

# Molecular systematics of mantelline frogs from Madagascar and the evolution of their femoral glands

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Several genera of frogs from Madagascar, classified in the family Mantellidae, subfamily Mantellinae, possess structures commonly called ‘femoral glands’ on the ventral side of their shanks. The question arises as to the origin and phylogenetic significance of these glands. A molecular phylogeny based on 3601 nucleotide DNA sequences of three mitochondrial and two nuclear genes of 30 mantellid species provided strong support for monophyly of the included mantellines, all characterized by enlarged femoral gland clusters, as well as for those with gland clusters of coordinated central arrangement of secretion ducts. However, the phylogeny also strongly supported the hypothesis of convergent evolution of structurally similar glands in unrelated frogs (*Indirana*, *Petropedetes*), and several trends of convergent evolution of gland structure within mantellines. We studied the light microscopic structure of the femoral glands in a representative array of 18 mantellid species. Males of all species of the subfamily Mantellinae were characterized by clusters of distinct single glands. Each was structurally similar to an enlarged granular gland and secreted independently, probably through a single duct. By contrast, the largely semi-aquatic frogs in the genus *Mantidactylus* had a specialized cluster of glands, in which the secretion ducts led into a macroscopically recognizable central depression. In *Boophis opisthodon*, a mantellid species of the subfamily Boophinae without externally recognizable femoral glands, we observed a large number of enlarged granular glands of various sizes in the ventral skin of the shank. This observation is consistent with the hypothesis that the large and more uniform organs of mantellines are derived granular glands. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, **92**, 529–539.

**ADDITIONAL KEYWORDS:** Amphibia – Anura – macroglands – Mantellidae – Mantellinae – phylogeny – Rag-2.

## INTRODUCTION

The presence of multicellular mucous and granular glands (the latter also termed serous or poison glands) in the skin is a synapomorphy of extant amphibians (Duellman & Trueb, 1986). Whereas the mucous glands secrete mucoproteins that lubricate and moisturize the skin (Houck & Sever, 1994;

Clarke, 1997), the granular glands serve a multitude of functions, such as deterrence of pathogens and predators and, in some cases, also producing mucus (Fontana *et al.*, 2006). Accordingly, they secrete a wide variety of noxious and/or toxic compounds: predominantly peptides.

Poison-secreting glands can occur singly or as large clusters, such as the parotoid glands of bufonid frogs (Duellman & Trueb, 1986), inguinal glands of *Physalaemus* (Lenzi-Mattos *et al.*, 2005) or the tibial gland of some myobatrachids (Crook &

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Tyler, 1981). Other macroglands of amphibians are involved in pheromone and toxin secretion (Conaway & Metter, 1967; Sever, 1976, 1989; Visser, Cei & Gutierrez, 1981), and often are interpreted as modified granular glands (Von Eggeling, 1914a, b; Delfino *et al.*, 2001; Lenzi-Mattos *et al.*, 2005), although, in many cases, this homology remains hypothetical.

Most of the sexually dimorphic skin glands of 14 species of extant amphibians examined histochemically by Thomas, Tsang & Licht (1993) were multicellular alveolar glands, in which the secretory cells were filled with eosinophilic granules. The femoral skin glands of *Mantidactylus betsileanus*, a mantellid frog from Madagascar, however, presented a completely different histological picture, consisting of macroglandular clusters of serous granular glands.

Mantellid frogs are a monophyletic and species-rich lineage endemic to Madagascar and the Comoro Islands (Vences *et al.*, 2003). Aspects of their biosystematic relationships, however, still need further clarification. One character complex that could be useful in this regard is their unique glands. Numerous mantellid genera, such as *Blommersia*, *Guibemantis*, *Gephyromantis*, *Mantidactylus*, *Mantella*, *Spinomantis*, and *Wakea*, which together form the subfamily Mantellinae in the family Mantellidae (Vences & Glaw, 2001; Glaw & Vences, 2006), possess distinct and morphologically diverse femoral glands (Blommers-Schlösser, 1979; Blommers-Schlösser & Blanc, 1991).

Glaw, Vences & Gossmann (2000) described a great macroscopic variability of these glands among species, which predisposes mantellids as a model group for better understanding patterns of femoral gland evolution in frogs. Systematic differences may also be present at the microscopic level: in two *Mantidactylus* species, the ducts of various large glands lead to the centre of the gland cluster where the secretion pori are concentrated (Von Eggeling, 1914a). Detailed histological studies of a representative number of mantellid species, however, are lacking.

To trace the evolution of the femoral gland structure at the macroscopic and microscopic level, a robust cladogram is required. In the present study, we therefore first review mantellid systematics, supplementing this with new molecular data. Subsequently, we provide histological data on the femoral glands of 18 mantellid species and two other anuran genera that display femoral glands (*Petropedetes* and *Indirana*). The aims of the study were: (1) to describe the microscopic structure of these organs and (2) to analyse how their structure varied in the course of mantellid evolution.

