum and Research Institute Senckenberg, Frankfurt/Main (SMF), and provenance

Species	Catalogue no.	Subfamily	Provenance
Himalayapotamon emphysetum (Alcock, 1909)	SMF 26069	Potaminae	Nepal, Gandaki Prov., Chundi Khola; leg. Brandis & Sharma, 2000
Parathelphusula panningi (Bott, 1966)	SMF 30193	Potaminae	Nepal, Jahapa, Deune Khola; leg. Brandis & Sharma, 2000
Potamon fluviatile (Herbst, 1785)	Collection R. Jesse	Potaminae	Greece, Arcadia, Gortys; leg. Jesse & Klaus, 2006
Socotrapotamon socotrense Apel & Brandis, 2000	SMF 25274	Potamiscinae	Yemen, Socotra, Wadi Ayhaft; leg. Apel, 2000

operculum thickness, and was thus excluded from principle component analysis.

In this study only museum material and a freezer-stored specimen were available (instead of fresh, glutaraldehyde-fixed tissue), and some deterioration of structures had occurred. This concerns mainly the nuclei and nuclear arms (compare Fig. 1B, G), and outer membranes, as well as the spermatophore walls. The acrosome largely stayed intact. The nomenclature for sperm and spermatophore description is based on that of Klaus *et al.* (2009a).

Phylogenetic hypothesis

16S rDNA sequences of potamoid species were obtained from GenBank (accession no. indicated in Fig. 2) or were newly sequenced. In the latter case, 16S rDNA primers and polymerase chain reaction conditions were used as described in Klaus *et al.* (2009b). A 523-bp fragment was aligned manually and analysed using Bayesian inference in BEAST 1.4.8 (Drummond & Rambaut, 2007). We applied a general time reversible substitution model accounting for site rate heterogeneity and for invariant sites, with a relaxed molecular clock allowing branch lengths to vary according to an uncorrelated lognormal distribution (chain length of 30 million generations, sampling every 1000 generations, and discarding the first 3000 trees as burn-in). The phylogenetic topology used for the subsequent ancestral character state reconstructions is the same as the maximum clade credibility tree generated by TreeAnnotator 1.4.8 (part of the BEAST package) displayed in Figure 2.

Ancestral character state reconstruction

Ancestral character state reconstruction was conducted for the following discrete sperm traits: operculum (perforate or imperforate); spermatophores (cleistospermia or coenospermia); acrosomal zonation (middle acrosomal zone absent or present); connection of the sperm cell operculum with the acrosomal zones ('tongue-and-groove' connection absent or present). Reconstructions were performed under a parsimony model with unordered states. Ancestral states were reconstructed over 1000 post-burn-in trees from the Bayesian inference in MESQUITE 2.6 (Maddison & Maddison, 2009). Parsimony reconstructs states at internal nodes by minimizing state changes that are needed to explain the observed character distribution. For this analysis the gecarcinucid out-group taxa were removed. At nodes, where alternative best states were reconstructed, the percentage of trees with the respective reconstructed state or percentage of equivocal reconstructions is given.

Ancestral states of acrosome morphometric data (PCA1) at internal nodes of the phylogeny and their standard deviation were reconstructed in ANCML under a likelihood model (Schluter *et al.*, 1997). Assuming a Brownian motion process for character evolution, the likelihood approach calculates the ancestral states by minimizing the sum of differences of the Brownian rate parameter along the branches (Schluter *et al.*, 1997). The ancestral reconstructions were plotted against the distance of the respective node from the phylogenies root to visualize the rate and direction of acrosome size changes in the two potamid subfamilies. Significance of evolutionary rate difference between the two subfamilies was tested with an ANCOVA using the distance from the root as covariate.

RESULTS

Parathelphusula panningi (Figs 3A, B, 4A; Table 2)

The acrosome of *Parathelphusula panningi* is nearly spherical with a mean acrosome width (AW) to acrosome length (AL) ratio of 0.9 ± 0.05 ($N = 5$). The acrosome ray zone appears in longitudinal sagittal section as prominent. The inner acrosomal zone is only clearly visible in cross section. The subacrosomal material is prominent. The proximal part of the outer acrosomal zone (towards the perforatorial chamber) is

