



## Descriptions of the tadpoles of two species of *Gephyromantis*, with a discussion of the phylogenetic origin of direct development in mantellid frogs

ROGER-DANIEL RANDRIANIAINA<sup>1</sup>, FRANK GLAW<sup>2</sup>, MEIKE THOMAS<sup>3</sup>, JULIAN GLOS<sup>4</sup>,  
NOROMALALA RAMINOSOA<sup>1</sup>, MIGUEL VENCES<sup>4\*</sup>

<sup>1</sup> Département de Biologie Animale, Université d'Antananarivo, Antananarivo, Madagascar

<sup>2</sup> Zoologische Staatssammlung, Münchenstr. 21, 81247 München, Germany

<sup>3</sup> University of Zurich, Institute of Zoology, Winterthurerstrasse 190, 8057 Zurich, Switzerland

<sup>4</sup> Technical University of Braunschweig, Spielmannstr. 8, 38106 Braunschweig, Germany

\* Corresponding author; m.vences@tu-bs.de

### Abstract

We describe the larval stages of two Malagasy frog species of the genus *Gephyromantis*, based on specimens identified by DNA barcoding. The tadpoles of *Gephyromantis ambohitra* are generalized stream-living Orton type IV type larvae with two lateral small constrictions of the body wall at the plane of spiracle. *Gephyromantis pseudoasper* tadpoles are characterized by totally keratinised jaw sheaths with hypertrophied indentation, a reduced number of labial tooth rows, enlarged papillae on the oral disc, and a yellowish coloration of the tip of the tail in life. The morphology of the tadpole of *G. pseudoasper* agrees with that of *G. corvus*, supporting the current placement of these two species in a subgenus *Phylacomantis*, and suggesting that the larvae of *G. pseudoasper* may also have carnivorous habits as known in *G. corvus*. Identifying the tadpole of *Gephyromantis ambohitra* challenges current assumptions of the evolution of different developmental modes in *Gephyromantis*, since this species is thought to be related to *G. asper*, a species of supposedly endotrophic direct development.

**Key words:** Amphibia; Anura; Mantellidae; *Gephyromantis ambohitra*; *Gephyromantis pseudoasper*; Madagascar; Larval morphology; Reproduction; Endotrophic development

### Introduction

In frogs, the conquest of a new adaptive zone via a highly derived suspension-feeding larva, the tadpole (Wassersug, 1975), may be seen as one of the factors favouring the evolution of the current enormous diversity, in terms of numbers of species and reproductive modes. A high diversity of eco-morphological adaptations of tadpoles is known (Altig and Johnston, 1989). Especially in the tropics, many lineages of frogs have independently evolved trends towards terrestriality, involving reduction of larval stages and direct development (Bogart, 1981; Duellman and Trueb, 1986; Thibaudeau and Altig, 1999). These endotrophic, direct developing anurans are independent of water bodies for breeding and therefore might not be restricted by some time and space constraints of pond-breeding or stream-breeding anurans.

Among the frogs of Madagascar, the family Mantellidae is by far the largest lineage, including 165 described and many undescribed species, with the subfamily Mantellinae being exceptionally diverse. Recently, a new classification divided the Mantellinae into eight genera, creating three new genera (*Boehman-tis*, *Tsingymantis*, *Wakea*) and raising four former subgenera of *Mantidactylus* to genus level (*Blommersia*, *Guibemantis*, *Spinomantis*, *Gephyromantis*) (Glaw and Vences, 2006; Glaw *et al.*, 2006).