## MATERIAL AND METHODS

### ANIMAL MATERIAL

We studied specimens from the collections in the Muséum National d'Histoire Naturelle, Paris (MNHN), Museo Regionale di Scienze Naturali, Torino (MRSN), Département de Biologie Animale, Université d'Antananarivo, Madagascar (UADBA), Zoologisches Forschungsmuseum Alexander Koenig, Bonn (ZFMK), Zoological Museum Amsterdam (ZMA), and the Zoologische Staatssammlung München (ZSM). For some specimens, which will be included in these collections later, we provide preliminary field numbers: FGMV, field number of F. Glaw and M. Vences; FGZC, zoological collection of F. Glaw; ZCMV, zoological collection of M. Vences.

### MOLECULAR SYSTEMATICS

DNA was extracted from muscle tissue stored at  $-80^{\circ}\text{C}$  or fixed in 70% ethanol. Tissue samples were digested using proteinase K (final concentration  $1\text{ mg mL}^{-1}$ ), homogenized and subsequently purified following a standard salt extraction protocol. We assembled a multigene dataset of DNA sequences fragments of the mitochondrial genes for cytochrome *b*, 16S rRNA, and 12S rRNA (the latter fragment including also part of the adjacent tRNA<sub>Val</sub> gene), and the nuclear genes for rhodopsin (exon 1) and Rag-2. Polymerase chain reaction (PCR) of the Rag-2 fragment was performed in 25- $\mu\text{L}$  reactions containing 0.5–1.0 units of REDTaq DNA polymerase (Sigma), 0.01 units of Pwo DNA polymerase (Roche), 50 ng of genomic DNA, 10 pmol of each primer, 15 nmol of each dNTP, 50 nmol of additional  $\text{MgCl}_2$  and the REDTaq PCR reaction buffer (end concentrations: 10 mM of Tris-HCl, pH 8.3, 50 mM of KCl, 1.1 mM of  $\text{MgCl}_2$  and 0.01% gelatine). Rag-2 fragments were amplified with a nested approach and a first PCR using external primers 31FN.Venk (Venkatesh, Erdmann & Brenner, 2001) and Rag2.Lung.460R GCA TYG RGC ATG GAC CCA RTG ICC (Brinkmann *et al.*, 2004), and a second PCR with internal primers (Rag2A.F35, Rag2.Lung.35F, Rag2.Lung.320R) (Hoegg *et al.*, 2004), which amplify a 829-bp fragment of the 5' end of the coding region. Cycle conditions included an initial denaturation step at  $94^{\circ}\text{C}$  for 5 min, 35 cycles with  $94^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 40 s and  $68^{\circ}\text{C}$  for 2 min. The final extension was carried out at  $68^{\circ}\text{C}$  for 5 min. PCR products were purified with spin columns (Qiagen). Rhodopsin exon 1 sequences were obtained using the forward primer Rhod.ma and the reverse primer Rhod.md (Hoegg *et al.*, 2004). Primers for 12S were 12SA-L and 12SR3 and, for the 16S (3') fragment, 16SA-L and 16SB-H of Palumbi *et al.* (1991).