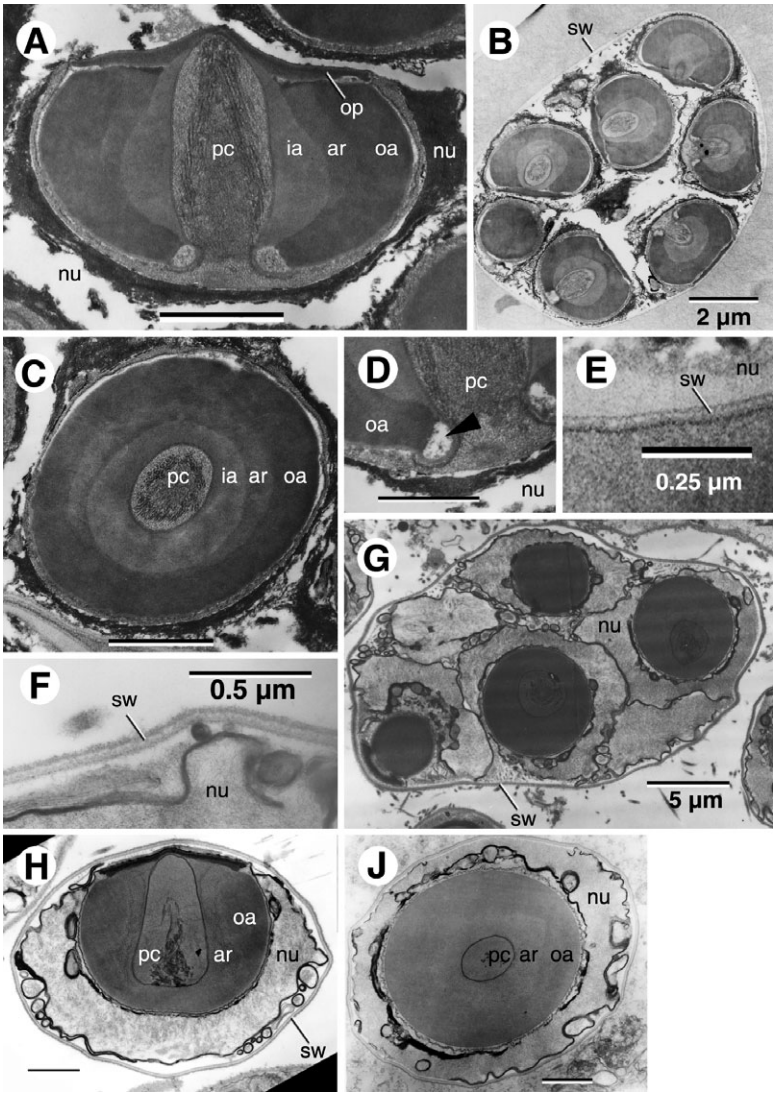

Figure 1. Transmission electron microscopy (TEM) of potamid spermatozoa. A–E, *Socotrapotamon socotrense*. A, longitudinal sagittal view; the white line points to the perforate operculum. B, coenospermic spermatophore. C, cross section. D, electron-lucent ring around the opening of the perforatorial chamber. E, spermatophore wall. F–J, *Potamon fluviatile*. F, spermatophore wall. G, coenospermic spermatophore. H, longitudinal sagittal section (slightly transverse) and cleistospermic spermatophore. J, cross section. Scale bars: 1 µm, or as indicated. Abbreviations: ar, acrosome ray zone; ia, inner acrosomal zone; nu, nucleus; oa, outer acrosomal zone; op, operculum; pc, perforatorial chamber; sw, spermatophore wall.

diffusely differentiated. *Parathelphusula panningi* has cleistospermic spermatozoa.

Himalayapotamon emphysetum (Figs 3C–F, 4B; Table 2)

In *Himalayapotamon emphysetum* the spermatozoa are less spherical than in *Parathelphusula panningi* (AW/AL = 0.8 ± 0.08, $N = 5$). The perforate operculum is centrally bulging and is connected to the acrosomal zones beneath by a 'tongue-and-groove' structure. The subacrosomal material and the acrosome ray zone are prominent. Basal to the perforatorial chamber, two parallel oriented centrioles can be recognized. Fragments of laterally situated nuclear arms can be identified. *Himalayapotamon emphysetum* has cleistospermia.

Socotrapotamon socotrense (Fig. 1A–E, 1C; Table 2)

Socotrapotamon socotrense has the smallest acrosome size of the Potamidae (AL = 2.76 ± 0.11 µm,