The diversity of frogs in the Mantellinae is not restricted to mere species number but is also reflected in their large variety of reproductive modes. As far as it is known, all mantellines lay their eggs outside the water, sometimes just above the water surface (Glaw and Vences, 2003, 2006). Mantellines contain terrestrial or semi-aquatic frogs, mainly in the genus *Mantidactylus*, that deposit their eggs near the water on the ground or close to the ground and have tadpoles that often develop on the bottom of slow-moving streams or adjacent pools. Other mantellines are arboreal and lay their eggs on leaves above stagnant (*Guibemantis*, *Blommersia*) or running water (*Spinomantis*), the hatching tadpoles dropping into the water and completing their development there (e.g., Vejarano et al., 2006). Some species of *Guibemantis* and one *Mantella* lay eggs into leaf-axils and tree holes where their tadpoles develop (Blommers-Schlösser, 1979; Glaw et al., 2000, Heying, 2001; Lehtinen, 2003; Lehtinen et al., 2004). Finally, the genus *Gephyromantis* contains many terrestrial to semiarboreal species that seem to represent different stages of a trend of becoming independent from water, although their reproductive biology is very insufficiently known (Blommers-Schlösser, 1979; Glaw and Vences, 1994, 2003).

Within the genus *Gephyromantis*, direct development seems probable for the species of the subgenus *Gephyromantis* since calling adults are found in widely dispersed sites in the forest and are not concentrated around water bodies (Vences and Glaw, 2001). Accordingly, direct development has been described for one species of this subgenus: according to Glaw and Vences (1994), *Gephyromantis eiselti* deposits a few large eggs in leaf litter, and after 3–4 weeks small froglets hatch, without undergoing any larval development. In contrast, *Gephyromantis corvus* (in the subgenus *Phylacomantis*) is known to have free-swimming tadpoles with enlarged jaw sheaths and reduced numbers of labial tooth rows, which are known to prey upon other tadpoles (Glaw and Vences 1994). For *Gephyromantis granulatus* (now in the subgenus *Duboimantis*), only single larvae in highly advanced metamorphic stages have been found in streams, indicating that this species may have tadpoles that undergo early phases of development in hidden nests, or show similar derived reproductive modes. A second species of the subgenus *Duboimantis*, *Gephyromantis asper*, has been reported to be a direct developer (Blommers-Schlösser, 1979).

In the framework of a survey of larval diversity of Madagascan frogs we have screened tadpoles from a variety of sites in northern Madagascar using a DNA barcoding approach as described by Thomas et al. (2005) and Vences et al. (2005). Surprisingly, several tadpole series were unambiguously identified as belonging to *Gephyromantis ambohitra*, a species thought to be closely related to the purportedly direct-developing *G. asper*. Here we describe the morphology of the tadpoles of this species, and of *Gephyromantis pseudoasper*, a species of the subgenus *Phylacomantis* which turned out to agree in larval morphology with the related *G. corvus*.

## Materials and Methods

Tadpoles were collected in the field, euthanised by immersion in chlorobutanol solution, and immediately sorted into homogeneous series based on morphological characters. From each series one specimen was selected and a tissue sample from its tail musculature or fin taken and preserved in 99% ethanol. This specimen is here named "DNA voucher". All detailed tadpole descriptions and drawings are based on this DNA voucher, whereas variation is described based on further specimens of the series. After tissue collection, all specimens were preserved in 4% formalin. Specimens were deposited in the Zoologische Staatssammlung München, Germany (ZSM). We give the ZSM catalogue number of voucher specimens as well as the field numbers (FG/MV) applied of the same series.

Tadpoles were identified using a DNA barcoding approach based on a fragment of the mitochondrial 16S rRNA gene, which is known to be sufficiently variable among species of Malagasy frogs (Thomas et al., 2005). The ca. 550 bp fragment was amplified using primers 16Sa-L and 16Sb-H from Palumbi et al. (1991) applying standard protocols, resolved on automated sequencers, and compared to a near-complete database of

sequences of adult Malagasy frog species. Identification was considered to be unequivocal when the tadpole sequence was 99-100% identical to an adult specimen from the same geographical region, and not more similar to any sequence from another species. DNA sequences were deposited in Genbank (accession numbers DQ987502-DQ987518; accession numbers of comparative adult specimens are included in the sequence sets AY847959-AY848683 and AJ315909-AJ315913).