**Table 1.** Voucher specimens of taxa used for histological study, and specific stains applied to each sample

Name	Family: subfamily	Voucher number	KKK stain	Periodic acid-Schiff stain	Toluidin blue stain	Fixation formalin/ethanol
<i>Blommersia wittei</i>	Mantellidae: Mantellinae	ZMA 7058 (761)	X			F
<i>Blommersia wittei</i>	Mantellidae: Mantellinae	ZFMK 52594	X			E
<i>Blommersia wittei</i>	Mantellidae: Mantellinae	ZFMK 52625	X			E
<i>Gephyromantis pseudoasper</i>	Mantellidae: Mantellinae	ZFMK 53706				E
<i>Gephyromantis cornutus</i>	Mantellidae: Mantellinae	MNHN 1972.1471	X	X	X	F
<i>Gephyromantis rivicola</i>	Mantellidae: Mantellinae	Not preserved		X		E
<i>Gephyromantis luteus</i>	Mantellidae: Mantellinae	MNHN 1972.1410	X			F
<i>Gephyromantis malagasius</i>	Mantellidae: Mantellinae	ZFMK 59897	X	X		E
<i>Gephyromantis malagasius</i>	Mantellidae: Mantellinae	ZFMK 59929	X			E
<i>Guibemantis liber</i>	Mantellidae: Mantellinae	ZMA 6658 (646)	X	X		F
<i>Guibemantis liber</i>	Mantellidae: Mantellinae	MRSN 437.1	X			F
<i>Guibemantis depressiceps</i>	Mantellidae: Mantellinae	ZMA 6972 (739)	X	X		F
<i>Guibemantis depressiceps</i>	Mantellidae: Mantellinae	ZMA 6972 (742)	X			F
<i>Guibemantis bicalcaratus</i>	Mantellidae: Mantellinae	ZFMK 53702	X	X		E
<i>Guibemantis bicalcaratus</i>	Mantellidae: Mantellinae	Not preserved				E
<i>Spinomantis aglavei</i>	Mantellidae: Mantellinae	ZFMK 60002	X			E
<i>Spinomantis aglavei</i>	Mantellidae: Mantellinae	ZFMK 46021				E
<i>Mantella ebenaui</i>	Mantellidae: Mantellinae	Not preserved	X			E
<i>Mantidactylus charlotteae</i>	Mantellidae: Mantellinae	ZMA 7001 (690)	X	X	X	F
<i>Mantidactylus brevipalmatus</i>	Mantellidae: Mantellinae	ZFMK 56163	X			E
<i>Mantidactylus femoralis</i>	Mantellidae: Mantellinae	MNHN 1973.853	X	X		F
<i>Mantidactylus lugubris</i>	Mantellidae: Mantellinae	ZFMK 47250		X		E
<i>Mantidactylus lugubris</i>	Mantellidae: Mantellinae	MNHN 1973.886	X			F
<i>Mantidactylus cf. ulcerosus</i>	Mantellidae: Mantellinae	MNHN 1975.632	X	X	X	F
<i>Mantidactylus ulcerosus</i>	Mantellidae: Mantellinae	Not preserved			X	E
<i>Mantidactylus grandidieri</i>	Mantellidae: Mantellinae	MRSN A1971.1	X	X		F
<i>Boophis opisthodon</i>	Mantellidae: Boophinae	ZMA 6851 (763)	X			F
<i>Petropedetetes cf. natalensis</i>	Petropedetidae	MNHN 1989.3999	X			F
<i>Indirana</i> sp.	Petropedetidae: Ranixalinae	MNHN 1985.623	X			F
<i>Aubria subgillata</i>	Dicroglossidae	ZFMK 68708			X	F?

KKK stain indicates nuclear fast red stain.

The 16S (3') fragment was amplified with 16SL3 and 16SA-H (Vences *et al.*, 2003). Cytochrome *b* was amplified with CBJ10933 and Cytb-c of Bossuyt & Milinkovitch (2000). For PCR of 12S, the two 16S fragments, cytochrome *b*, and rhodopsin, the denaturation step was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s. Sequencing was performed directly using the corresponding PCR primers (forward and reverse). DNA sequences of both strands were obtained using the BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems Inc.) on an ABI 3100 capillary sequencer using the manufacturer's instructions. New sequences gathered in the present study are deposited in GenBank (accession numbers EF100456-EF100512; for voucher specimens, see Supplementary Material, Table S1).

Rag-2 and rhodopsin sequences were aligned manually; 12S and 16S sequences were aligned with ClustalW (Thompson, Higgins & Gibson, 1994) and manually refined. Positions that could not be aligned were excluded from further analyses. MrModeltest (Nylander, 2004) was run on the single datafiles to estimate the model and parameter for the partitions, which resulted in the general time reversible model (GTR) + I + G for all datasets. Bayesian inference was performed using MrBayes, version 3.0 (Huelsenbeck & Ronquist, 2001) defining each gene as separate partition, running 1 000 000 generations, sampling every tenth, with a burn in of 5000 trees.

#### HISTOLOGICAL METHODS

Most specimens had been fixed, upon collection, in diluted formaldehyde (presumably approximately 5%), and subsequently preserved in 70% ethanol. Other specimens had been fixed in 95% ethanol and preserved in 70% ethanol. Most histological analyses were based on formalin-fixed material. Species used for

histological analysis and the corresponding voucher specimens are listed in Table 1.

Samples were re-fixed using a Susa mixture after Heidenhain (Romeis, 1968), alcohol dehydrated and embedded in paraffin or glycol metacrylate (Kulzer Hereaus). Serial sections were cut at 7 µm (paraffin) or 1–2 µm (metacrylate). A few samples were embedded in paraffin or metacrylate without re-fixation, to avoid loss of water-soluble polysaccharids. Sections were stained using: (1) nuclear fast red combination (Anken & Kappel, 1992) for general histology; (2) toluidine blue to detect acid mucopolysaccharids; and (3) periodic acid-Schiff to visualize neutral polysaccharides and glycoproteins.