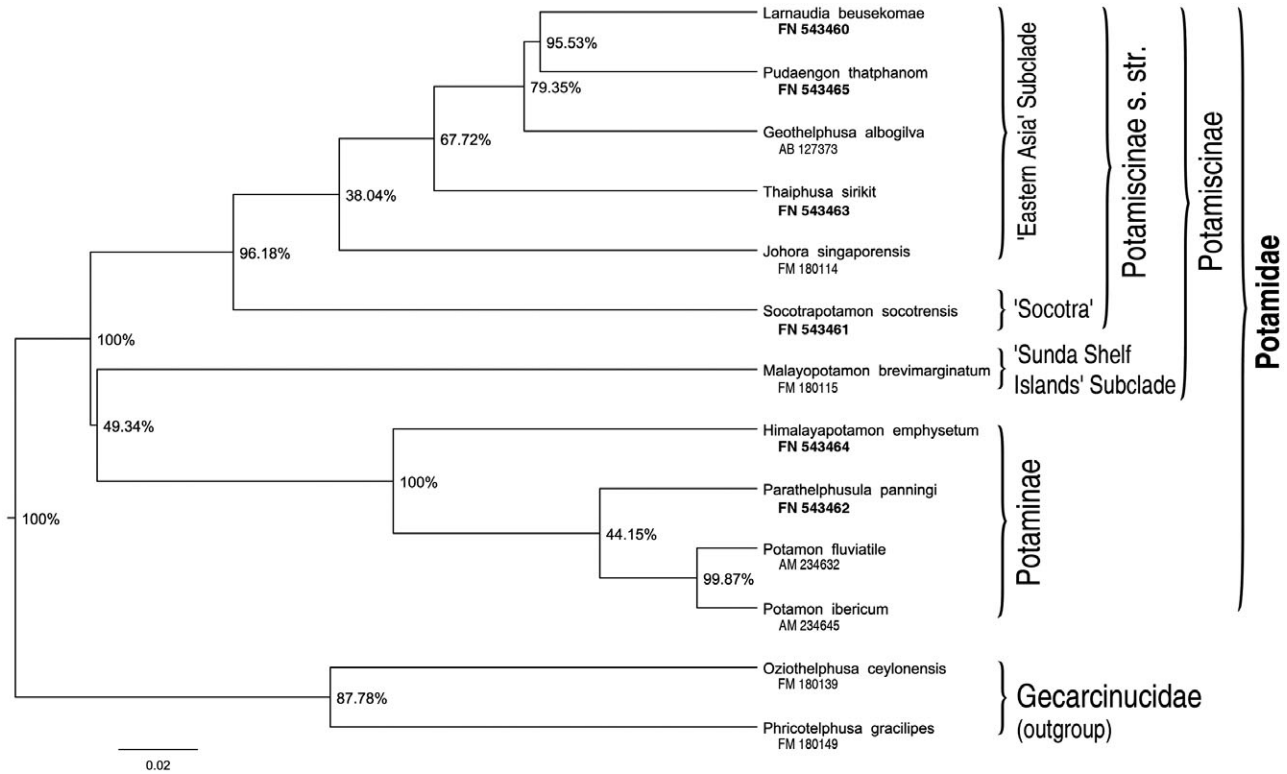

Figure 2. Maximum clade credibility tree based on Bayesian inference (BEAST 1.4.8) of a 16S rRNA fragment (523 bp) including potamid species with known sperm ultrastructure and Gecarcinucidae as the out-group. The nomenclature of subclades is as suggested by Shih *et al.* (2009). European Molecular Biology Laboratory (EMBL) accession numbers of sequences obtained in this study are shown in **bold**.

AW = 1.90 ± 0.16 μm, $N = 7$). The acrosome is strongly depressed (AW/AL = 0.7 ± 0.05). The centrally bulging operculum is perforate and not connected via tongue and groove to the acrosomal zones beneath. The very prominent inner acrosomal zone and the distinct acrosome ray zone bulge outwards. Apically, towards the operculum, the acrosome ray zone is covered with electron-dense material. There is an electron-lucent ring that surrounds the basal opening of the perforatorial chamber. The coenospermic spermatozoa are densely packed into spermatophores. The spermatophore wall is formed by two layers: an outer, electron-dense layer, and an inner, less electron-dense layer (Fig. 1E).

The wide inner acrosomal zone, the electron dense 'cap' on the acrosome ray zone, and the electron-lucent ring surrounding the proximal end of the perforatorial chamber represent unique sperm characters within the Potamidae.

Potamon Fluviatile (Fig. 1F–J; Table 2)

As stated above, this specimen of *Potamon fluviatile* was frozen after death, which elicited acrosome reaction. However, there were still a few sperm cells that remained intact. The operculum is rather thin. Lateral of the operculum there are electron-lucent structures that resemble a vestigial peripercular rim as it occurs in Potamonautidae and Gecarcinucidae. The acrosome ray zone is prominent and bulging outwards. There are many laterally situated nuclear arms. *Potamon fluviatile* shows both coenospermia and cleistospermia, with the coenospermic spermatophores being identical to those of the Potamiscinae.