Tadpoles of *Gephyromantis ambohitra* were collected on 17 February 2003 at Montagne d'Ambre National Park. They were found in a slow-running shallow (depth about 20 cm) stream which crosses the way to the "gîte d'étape", near the road in open canopy (coordinates at stream not taken, but not far from 12°31'37"S, 49°10'19"E, 1050 m above sea level). Tadpoles of *Gephyromantis pseudoasper* were collected on 01 February 2003 at Manongarivo Special Reserve, at a campsite named by us campsite 0 (13°58'32"S, 48°23'36"E; 688 m). They were found in a shallow small pond downstream of a waterfall. The pond had a maximum depth of 30 cm and a surface of about 2 m<sup>2</sup>, and its bottom was rocky. Additional specimens of this species came from Manongarivo Special Reserve, campsite 1 (13°58'37"S, 48°25'19"E, 751 m), and Ankarana Special Reserve (coordinates not taken).

Developmental stages are described following Gosner (1960). Morphological measurements were taken by using a graduated ocular attached to a stereomicroscope, following landmarks, terminology and definitions of Altig and McDiarmid (1999). The formula of labial tooth rows (LTRF) is given according to Altig and McDiarmid (1999) and Dubois (1995).

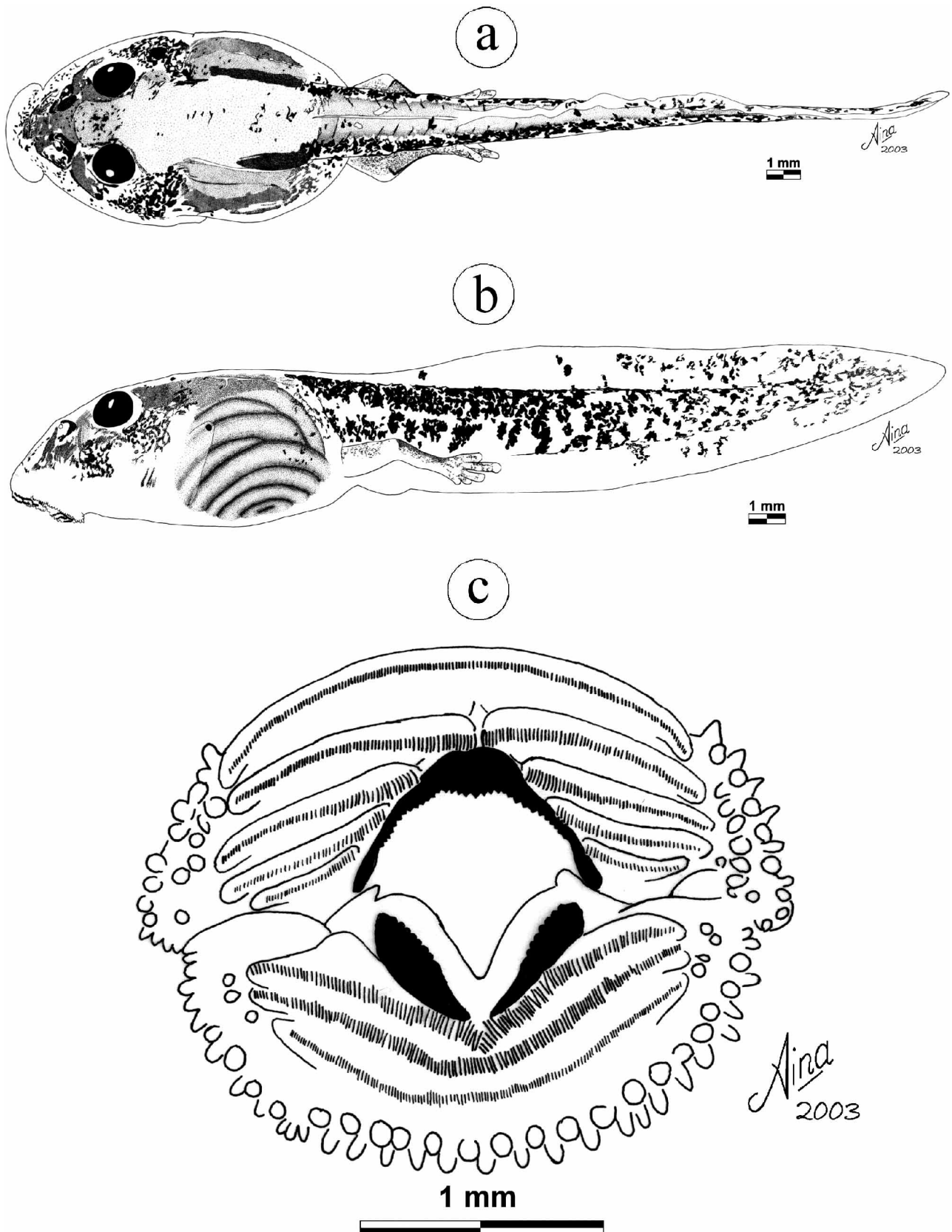
The following abbreviations were used: BH (maximum height of body), BL (body length), BW (maximum width of body), DG (maximum size of dorsal papilla gap), ED (maximum diameter of eye), MTH (maximum tail height), NN (internarial distance), NP (distance from centre of nares to anterior margin of eye), ODW (oral disc width), PP (interpupilar distance), RN (rostronarial distance), SS (distance from tip of snout to opening of spiracle), TMH (tail muscle height), TMW (tail muscle width), TL (total length).