## RESULTS

#### MOLECULAR PHYLOGENY OF MANTELLIDS

After exclusion of hypervariable sections of the 12S and 16S genes, the total data set comprised 3601 nucleotide positions. Of these, 1606 were constant and 1531 were parsimony-informative. The molecular tree obtained (Fig. 1) confirms previous topologies (Vences *et al.*, 2003; Glaw & Vences, 2006; Glaw, Hoegg & Vences, 2006) that have been obtained using largely different mantellid species, and/or a smaller set of genes and sequences. Following the taxonomic scheme of Glaw & Vences (2006), and focusing on the clades relevant for the understanding of femoral gland structure, the data unambiguously support monophyly of the subfamily Mantellinae. Within the Mantellinae, the data also support a clade consisting of the monophyletic genera *Gephyromantis* and *Mantidactylus*. In addition, the representatives of all genera recognized by Glaw & Vences (2006) and included in the present study formed monophyletic clades.

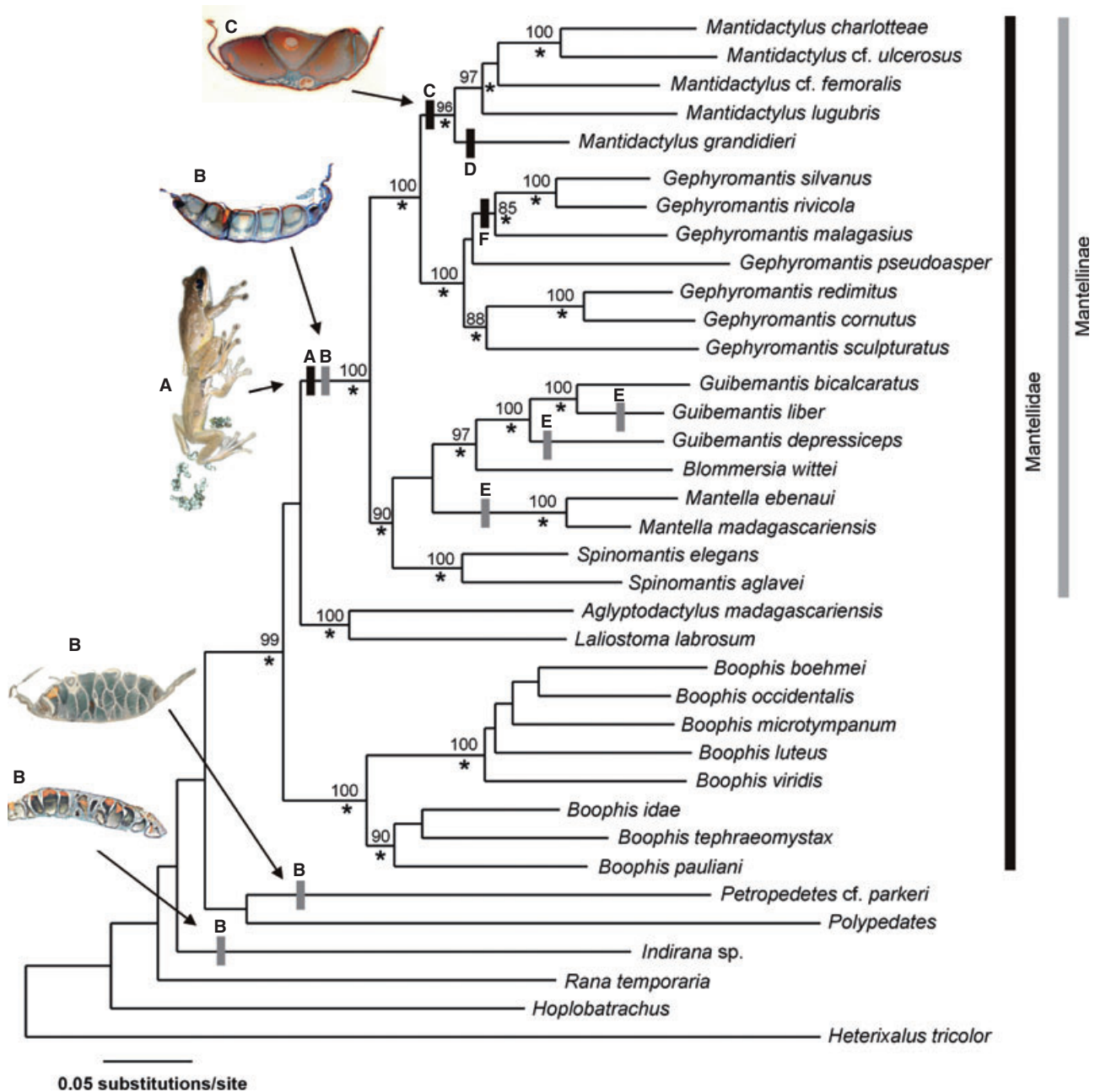
**Figure 1.** Maximum likelihood phylogram of mantellid frogs and a number of outgroup taxa, based on 3601-bp sequences of the mitochondrial 12S rRNA, 16S rRNA and cytochrome *b* genes, and the nuclear Rhod and Rag-2 genes, calculated under a GTR + I + G substitution model suggested by Modeltest. Numbers are support values in percent from a maximum likelihood bootstrap analysis (120 replicates). Asterisks indicate Bayesian posterior probabilities of 0.99–1 from a partitioned Bayesian analysis. Bars indicate relevant character state changes in femoral gland morphology (black, putative unique synapomorphies in the taxon set included here; grey, convergent character state changes); A, loss of strong mating amplexus (here documented in *Guibemantis tornieri*; also known from the femoral-gland bearing, unrelated, *Nyctibatrachus* (Nyctibatrachidae), not included here; unknown in *Indirana*); B, enlargement of some granular glands on the underside of shanks to form a macrogland patch ('femoral gland'; pictures show macroglands of *Blommersia wittei*, *Petropedetes cf. parkeri*, and *Indirana* sp.) composed of rather evenly sized glands; C, centripetal arrangement of most secretion ducts in macrogland, concentrated secretion in external central depression (picture shows macrogland of *Mantidactylus brevipalmatus*); D, loss of last enlarged glands with independent orientation of secretion ducts; E, size reduction of glands, and less compact arrangement of macrogland; many single glands, often rosette-like arranged; F, reduction in number of individual glands; macroglands often composed by less than ten relatively large single glands, but all with independent secretion. An alternative interpretation would assume that character E is acquired once in the *Mantella*–*Blommersia*–*Guibemantis* clade, and lost twice within the clade.