Phylogeny of the Potamidae

Here, phylogenetic inference is not performed to reveal potamid relationships, but to be used in the subsequent ancestral character state reconstruction. Thus, it is important to note that the topology of the 16S rDNA analysis (although several splits have only minor support; Fig. 2) is widely in agreement with that of Shih *et al.* (2009), which was based on a large data set of 72 species and 49 genera. The major difference is the position of *Malayopotamon brevimarginatum* (representing the 'Sunda Shelf islands' subclade), which clusters in our analysis with a very short branch and low posterior probability as sister group to the subfamily Potaminae, whereas in the

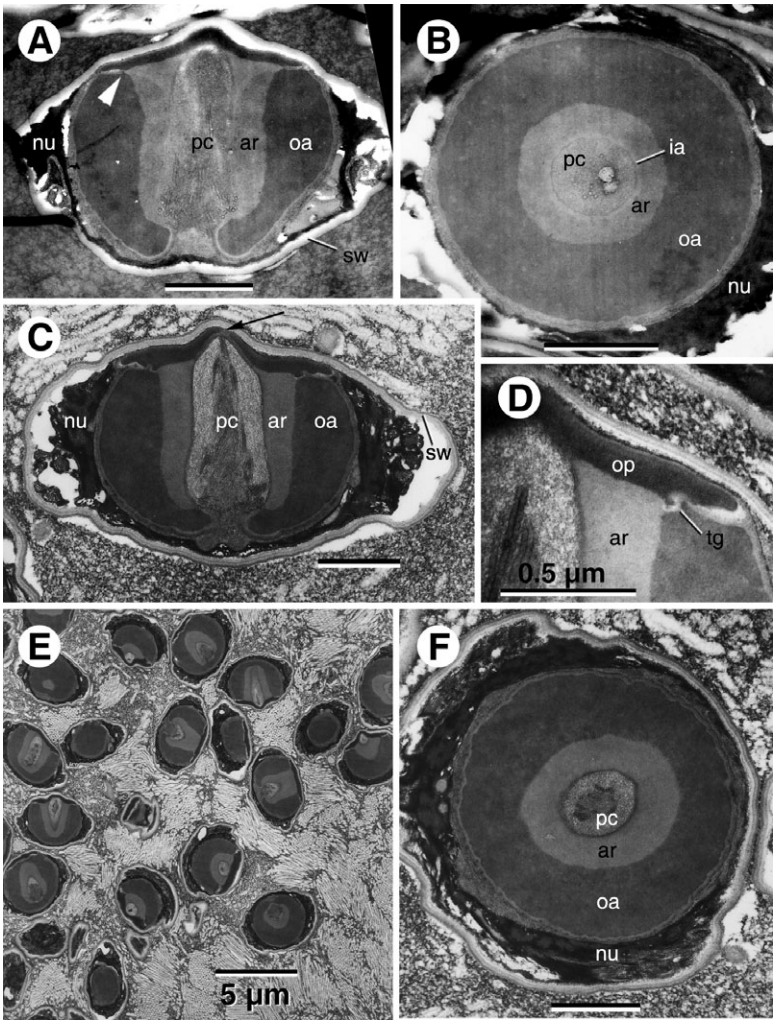

Figure 3. Transmission electron microscopy (TEM) of potamid spermatozoa. A, B, *Parathelphusula panningi*. A, longitudinal sagittal view. B, cross section. C–F, *Himalayapotamon emphysetum*. C, longitudinal sagittal view; the arrowhead indicates the perforate operculum. D, 'tongue-and-groove' connection between the operculum and the acrosomal zones beneath. E, overview of cleistospermic spermatophores. F, cross section. Scale bars: 1 µm, or as indicated. Abbreviations: arrowhead, vestigial 'tongue & groove connection'; ar, acrosome ray zone; ia, inner acrosomal zone; nu, nucleus; oa, outer acrosomal zone; op, operculum; pc, perforatorial chamber; tg, 'tongue-and-groove' connection; sw, spermatophore wall.

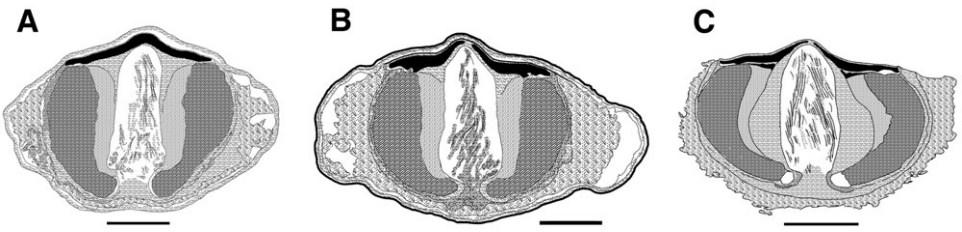

Figure 4. Diagrammatic drawings of potamine spermatozoa. A, *Parathelphusula panningi* (with cleistospermic spermatophore wall). B, *Himalayapotamon emphysetum* (with cleistospermic spermatophore wall). C, *Socotrapotamon socotrense*. Scale bars: 1 µm.