## Results

### *Gephyromantis ambohitra* (Vences and Glaw, 2001)

The following description refers to one tadpole in developmental stage 37 (field number FG/MV 2002.1963, catalogued as ZSM 790/2004, TL 26.6 mm, BL 9.5 mm) (Fig. 1), from Montagne d'Ambre National Park. Generalized tadpole of type IV of Orton (1953). In dorsal view, body ovoid with small constrictions of the body wall at the plane of spiracle. Snout large, edge of anterior labium in front of the snout. In lateral view, body depressed, maximum width attained behind midbody, BW 122 % of BH. Eyes of moderate size, ED 12 % of BL, not visible in ventral view, positioned dorsally and directed dorsolaterally situated at about 1/3 of body length. Nares elliptical, moderately sized, with dark spots, positioned dorsally, oriented anterolaterally and equidistant between snout tip and eye. NN 64 % of PP. Spiracle sinistral with its inner wall free and formed such that the aperture opens laterally instead of posteriorly, visible in dorsal view. Spiracular opening round, positioned laterally, directed dorsolaterally, situated at the height of the longitudinal axis of caudal musculature and much closer to the end of the body than to the snout, SS 55 % of BL. Medial vent tube tubular, medial, with both walls attached directly to ventral fin. Caudal musculature moderately developed, myosepta visible in dorsal view in the proximal 3/5 of the tail. TMH 42 % of BH and 49 % of MTH, TMW 36 % of BW; its height 2/5 of total height at midlength of tail. Dorsal fin inserts at the tail muscle posterior to the dorsal tail-body junction, increases quickly to attain the maximum height, then diminishes gradually to the tail tip. Ventral fin begins at the ventral terminus of the body, is constant until midtail, diminishes quickly towards tail tip. Tail tip pointed. MTH 86 % of BH, lateral line visible on the proximal 2/5 of the tail. Oral disc relatively large, ODW 29 % of BL and 51 % of BW, positioned anteroventrally, directed anteroventrally, emargin-

ated. Two rows of marginal papillae, large medial gap anteriorly (DG 72 % of ODW), no medial gap on the posterior labium; total number of marginal papillae 79, submarginal papillae positioned in the lateral parts of