## SECRETION DUCTS AND PORUS ORIENTATION

The 'femoral glands' of mantellids consist of separate individual gland units, as is visible from external view (Fig. 2), and histological examination revealed that these differences extend beyond the number and size of individual glands. Our comparative data indicate the existence of two fundamentally different types of arrangement of the secretion ducts of each individual gland in mantellids. In most species, each gland functions as an independent secretion unit, which opens to the ventral surface of the shank in a separate porus (Fig. 3A, B). Each of these indepen-

dent glands corresponds to one macroscopic 'granule' in glands of types 1 and 2 as defined by Glaw *et al.* (2000). The histologically estimated diameter of glands corresponded with the measurements of 'granules' (0.13–1.5 mm; Glaw *et al.*, 2000). The number and size of these functionally independent glands were very variable. For example, in some species of *Gephyromantis*, they were reduced to two or three relatively large single glands on each shank, and they differed greatly in their arrangement into densely packed macroglands (gland type 2; Glaw *et al.*, 2000) or rosette-like groups (gland type 1;







**Figure 2.** Photographs of femoral glands in living mantelline frogs. The photos (not to scale) are ventral views of the thighs. The left row shows species in which the glands are composed by usually small single 'granules', each of which is a separate secretory unit according to histological data presented herein. The right row shows species in which the major or sole part of the gland (structure A; Glaw *et al.*, 2000) is a rounded structure in which circularly arranged granula secrete into a central external depression. A, *Mantella aurantiaca*; B, *Guibemantis liber*; C, *Guibemantis bicalcaratus*; D, *Gephyromantis pseudoasper*; E, *Gephyromantis cornutus*; F, *Gephyromantis luteus*; G, *Gephyromantis malagasius*; H, *Mantidactylus cf. ulcerosus*; I, *Mantidactylus cf. betsileanus*; J, *Mantidactylus albofrenatus*; K, *Mantidactylus brevipalmatus*; L, *Mantidactylus cf. femoralis*; M, *Mantidactylus argenteus*; N, *Mantidactylus grandidieri*.

Glaw *et al.*, 2000). The femoral glands of the nonmantellid frogs *Indirana* and *Petropedetes* corresponded to these general functional types as well. Figure 3C shows the secretion ducts of single glands of *Petropedetes*.

In a second type of macroglands, the single glands are circularly arranged around an externally recognizable central depression. Histological examination confirmed that each of the single glands ends in a particularly elongated duct leading to this central structure (Fig. 3D). Scanning electron microscopy pictures clearly reveal that the external pori in this kind of macroglands are all concentrated in the central external depression (Fig. 4). In conclusion, in this type of macrogland, the single glands probably together form a functional unit, all of them secreting through inward-directed ducts into one spot on the ventral shank. This kind of macrogland is accompanied, in most species, by additional glands (more towards the cloacal region) with independent secretion pori (structure 'B'; Glaw *et al.*, 2000; see also Von Eggeling, 1914a). Our data confirm the presence of such a macrogland secreting into one central area in all species of the genus *Mantidactylus* studied (Table 1), whereas no such structure was observed in any other species.

Most interestingly, the ventral skin of the shanks of *Boophis opisthodon*, a mantellid frog of the subfamily Boophinae that has no femoral glands visible in external or internal view, turned out to be rich in granular glands of very similar general structure, although of highly variable and generally smaller size than the glands in femoral gland clusters of mantellines (Fig. 5).