analysis of Shih *et al.* (2009) it is monophyletic with the other Potamiscinae (however, only weakly supported in their minimum-evolution approach). Apparently the 'Sunda Shelf islands' subclade corresponds to the Isolapotamidae of Bott (1970a), and might be recognized as a third potamid subfamily Isolapotaminae (see Shih *et al.*, 2009). We did not force monophyly on the Potamiscinae in the phylogenetic analysis, but kept this incongruence during the ancestral state reconstructions as none of the two topologies can currently be excluded. In summary, we can recognize the 'Potamiscinae *s.s.*' (including the genus *Socotrapotamon*, but excluding *Malayopotamon*), the subfamily Potaminae, and a group [*Malayopotamon* + Potaminae], with the possibility that the latter is not monophyletic (the respective node is present in 49% of the 1000 trees fed into the ancestral character state analysis).

Ancestral character state reconstruction

Reconstructions of ancestral character states under a parsimony model are shown in Figure 5A–D. The ancestral character state of the operculum (perforate or imperforate) at the base of the Potamidae remains equivocal. In total, 25.2% of reconstructions support the imperforate state and 19.5% of reconstructions support the perforate state at this node (Fig. 5C). The same ambiguous reconstruction of operculum states occurs in the 'Potamiscinae *s.s.*', the Potaminae, and the [Potaminae + *Malayopotamon*].

A true middle acrosomal zone only occurs in the Potamiscinae and in the genus *Malayopotamon* (Fig. 5D). Consequently, the absence of the middle acrosomal zone belongs to the ground pattern of the Potaminae. However, because of its absence in *Socotrapotamon*, the ancestral state reconstruction finds this character in only 58% of the analysed trees at the base of the 'Potamiscinae *s.s.*'.

The presence of the 'tongue-and-groove' connection that connects the operculum and acrosomal zones situated beneath is most probably an apomorphy for the whole Potamidae (occuring in 71.1% of