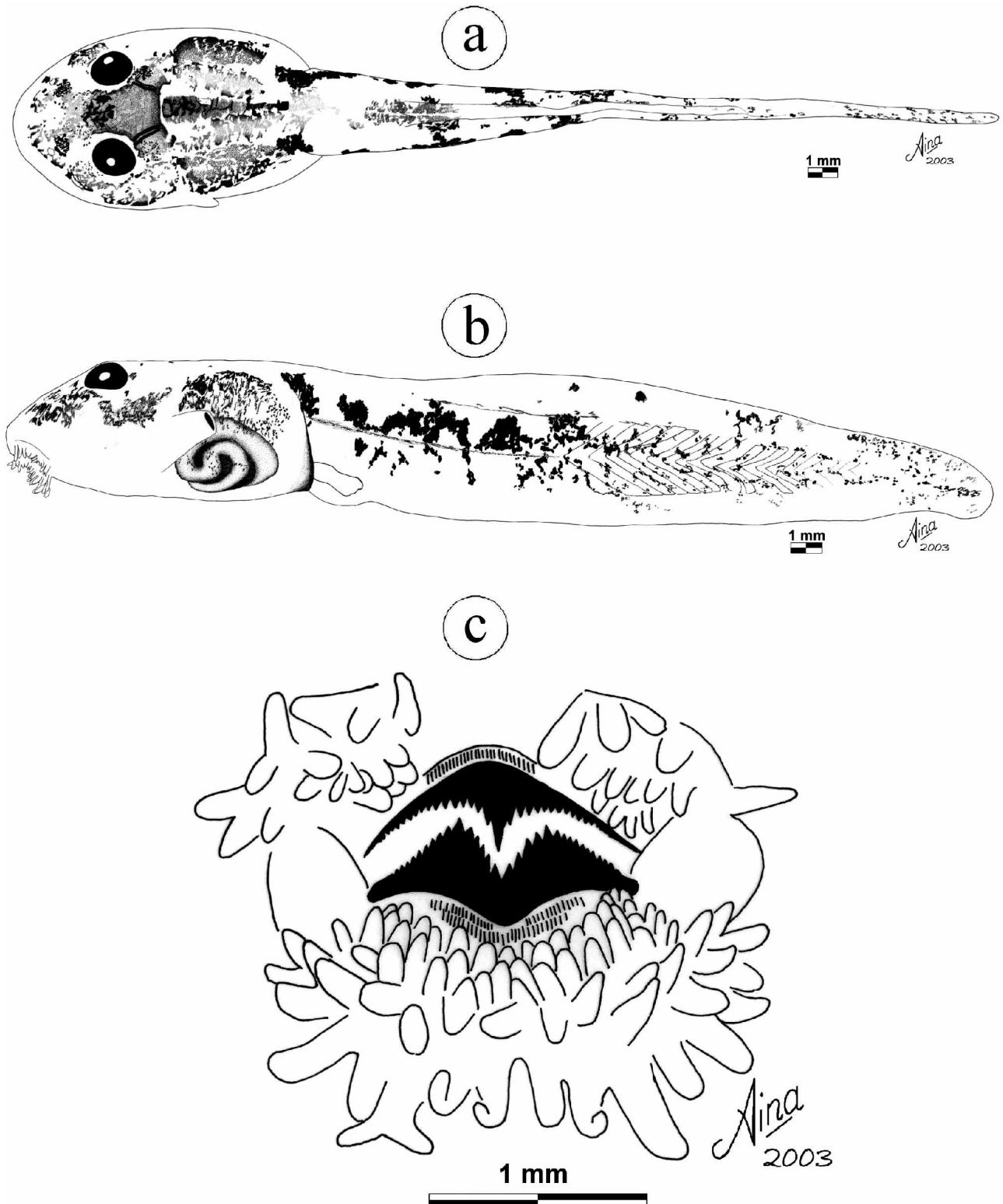
**FIGURE 1.** Drawings of preserved tadpole specimen of *Gephyromantis ambohitra* from the Montagne d’Ambre National Park (developmental stage 37). (a) dorsal view; (b) lateral view; (c) oral disc.