The observed femoral glands were multicellular, characterized by distinct myoepithelial cells and secretion through an apparently continuous duct. We found no evidence for bulk discharge in the glands examined, and therefore consider a merocrine/apocrine secretion mechanism as more likely in these glands.

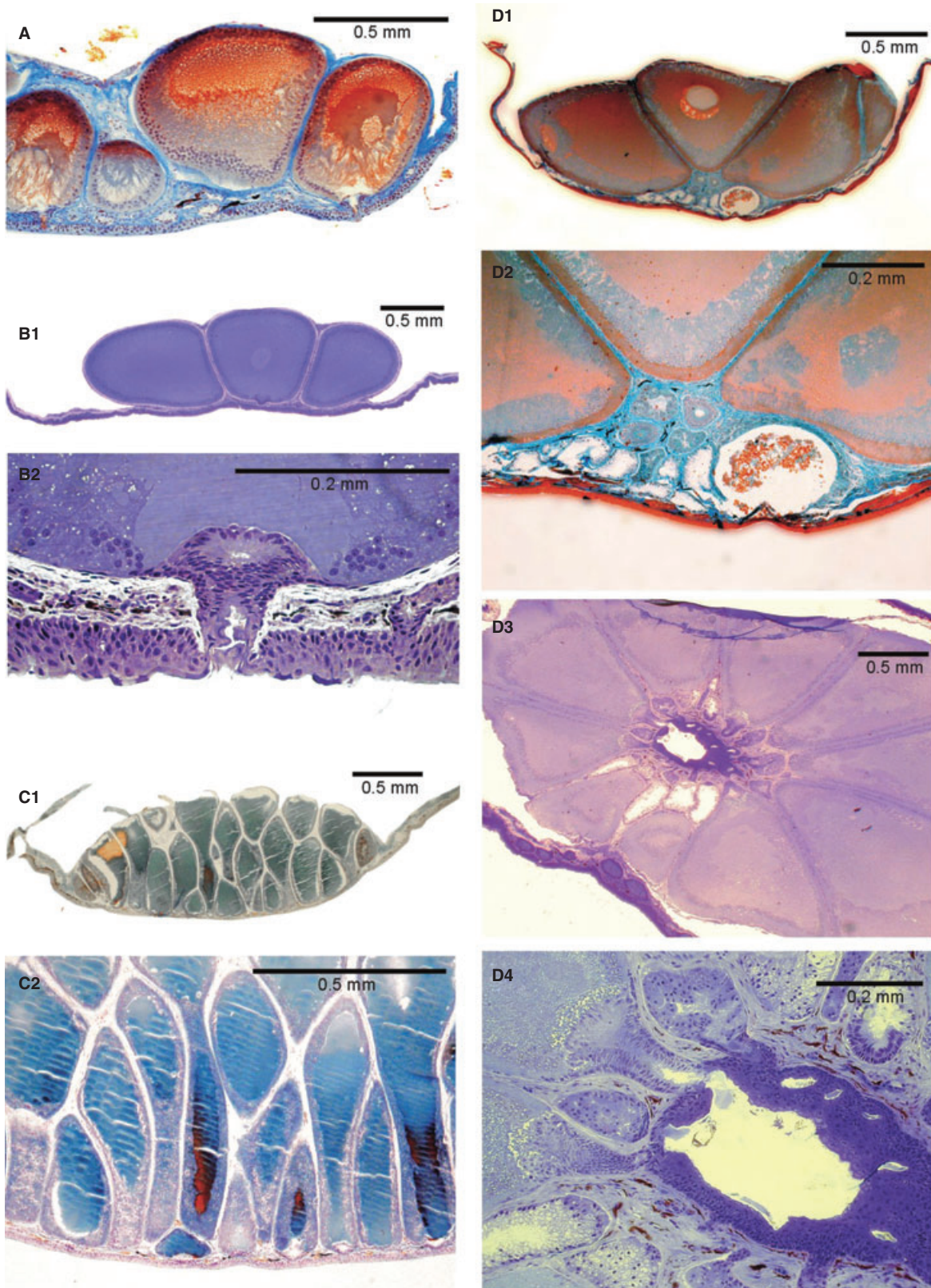
In our dataset, the appearance of the secretion product within glands of mantelline frogs was very different among taxa. Some congruence was observed among all studied species in the genus *Mantidactylus*, all of which were also characterized by a derived

arrangement of the secretory ducts (see below). These species showed in all cases within each gland a differently stained central mucous sphere. In all cases, the colloid-like secretion material showed less metachromasia with toluidine blue than did the secretory epithelium. Thus, it is highly unlikely that the material contained within the femoral glands of mantellid frogs contains acid mucopolysaccharides.

