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The radiation of microhylid frogs (Amphibia: Anura) on New Guinea: A mitochondrial phylogeny reveals parallel evolution of morphological and life history traits and disproves the current morphology-based classification

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Abstract

Microhylidae account for the majority of frog species on New Guinea and have evolved an extraordinarily wide range of ecological, behavioural, and morphological traits. Several species are known for their unique paternal care behaviour, which includes guarding of clutches in some and additional froglet transport in other species. We sampled 48 out of 215 New Guinean microhylid species and all but two (*Mantophryne* and *Pherohapsis*) of 18 New Guinean genera and analysed a concatenated data set of partial sequences of the mitochondrial genes 12S and 16S, which comprises 1220 aligned nucleotide positions, in order to infer the phylogenetic relationships within this diverse group of frogs. The trees do provide resolution at shallow, but not at deep branches. Monophyly is rejected for the genera *Callulops*, *Liophryne*, *Austrochaperina*, *Copiula*, and *Cophixalus* as currently recognized. Six clades are well supported: (1) *Hylophorbus* and *Callulops* cf. *robustus*, (2) its sister taxon comprising *Xenorhina*, *Asterophrys turpicola*, and *Callulops* except for *C. cf. robustus*, (3) *Liophryne rhododactyla*, *L. dentata*, *Oxydactyla crassa*, and *Sphenophryne cornuta*, (4) *Copiula* and *Austrochaperina*, (5) *Barygenys exsul*, *Cophixalus* spp., and *Oreophryne*, (6) *Cophixalus sphagnicola*, *Albericus laurini*, and *Choerophryne*. The phylogenies provide evidence for the parallel evolution of parental care modes, life styles, and morphological traits that have thus far been emphasized in recent classifications.

Keywords: Microhylidae; Asterophryinae; Systematics; Parallelism; Paternal care; Life style

1. Introduction

Microhylidae represent one of five families of native New Guinean anurans, but account for the majority of frog species on this landmass and its satellite islands. Current treatments recognize 18 microhylid genera, of which only 4–5 are not endemic in New Guinea (Frost, 2007). This classification rests, however, exclusively on morphological and behavioural characters (Zweifel, 1972, 2000; Burton, 1986; Zweifel et al., 2003, 2005; Menzies, 2006). Because New Guinean microhylids have evolved an extraordinarily wide range of ecological and morphological adaptations in association with various life styles from burrowing in the ground to dwelling in canopy habitats, it remains doubtful if the morphology-based classification truly reflects their phylogenetic relationships. In other anuran groups it was found that morphological characters are particularly prone to homoplasy when they are associated with the possession of distinct life styles that may have been acquired in parallel (Emerson, 1986; Bossuyt and Milinkovitch, 2000). In addition, purely morphology-based classifications of amphibians have frequently been misled by plesiomorphic traits, such as in salamanders (Wiens et al., 2005) or gymn-

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ophionans (San Mauro et al., 2004). Wake (1991) discussed design limitations in amphibians as one possible reason for this phenomenon.

Australopapuan microhylid frogs are of special interest because they possess unique and derived forms of reproduction. All species develop directly from eggs into four-limbed froglets, skipping the aquatic tadpole stage. Many species deposit their eggs either in holes in the ground, among leaf litter, in funnels of epiphytes, or attach their clutch to leaves (Zweifel, 1972; Bickford, 2002; Günther, 2006). In addition, several species developed parental care. Simpler forms involve guarding of terrestrial or arboreal clutches in species of the genera *Oreophryne*, *Callulops*, *Cophixalus*, *Hylophorbus*, and *Xenorhina* (Simon, 1983; Price, 1992; Johnston and Richards, 1993; Günther, 2006), while in some remarkable cases hatchlings are carried thereafter by their father (Günther et al., 2001; Bickford, 2002, 2004; Günther, 2006). Froglet transport was reported from several species, such as *Oreophryne* cf. *wapoga*, *Sphenophryne cornuta*, *Aphantophryne pansa*, *Liophryne schlaginhaufeni*, *Callulops pullifer*, as recently reviewed by Günther (2006). Our knowledge of the mating behaviour and reproductive strategies of Papuan microhylids is, however, still sketchy and our understanding of the factors that drive evolution of parental care remains unsatisfactory. In addition, hypotheses of the evolution of different behavioural and morphological traits suffer badly from the absence of a well-resolved phylogeny of this neglected group.