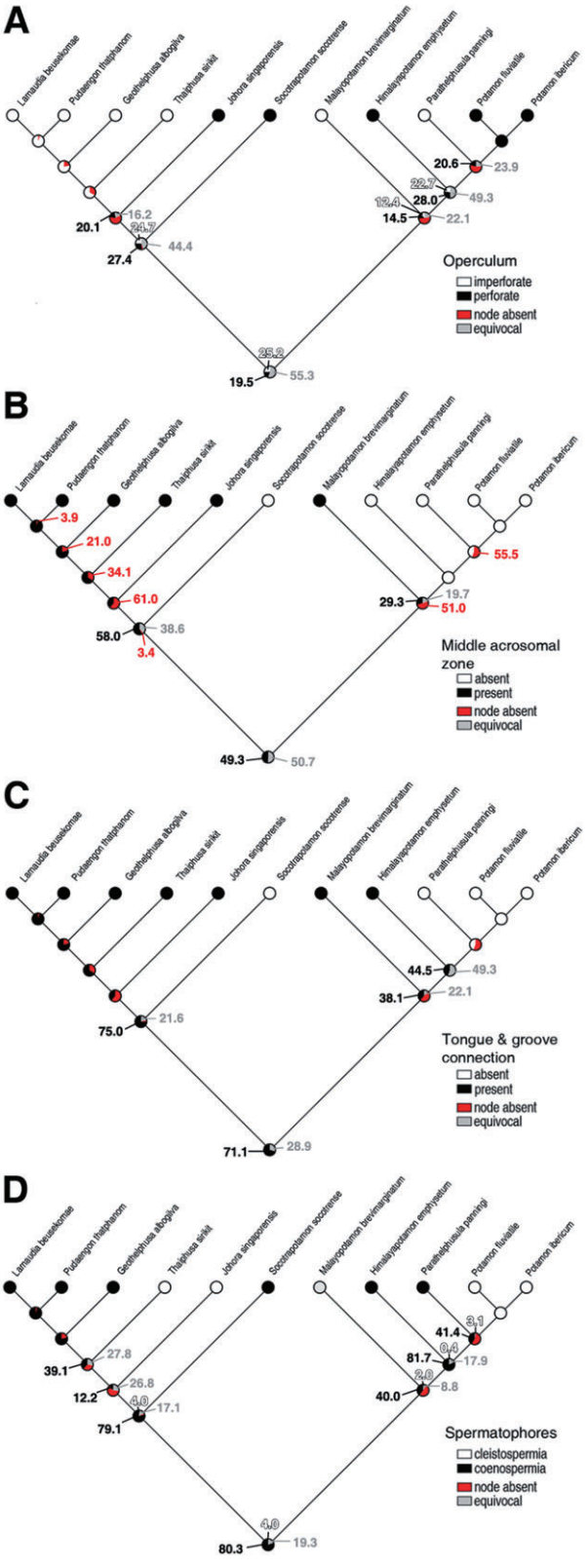

Figure 5. Reconstruction of ancestral sperm characters under a parsimony model over 1000 trees from the Bayesian inference (MESQUITE 2.6). A, operculum perforate or imperforate. B, 'middle acrosome zone' present or absent. C, 'tongue-and-groove' connection present or absent. D, coenospermia present or absent. Values at nodes indicate the percentage of trees with the respective state reconstructed or percentage of equivocal reconstructions. The proportion of trees that failed to find a respective node is the same for all four reconstructions, and the exact values are given in Figure 5B.

reconstructions; Fig. 5A). Secondary reduction of this character took place in *Socotrapotamon socotrense*, and in the lineage leading to *Parathelphusula panningi* and the genus *Potamon* within the Potaminae.

The coenospermic spermatophores are reconstructed as the ground pattern of the Potamidae (reconstructed in 80.3% of the trees analysed; Fig. 5B). Several reductive events led to cleistospermia in the genus *Potamon*, in *Johora singaporensis*, and in *Thaiphusa sirikit*. Also for the 'Potamiscinae *s.s.*' (79.1%), the [Potaminae + *Malayopotamon*] (40.0%), and for the Potaminae (81.7%), coenospermia most probably represents the ancestral character state.

Evolution of acrosomal size

The acrosomal size of *Himalayapotamon emphysetum*, *Parathelphusula panningi*, and *Malayopotamon brevimarginatum* overlaps with the size of gecarcinucid acrosomes. Within the Potamiscinae, *Larnaudia beusekomae*, *Geothelphusa albogilva*, *Pudaengon thatphanom*, and *Thaiphusa sirikit* have wider and longer acrosomes than the other investigated potamid species (Fig. 6A). Although Potamidae and Gecarcinucidae generally overlap in operculum width, these potamiscine species also have much larger operculi (Fig. 6B). Operculum height alone does not allow discrimination between the families Potamidae and Gecarcinucidae, or between the potamid subfamilies. *Potamon fluviatile* has the largest acrosomes within the Potaminae. However, only one sperm cell could be investigated because of the poor preservation of the specimen, and it cannot be excluded that its acrosome size was perhaps affected by the freezing of the original specimen.

Data reduction by principle component analysis leads to one factor (PCA1) that explains 86.4% of the total variation, thus being a good proxy for acrosome size. Evolution of acrosome size is visualized by plotting the ancestral reconstructions of PCA1 against the nodes' distance from the root (Fig. 6C). The graph shows differences in the evolution of acrosome size between the [Potaminae + *Malayopotamon*] and the 'Potamiscinae *s.s.*' (indicated by different slopes between nodes). ANCOVA found a significant effect of slope heterogeneity ($P = 0.004$, d.f. = 1, mean square = 0.592, $F = 17.675$). In the 'Potamiscinae *s.s.*' a strong evolutionary trend towards larger acrosomes