## DISCUSSION

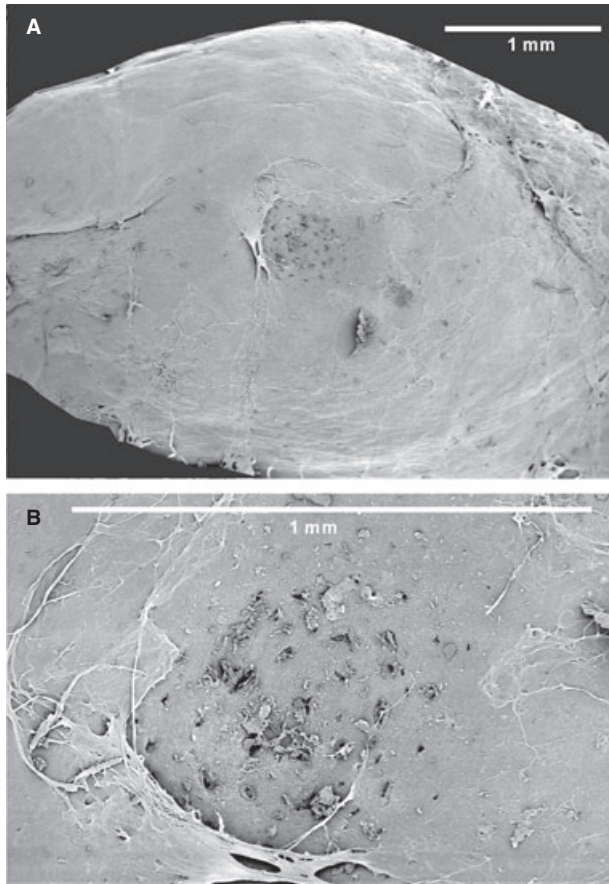
The only nuclear DNA sequences so far used to reconstruct intrafamilial relationships of mantellids were those of the approximately 300 bp portion of exon 1 of the rhodopsin gene (Vences *et al.*, 2003; Glaw & Vences, 2006; Glaw *et al.*, 2006). Although they provided some support for several deeper splits within the family, they contained an insufficient amount of informative sites to be phylogenetically unambiguous below the family level (M. Vences, unpubl. data). Hence, the molecular data presented in the present study add to the robustness of the phylogenetic tree of mantellids. The topology of the combined tree (Fig. 1), based on mitochondrial and nuclear DNA sequences, is largely congruent with that reconstructed on the basis of the newly-obtained Rag-2 sequences only (816 nucleotide positions; tree not shown).

Combining the molecular tree with the structural differences in femoral glands of mantellid and other frogs, several evolutionary conclusions and hypotheses can be drawn. On a deep phylogenetic level, all data available so far (Vences *et al.*, 2003; Roelants, Jiang & Bossuyt, 2004; Van der Meijden *et al.*, 2005; present study) are unambiguous in placing the femoral gland-bearing *Indirana* and *Petropedetes* into clearly different clades among ranoid frogs, not closely related to each other or to mantellids. This confirms that the glands in these three groups have evolved convergently. For at least one further unrelated, femoral gland-bearing group of frogs, the genus *Nyctibatrachus* (Nyctibatrachidae), the observations of Kunte (2004) indicate a mating behaviour very similar to that of mantellids (absence of strong mating amplexus, with the male positioned above the female on vertical leaves), which supports the hypothesis that the femoral glands may be related to this particular





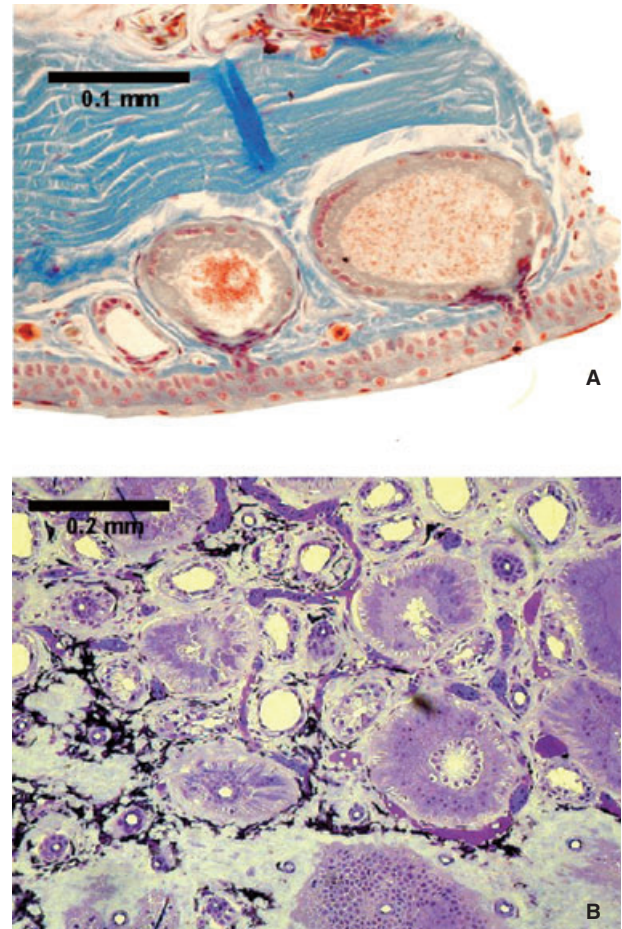
**Figure 3.** Histological sections of femoral macroglands in mantellid frogs and *Petropedetes*. The left column (A–C) shows the glands of species where each gland secretes independently; the right column (D) shows the derived glands of a species where glands secrete into a central external depression. All sections (except D3–D4) are transversely orientated with the ventral epidermis of the thigh orientated to the bottom. A, glands of *Blommersia wittei*, with secretion porus visible in the right gland. B, glands of *Gephyromantis rivicola*, with the secretion porus of the central gland enlarged in B2. C, glands of *Petropedetes* cf. *natalensis*. D, glands of *Mantidactylus brevipalmatus* (D1) representing an enlarged view of the area of the central external depression where the secretion pori of all glands converge. D1–D2, showing the entire macrogland and an enlarged view of the central depression with secretion pori in coronal view. A, C1–C2, D1–D2, after nuclear fast red (KKK) staining; B1–B2, D3–D4, after toluidine blue staining.