Bickford (2004) suggested that microhabitat-specific selection pressures cause the evolution and maintenance of parental care in these frogs. However, we do not attribute the evolution of parental care in the New Guinean Microhylidae to the habitat alone. Froglet transport is known from several New Guinean species that are apparently not closely related to each other. Therefore, we hypothesize that parental care has evolved several times in parallel. This implies that as well as environmental factors, certain intrinsic factors inherent to all or most Australopapuan microhylids are also important. The identity of these factors, however, remains equivocal.

It is the goal of the present study to uncover the phylogenetic relationships among the New Guinean Microhylidae by analysing mtDNA trees that are based on a concatenated data set of partial sequences of the ribosomal genes 12S and 16S. In particular, we want to evaluate the value of certain morphological and behavioural traits with regard to their suitability for the delimitation of taxa and to scrutinize whether the current morphology-based classification also receives support from a molecular perspective. In addition, we address the question of whether certain modes of parental care are indeed randomly distributed across the phylogenetic tree, as suggested by the current systematics, or if the development of particular strategies is perhaps restricted to certain (as yet unrecognized?) lineages. Answering this question will help to understand the evolutionary mechanisms that led to the development of these remarkable behaviours.

2. Materials and methods

2.1. Examined material

The study is based on specimens collected at various localities in the Indonesian part of New Guinea (Papua Province) between 1997 and 2003 by Rainer Günther (details on localities and circumstances in Günther, 2001, 2002; Günther and Richards, 2005; Günther and Knop, 2006). At present the specimens are housed in the Herpetological Collection of the Museum für Naturkunde, Humboldt-Universität, Berlin (ZMB). Additional tissue samples were obtained from Fred Kraus (Bishop Museum, Honolulu) and Stephen Richards (University of Adelaide). This data set of our own sequences was complemented by sequences obtained from GenBank (Table A.1, Appendix). The use of taxonomical names follows the classification suggested by Frost (2007).

2.2. Codens of museum repositories and field codes

ABTC—Australian Biological Tissue Collection, South Australian Museum, Adelaide; AMCC—Ambrose Monell Cryo-Collection, American Museum of Natural History, New York; AMNH—American Museum of Natural History, New York; AMS—Australian Museum, Sydney; ATH—Andrew T. Holycross field series; BPBM—Bishop Museum, Honolulu; CFBH-T—Célio F.B. Haddad tissue collection; CMNH—Cincinnati Museum of Natural History; FK—Fred Kraus collection field numbers; FMNH—Field Museum, Chicago; RdS—Rafael de Sá collection; RG—Rainer Günther collection field numbers; SR—Stephen Richards collection field numbers; TNHC—Texas Natural History Collections, Texas Memorial Museum, Austin; USNM—United States National Museum, Smithsonian Institution, Washington DC; ZMB—Museum für Naturkunde, Humboldt University, Berlin.

2.3. DNA isolation and sequencing

Pieces of muscle tissue taken from specimens in the field were preserved in 75% ethanol. DNA was extracted from tissues that were soaked in water overnight, dried, and macerated in 300 µl lysis buffer containing 10 µl Proteinase K. This solution was incubated for 4 h at 60 °C. Total DNA was extracted by use of a Qiagen DNA extraction kit following the standard protocol for animal tissues. PCR amplifications were conducted in 25 µl volumes containing 1× PCR buffer, 200 µM each dNTP, 2.0 mM $MgCl_2$, 0.5 µM each Primer, 1.25 U of Taq polymerase (Invitek), and approximately 50 ng of DNA.

After an initial denaturation step of 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C were performed, followed by a final extension step of 5 min at 72 °C.

The two fragments used, the mitochondrial 12S and 16S genes, were amplified and sequenced using the primers 12SA-L (Palumbi et al., 1991) and L2519 (Richards and Moore, 1996) for 12S and 16S-L (TCGAACTTAGAGAT AGCTGGTT) and 16S-H (GCGAATGTTTTTGGTAA ACA) for 16S. PCR products were directly sequenced using PCR primers and BigDye terminator chemistry on a 3130XL Genetic Analyser (Applied Biosystems).