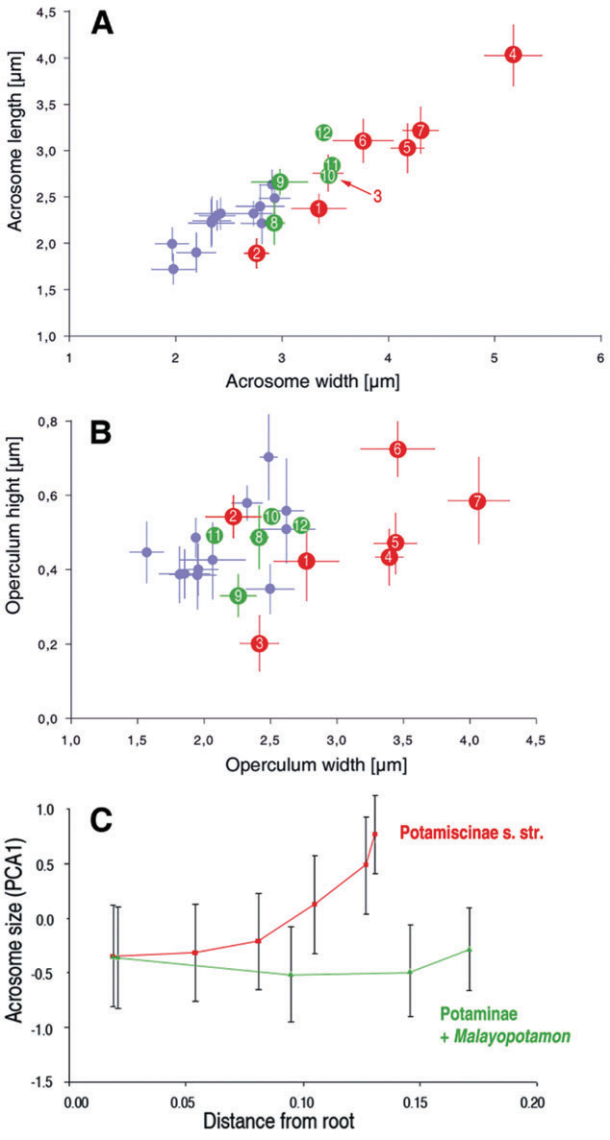

Figure 6. A, B, acrosomal measurements of potamid/potamiscine (1–7) and potamid/potamine (8–12) sperm cells, with each label representing one species. For comparison, species of the Gecarcinucidae (data points without numbers) are included. A, acrosome length plotted against acrosome width. B, operculum height plotted against operculum width. Species: 1, *Malayopotamon brevimarginatum*; 2, *Socotrapotamon socotrense*; 3, *Johora singaporensis*; 4, *Thaiphusa sirikit*; 5, *Geothelphusa albogilva*; 6, *Larnaudia beusekomae*; 7, *Pudaengon thatphanom*; 8, *Himalayapotamon emphysetum*; 9, *Parathelphusula panningi*; 10, *Potamon ibericum*; 11, *Potamon fluviatile*; 12, *Potamon fluviatile* (this study, only one sperm cell measured). Data for 1, 3–7 and the Gecarcinucidae are derived from Klaus *et al.* (2009a); data of 10 and 11 are from Guinot *et al.* (1997) (standard deviation not indicated). C, ancestral reconstruction of acrosome size (PCA1, incorporating acrosome length, width, and operculum width) at internal nodes plotted against the nodes' distance from the root of the phylogeny (Fig. 2) for the Potamiscinae and for [Potaminae + *Malayopotamon brevimarginatum*].

is apparent, whereas in the Potaminae the ancestral acrosome size is retained.

DISCUSSION

Phylogenetic hypotheses derived from molecular data can contribute to the correct determination of morphological ground patterns. Ancestral character state reconstruction as conducted here takes into account ambiguous reconstructions at internal nodes and uncertainties of phylogenetic inference. This method is strongly dependent on the availability of a robust molecular data set. Concerning the Potamidae, the uncertainty of the phylogenetic position of the 'Sunda Shelf islands' subclade also restricts the inference of ancestral character states. A better resolution of the phylogenetic position might be achievable using additional molecular markers. However, confirmation of potamiscine monophyly, including the 'Sunda Shelf islands' subclade (as suggested by Shih *et al.*, 2009), would presumably have only minor effects on the present analysis of discrete ancestral states. Its major impact would probably be the unambiguous recognition of the middle acrosomal zone as an apomorphy of the Potamiscinae.

Acrosome morphometrics do not unequivocally discriminate between the families Gecarcinucidae and Potamidae, although the potamids tend to have larger and more depressed acrosomes. Also, within the family Potamidae, acrosome width and length, and especially operculum width, broadly overlap between the subfamilies Potaminae and Potamiscinae. The species belonging to the 'Eastern Asia' subclade are at the upper end of acrosome size and operculum width (excluding *Johora singaporensis*, which has smaller acrosomes) (Fig. 6A, B). The reconstruction of acrosome size evolution shows that the Potamiscinae, and especially the 'Eastern Asia' subclade, increasingly developed a larger acrosome compared with the Potaminae (Fig. 6C). However, sampling within different groups of the Potamiscinae is far from complete, and the increase in acrosome size possibly occurred in only part of the 'Eastern Asia' subclade. We hypothesize that this change in size might be the result of sperm competition, which is common in brachyuran crabs (see Diesel, 1991). Freshwater crabs, like other Brachyura (Diesel, 1991), store spermatozoa of several copulations in their spermathecae [*Sayamia bangkokensis* (Nayanetr, 1982), *Syntripsa matannensis* (Schenkel, 1902), both Gecarcinucidae; (S. Klaus, unpublished data)]. Thus, if larger acrosomes would give an advantage in the fertilization of oocytes, selection could drive such an increase in size.

For the investigated sperm traits the present study revealed a complex character distribution within the Potamidae (Fig. 5A). The ancestral character state reconstruction indicates that character reversal and convergent evolution have occurred frequently within the Potamidae. None of the characters investigated here can unequivocally be claimed as apomorphic for one of the two potamid subfamilies. The analysis even fails to reconstruct reliably the ancestral character state of the operculum and middle acrosomal zone for the Potamidae as a whole.