**Figure 4.** Scanning electron microscopy image of femoral macrogland of *Mantidactylus* cf. *ulcerosus* (specimen ZCMV 807) in external view, showing the concentration of secretion porus in a central depression. The second image (B) is an enlargement of the central depression (A).

mating behaviour that has first been described by Blommers-Schlösser (1975). Evolutionary shifts in the expression of the pheromone-producing mental glands, and associated behaviour, are also known in plethodontid salamanders (Houck & Sever, 1994).

Within the Mantellidae, our observations of the structural similarity of the femoral glands in the subfamily Mantellinae to the smaller and irregularly sized granular glands in the ventral skin of the shank



**Figure 5.** Histological sections of ventral skin of the shank of *Boophis opisthodon*, a mantellid frog of the subfamily Boophinae that is not characterized by obvious femoral glands in macroscopic view. The transverse (A) and frontal (B) sections show the presence of numerous granular glands of variable size, all very similar in general structure but distinctly smaller compared to those of which femoral macroglands are composed (Fig. 2). A, after nuclear fast red (KKK) staining; B, after toluidine blue staining.

of *Boophis* (subfamily Boophinae) support the hypothesis that the femoral glands evolved through size increase and possibly functional specialization of these granular glands. The general similarities in

histological structure of these glands with other amphibian macroglands (e.g. the probably poison-secreting tibial glands of the Australian *Limnodynastes*) (Crook & Tyler, 1981), corroborates that macroglands of different functions may have evolved numerous times from generalized granular glands.

The femoral glands of mantellids, unlike other sexually dimorphic amphibian skin glands, are structurally similar to granular glands, thus confirming the findings of Von Eggeling (1914a) and Thomas *et al.* (1993). Granular glands can be holocrine or merocrine (Delfino, Brizzi & Melis, 1996). However, due to their probable function during reproduction, it would be necessary to examine glands of males just before and after mating to fully understand the secretion mechanism.

According to the molecular phylogeny presented in the present study, *Guibemantis liber*, *Guibemantis depressiceps*, species of *Mantella*, and several related taxa are not basal within mantellines, and their femoral gland morphology (gland type 1; Glaw *et al.*, 2000), with single glands less compactly but rosette-like arranged, and smaller sized, must be considered as derived. This type of gland arrangement probably arose more than once in the evolution of this clade or underwent several reversals (Fig. 1). The opposite trend (increased size and reduced number of glands), however, is also observed in some taxa of the genus *Gephyromantis*.

Virtually all species of the genus *Mantidactylus* are semi-aquatic frogs and, according to our data, they are characterized by the most specialized femoral gland morphology. They appear to be unique in that their secretory ducts converge towards an external central depression. If femoral glands in mantellines are indeed related to the production of pheromones or other reproduction-related substances during mating, as is the case with the structurally not homologous femoral glands of lizards (Von Eggeling, 1914b; Cole, 1966; Alberts, 1991), then their special and apparently unique structure in *Mantidactylus* may allow the more precise and concentrated application of these substances under semi-aquatic conditions. More detailed behavioural studies on these frogs, and analyses of the functional compounds in the secretions of femoral glands, are the most crucial data needed to fully understand the evolutionary significance of these organs.

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## SUPPLEMENTARY MATERIAL

The following material is available for this article online:

**Table S1.** Voucher specimens and GenBank accession numbers of taxa used for molecular phylogenetic analysis. For collection acronyms, see Material and methods.

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