the anterior and posterior labia (9 on the right and 13 on the left side). Papillae of moderate size, round or conical with rounded tip. No denticulate papillae. Labial tooth row formula (LTRF) after McDiarmid and Altig (1999) 5(2-5)/3(1), after Dubois (1995) 1:4+4/1+1:2. The density of keratodonts on A1 is about 57 per millimetre (a total of about 100). The length of interrupted anterior keratodont rows (A2, A3 and A4) decreases gradually towards the centre of the disc. Rows A-1 and P-3 have small keratodonts, in the other rows, keratodont size declines from the centre to the periphery. Both jaw sheaths coarsely serrated, with three parts having different colorations: edge, totally keratinised (black); medial part, partially keratinised (brown); base, not keratinised (white). Upper jaw sheath medially with pointed serrations, each side of the sheath smoothly curved, inversed U-shaped opening with medial convexity. Lower jaw sheath V-shaped, rounded serration on each edge, medial smoothly curved arc. General coloration in life brownish. Coloration in preservative: Dorsally body with two different pigmentation patterns: first, speckled with differently sized and irregularly formed brown spots; second, irregular pigmentation that forms a network of patches in some areas; intestinal coils and spiracle well visible; tail musculature speckled with brown spots that are irregularly distributed on each side, forming a network of spots; laterally body speckled brownish with network of brown pigments below the eyes. Caudal musculature whitish, with large networks of irregularly distributed brown spots, size and density of spots diminishing towards the tail tip. Fins translucent, with brown variable sized spots that are irregularly distributed on the fins, density of spots higher in the upper fin. Ventrally translucent over the whole surface. Variation: TL and BL of 90 tadpoles at stages 30-44, all from Montagne d'Ambre National Park (field numbers: FG/MV 2002.1926, 1940, 1946-1949, 1954-1956, and 1963-1964, catalogued as ZSM 717, 743-746, 756-761, 769-773, 790-795/2004) are 18.7-30.8 mm and 5.4-9.7 mm, respectively. The ratios vary in the following proportions: BW 106-134 % of BH; ED 11-24 % of BL; SS 14-72 % of BL; TMH 45-96 % of MTH; TMW 34-72 % of BW; MTH 70-114 % of BH; ODW 17-33 % of BL; ODW 33-60 % of BW.

### ***Gephyromantis pseudoasper* Guibé, 1974**

The following description refers to one tadpole in developmental stage 34 (field number FG/MV 2002.1920, catalogued as ZSM 709/2004, TL 34.0 mm, BL 10.7 mm) (Fig. 2), from campsite 0 in Manongarivo Special Reserve. Tadpole of type IV of Orton (1953). In dorsal view, body ovoid elongated. Snout large. In lateral view, body depressed, maximum width attained behind midbody, BW 133 % of BH. Eyes of moderate size, ED 14 % of BL, not visible in ventral view, positioned dorsally and directed dorsally, situated at about 1/3 of body length. Nares elliptical, moderately sized, with dark spots, positioned dorsally, oriented dorsolaterally and closer to snout than to eye. NN 68 % of PP. Spiracle sinistral, inner wall free from body and formed such that aperture opens posteriorly, visible in dorsal view, spiracular opening oval, positioned laterally, directed dorsolaterally, situated at the height of the longitudinal axis of caudal musculature and much closer to the end of the body than to the snout, SS 65 % of BL. Medial vent tube with tissue between tube and fin, tubular. Caudal musculature well developed, myosepta visible in lateral view in the posterior half of the tail, not reaching the tail tip. TMH 69 % of BH and 66 % of MTH, TMW 46 % of BW; its height about 50 % of total height at midlength of tail. Dorsal fin height about 1/6 of tail muscle height at tail muscle insertion, getting wider quickly to maximum height, then diminishing gradually towards the tail tip. Ventral fin begins at the ventral terminus of the body; increases meticulously, then constant up to 3/4 of its length, finally diminishes meticulously towards the tail tip, tail tip rounded. MTH 105 % of BH, lateral line visible in the proximal half. Oral disc of moderate size, ODW 26 % of BL and 43 % of BW, positioned anteroventrally, directed anteroventrally, not emarginated, general shape is an open square. Marginal papillae dorsally interrupted by a small gap on the upper labium (DG 27 % of ODW), ventrally uniserial, no medial gap on the lower labium; total number of marginal papillae about 30. Many submarginal papillae positioned in the dorsolateral parts of the anterior labium and in the ventral part of the posterior labium. Papillae large and elongated with rounded tip. No den-

ticulate papillae. LTRF after McDiarmid and Altig (1999) 1/2(1), 1/1+1:1 after Dubois (1995). The density of keratodonts on A1 is about 46 per millimetre (a total of 28); keratodonts of similar size. Both jaw sheaths totally keratinised with hypertrophied indentation. Upper jaw sheath wide, strongly curved with strong medial