The ground pattern of the operculum is not resolved at all deeper nodes. A similar situation appears in the reconstruction of presence or absence of the middle acrosomal zone, as reconstructions of the basal nodes for the Potamidae and both subfamilies remain ambiguous.

The character state distribution of the middle acrosome zone corresponds to the occurrence of the transverse sternal ridge, the diagnostic state for the Potaminae according to Yeo & Ng (2003). The absence of a middle acrosomal zone in *Socotrapotamon socotrense* would support a closer relationship with the Potaminae, as initially proposed by Yeo & Ng (2003). However, the strongly depressed acrosome, and the 16S rDNA data both in the present analysis and in the study of Shih *et al.* (2009) argue against this relationship (Fig. 2). Ancestral character state reconstruction favours a secondary reduction of the middle acrosomal zone in *Socotrapotamon socotrense* (Fig. 5B). Considering the only weakly supported phylogenetic position of the 'Sunda Shelf islands' clade (*Malayopotamon brevimarginatum*) in our molecular phylogeny (Fig. 2), the occurrence of a middle acrosomal zone could support the monophyly of the Potamiscinae.

Contrary to previous expectations (Klaus *et al.*, 2009a), coenospermia also occurs in the Potaminae. Their spermatophores have the same morphology as described for the Potamiscinae, with very densely packed sperm cells. In the genus *Potamon*, so far claimed to have only cleistospermia (Guinot *et al.*, 1997), coenospermic and cleistospermic spermatophores occur, even in the same individual (*Potamon fluviatile*). The absence of coenospermia in Potamon fluviatile in the study of Guinot *et al.* (1997) possibly reflects seasonal changes in sperm packing (see Moriyasu & Benhalima, 1998).

Coenospermia is reconstructed as the ancestral character state of the Potamidae (Fig. 5B). It probably represents an apomorphy of this group, as in the Gecarcinucidae only spermatophores of the mucous type occur (Klaus *et al.*, 2009a), which are probably not homologous with the potamid spermatophores. For the third Old World freshwater crab family, the Potamonautidae, coenospermia has not yet been described (*Potamonautes sidneyi*: Jamieson, 1993; *Hydrothelphusa madagascariensis*: Klaus *et al.*, 2009a).

The homology of the spermatophores of Potaminae and Potamiscinae is also supported by ultrastructure. The morphology of the spermatophore wall of *Potamon fluviatile* (Fig. 1F, G, H; both cleistospermic and coenospermic spermatophores) is identical to the spermatophore pellicle of the coenospermic spermatophores of the Potamiscinae, with an outer electron-dense layer, and an inner, less electron-dense, layer (see Klaus *et al.*, 2009a). The spermatophore wall of the cleistospermic spermatophores of *Parathelphusula panningi* and *Himalayapotamon emphysetum* are not well preserved, and it is not evident if they have a complex spermatophore wall consisting of five layers, as in the potamiscine species that have cleistospermia (*Johora singaporensis* and *Thaiphusa sirikit*; see Klaus *et al.*, 2009a). Although *Johora singaporensis* and *Thaiphusa sirikit* share this character, and in general have a very similar and characteristic shape of acrosomal zones (Klaus *et al.*, 2009a), their phylogenetic relationship would argue for convergent character evolution, as they are not sister species (Fig. 5B).

The 'tongue-and-groove' connection was initially claimed to be an apomorphy for the subfamily Potamiscinae (Klaus *et al.*, 2009a). However, the present investigation of *Himalayapotamon emphysetum* and *Parathelphusula panningi* of the Potaminae clearly indicates that this character is an apomorphy for the whole Potamidae. It is absent in the Gecarcinucidae and has not yet been detected in the Potamonautidae.

In this study, we exemplify the difficulties associated with the phylogenetic interpretation of brachyuran sperm characters on the family level. Apparently, the most challenging issues are small sample size and convergent character evolution. The number of investigated species in ultrastructural studies is generally low, because of the difficulties in obtaining adequately fixed material, in this case especially for the more enigmatic lineages (e.g. the genus *Isolapotamon* and relatives). The risk of reconstructing false ground patterns, and of identifying false apomorphies, is apparent for the subfamily Potamiscinae because increased sample size reveals that characters such as the 'tongue-and-groove' connection and coenospermia do not represent apomorphies for this subfamily. Small sample sizes also obscure cases of convergent evolution. For example, perforation of the operculum and cleistospermia occur frequently in the Potamidae, and thus can not be homologized a priori without considering an independent phylogenetic hypothesis (Fig. 5B, C). For as long as we do not understand the selective regimes that shape brachyuran acrosome morphology (leading to independent reduction of characters and convergent evolution of complex character patterns), any hypotheses of homology at the family level must be treated with caution.

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