**FIGURE 2.** Drawings of preserved tadpole specimen of *Gephyromantis pseudoasper* from Manongarivo Special Reserve (developmental stage 34). (a) dorsal view; (b) lateral view; (c) oral disc.

convexity. Lower jaw sheath M-shaped. General coloration in life: brownish, distal half of tail yellowish. Coloration in preservative: Dorsally body with two different pigmentation patterns: first, small round brownish spots that are irregularly distributed and condensed in some areas creating a network of spots, second, dark brownish and variable formed spots that are irregularly distributed; tail musculature speckled brownish at the proximal part of the fin, large brown networks of spots irregularly distributed on each side. Laterally: body speckled brownish and with networks of brown spots. Intestinal coils and spiracle well visible; caudal musculature whitish, with networks of brown spots that are irregularly distributed in the tail musculature but condensed in the proximal half of the tail, size and density diminishing towards tail tip. Fins transparent with different sized networks of brown spots that are irregularly distributed on the fins, spots more abundant on the dorsal fin. Ventrally: translucent. Variation: TL and BL of 56 tadpoles at stages 27-44, from campsite 0 (field numbers: FG/MV 2002.1915, and 1918-1921, catalogued as ZSM 698, 706-711/2004) and campsite 1 in Manongarivo Special Reserve (FG/MV 2002.1932, catalogued as ZSM 729-730/2004), and from Ankarana Special Reserve (FG/MV 2002.1937, catalogued as ZSM 734, 736/2004) are 15-49 mm and 5.2-13.5 mm, respectively. The ratios vary in the following proportions: BW 87-146 % of BH; ED 12-21 % of BL; RN 20-95 % of NP; NN 44-79 % of PP; SS 48-73 % of BL; TMH 39-75 % of MTH; TMW 37-65 % of BW; MTH 60-120 % of BH; ODW 18-43 % of BL; ODW 31-70 % of BW.

## Discussion

### *Tadpole morphology in Gephyromantis*

The larvae of *Gephyromantis ambohitra* and *G. pseudasper* show a generalized external morphology of their body. The oral disc is generalized in *G. ambohitra* but more specialized in *G. pseudoasper*. The two species differ in the number of tooth rows on the upper and lower labium (LTRF of *G. pseudoasper* 1/1+1:1; *G. ambohitra* 1:4+4/1+1:2; after Dubois 1995). In both species we found no evidence for ontogenetic change of tooth row number during larval development. Furthermore, the tadpoles of *G. ambohitra* have two lateral small constrictions of the body wall at the plane of spiracle. *Gephyromantis pseudoasper* tadpoles are distinguishable by jaw sheaths that are totally keratinised with hypertrophied indentation and by the conspicuous yellow coloration of the tip of tail in life. The latter character is most striking in living animals. It resembles the remarkable tail coloration in *Gephyromantis corvus*, the second species included in the subgenus *Phylacomantis* according to Glaw and Vences (2006) and for which the larvae have been described previously (Glaw and Vences 1994). Although this previous description is relatively brief and less detailed than our description of tadpoles of *G. pseudoasper*, a high general morphological similarity is obvious among both species. However, they differ in their tooth row formula (*G. corvus*: 1:1+1/1+1:1). The general similarity in larval morphology of the two species included in *Phylacomantis* supports the subgeneric grouping, whereas the apparent differences in tooth row formula confirms the specific status of these probably allopatrically distributed species (*G. corvus* occurring in Isalo, south-western Madagascar, and *G. pseudoasper* occurring in northern Madagascar).

### *Larval adaptations in the subgenus Phylacomantis*

So far, free-swimming tadpoles in *Gephyromantis* were only known from *G. corvus*, a species of the subgenus *Phylacomantis*, and our description of the tadpole of *G. pseudoasper* confirms the particular oral morphology in this lineage. Glaw and Vences (1994) observed that *G. corvus* tadpoles reacted aggressively towards approaching conspecific tadpoles, and that they consumed tadpoles of other anuran species as food. The detailed description of external oral morphology of the tadpole of *G. pseudoasper* suggests that this species is characterized by similar eco-ethological adaptations. Although relations with carnivorous habits are likely for the enlarged jaw sheaths with strong and distinct serrations, and possibly for the reduction of labial

tooth rows, the function of the enlarged papillae in the oral disc is less obvious and can only be elucidated through more detailed behavioural observations.

#### *Evolution of direct development in mantellid frogs*

In mantellids, earlier works have assumed that most *Gephyromantis* have direct development (e.g., Blommers-Schlösser and Blanc, 1991, for the species included in the *Mantidactylus granulatus* species group by these authors). Thibaudeau and Altig (1999) distinguish six guilds of endotrophic anurans and conclude that, if endotrophy occurs in a genus, usually all species involved are in the same guild. *Gephyromantis*, as far as known, is the only genus in the Mantellidae with endotrophic species. It contains both direct developing species and species with a free-swimming larval stage, and is a monophyletic group (Glaw and Vences, 2006; Glaw *et al.*, 2006). Therefore it can be concluded that the evolution of endotrophy in the Mantellidae took place within this lineage, but at present we cannot trace any evolutionary scenario due to the uncertainties surrounding the identification of several of the described embryos and larvae, and the only weakly supported phylogenetic data available.

Blommers-Schlösser (1979) described endotrophic development in *Gephyromantis asper* based on direct-developing eggs found on a branch next to a calling male of this species. Therefore, the attribution of the eggs to *G. asper* requires confirmation. The same applies to the record of direct development in *G. eiselti* by Glaw and Vences (1994) where a clutch was found close to calling males. The attribution of larval stages to *G. granulatus* and *G. corvus* (Glaw and Vences, 1994) was based on morphological similarity of the reared juveniles to the adults, and is probable because they were collected in areas where only a relatively limited numbers of mantellid species co-occur. However, except for cases where eggs from well-identified reproducing adults are reared, molecular genetic methods of identification provide the most reliable means to identify tadpoles. DNA barcoding has proven to be an excellent method to identify larval stages of a variety of organisms to species (e.g., Hebert *et al.*, 2004), and in the present paper we used this technique to ascertain the larval morphology of a species belonging to the subgenus *Phylacomantis* (*G. pseudoasper*), and of a representative of *Duboimantis* (*G. ambohitra*). These attributions are therefore to be considered as the most reliable ones within the genus *Gephyromantis*.

According to the molecular phylogeny of Glaw *et al.* (2006) based on multiple gene sequences, *G. pseudoasper* is not the most basal representative of *Gephyromantis*, although bootstrap support for this conclusion was low. The phylogenetic hypothesis of Vences and Glaw (2001), based on a single mitochondrial gene only, indicated that *G. ambohitra* is relatively basal in the clade that is now considered to be the subgenus *Duboimantis*, but a clade containing the putatively endotrophic *G. asper* splits off the tree before. The available data therefore does not allow for a conclusive evolutionary scenario for the origin of endotrophy in mantellids, but the weak evidence available so far would be rather in accordance with a scenario of homoplastic evolution with parallel evolution and/or reversals of endotrophy, or at least of reproductive modes that include non-free-swimming tadpoles.

The generalized free-swimming tadpole in *G. ambohitra* is the first such larva reported in *Gephyromantis*. Whether endotrophy in mantellids evolved from ancestors with such unspecialized larvae, or via tadpoles with carnivorous adaptations such as those of *G. pseudoasper* and *G. corvus*, can only be inferred by better supported molecular phylogenies in conjunction with the discovery of reproductive mode and larval morphology of more species in the genus.

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