2.4. Sequence alignment and phylogenetic analyses

Sequences were aligned using ClustalX version 1.8.1 (Thompson et al., 1994) in the multiple alignment routine using default settings and 'accurate search'. Obvious alignment errors in this computerized alignment were manually corrected. Substitution saturation was estimated by plotting pair-wise rates of transitions and transversions against sequence divergence using the software DAMBE version 4.1.19 (Xia and Xie, 2001). Phylogenetic trees were reconstructed by employing Maximum Parsimony (MP, e.g. Fitch, 1971) using PAUP* version 4.0 b12 (Swofford, 2002), Maximum Likelihood (ML, Felsenstein, 1981) using Treefinder version June 2007 (Jobb et al., 2004), and Bayesian Inference (BI, e.g. Yang and Rannala, 1997) using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). Sequenced fragments of the mt 12S and 16S rRNA genes were merged into one concatenated sequence file and analyses were conducted for two differently composed data sets. First, a more comprehensive data set was analysed that comprised sequences of the New Guinean taxa as well as of microhylid frogs of other subfamilies from areas outside New Guinea, i.e. Microhylinae (*Ctenophryne*, *Dasyops*, *Elachistocleis*, *Hamptophryne*, *Kaloula*, *Kalophrynus*, *Gastrophryne*, *Microhyla*), Dyscophinae (*Caluella*, *Dyscophus*), Phrynomerinae (*Phrynomantus*), Cophylinae (*Platypelis*, *Stumpffia*), and Brevicipitinae (*Callulina*). These additional sequences were used as outgroups to root the tree. A second, more restricted data set contained sequences of only the New Guinean microhylids and *Kaloula* as an outgroup representative. The parsimony analyses were conducted under the option 'heuristic search' with 10 random stepwise additions and TBR branch swapping. Zero-length branches were collapsed and gaps were treated as a fifth base. Subsequently, bootstrap analyses (Felsenstein, 1985) with 5000 replicates were performed under the option 'fast and stepwise addition' to evaluate the robustness of the MP trees.

Prior to the model-based analytical approaches, the model of DNA evolution that best fits the sequence data was explored. For this purpose, a hierarchical likelihood ratio test using log likelihood scores to test the goodness-of-fit of nested substitution models was performed as implemented in the software MrModeltest (Nylander et al., 2004). In the following ML and BI analyses the substitution models and parameters were adjusted according to the estimates of MrModeltest. Bayesian Inference was used to estimate the posterior probabilities of phylogenetic trees by employing a 5,000,000 generations Metropolis-coupled Markov chain Monte Carlo (4 chains, chain temperature = 0.2) as implemented by MrBayes with the model specifications as indicated by MrModeltest and the parameters estimated from the data sets. Sampling rate of the trees was 100 generations. The Bayesian trees sampled for the last 500,000 generations were used to construct a 50%-majority rule consensus cladogram. The proportion of bifurcations found in this consensus tree are given as posterior clade probabilities (bpp, Larget and Simon, 1999) as an estimator of the robustness of the BI trees. Maximum likelihood trees were computed using the model of sequence evolution revealed by MrModeltest. 500 ML bootstrap replicates were performed to evaluate the nodal support of the ML topology.

3. Results

With respect to the taxon sampling, two differently composed data sets were analysed. A more comprehensive data file consisted of 145 aligned sequences that represent six different subfamilies as mentioned above. Twenty-one of these represented species from outside New Guinea that were used as outgroups to root the trees (Table A.1). All phylogenetic analyses (ML, MP, BI) conducted for this data set revealed a New Guinean clade (trees not shown). In order to reduce the degree of homoplasy within the sequence alignment, all analyses were repeated using a more restricted data set that contained 127 aligned sequences of only New Guinean species including three sequences of *Kaloula* as an outgroup. This data set had a total length of 1220 characters (687 bp of 12S and 533 bp of 16S). Inspection of the alignment led to the identification of 77 ambiguous alignment positions that were omitted prior to the phylogenetic analyses. Thus, the analysed data set contained 1043 characters of which 443 were constant and 532 variable and parsimoniously informative. A hierarchical likelihood ratio test revealed the generalized time reversible model (GTR + I + Γ) as the best fit model of sequence evolution. Plotting pair-wise rates of transitions and transversions against sequence divergence calculated under the most complex model revealed a nearly linear regression and thus did not indicate a considerable level of sequence saturation.

The trees gained by application of the different analytical methods (ML, BI, MP) were widely consistent with respect to delimitation of various clades among the New Guinean Microhylidae. In this respect, the topologies of the ML tree and the BI consensus tree were more similar

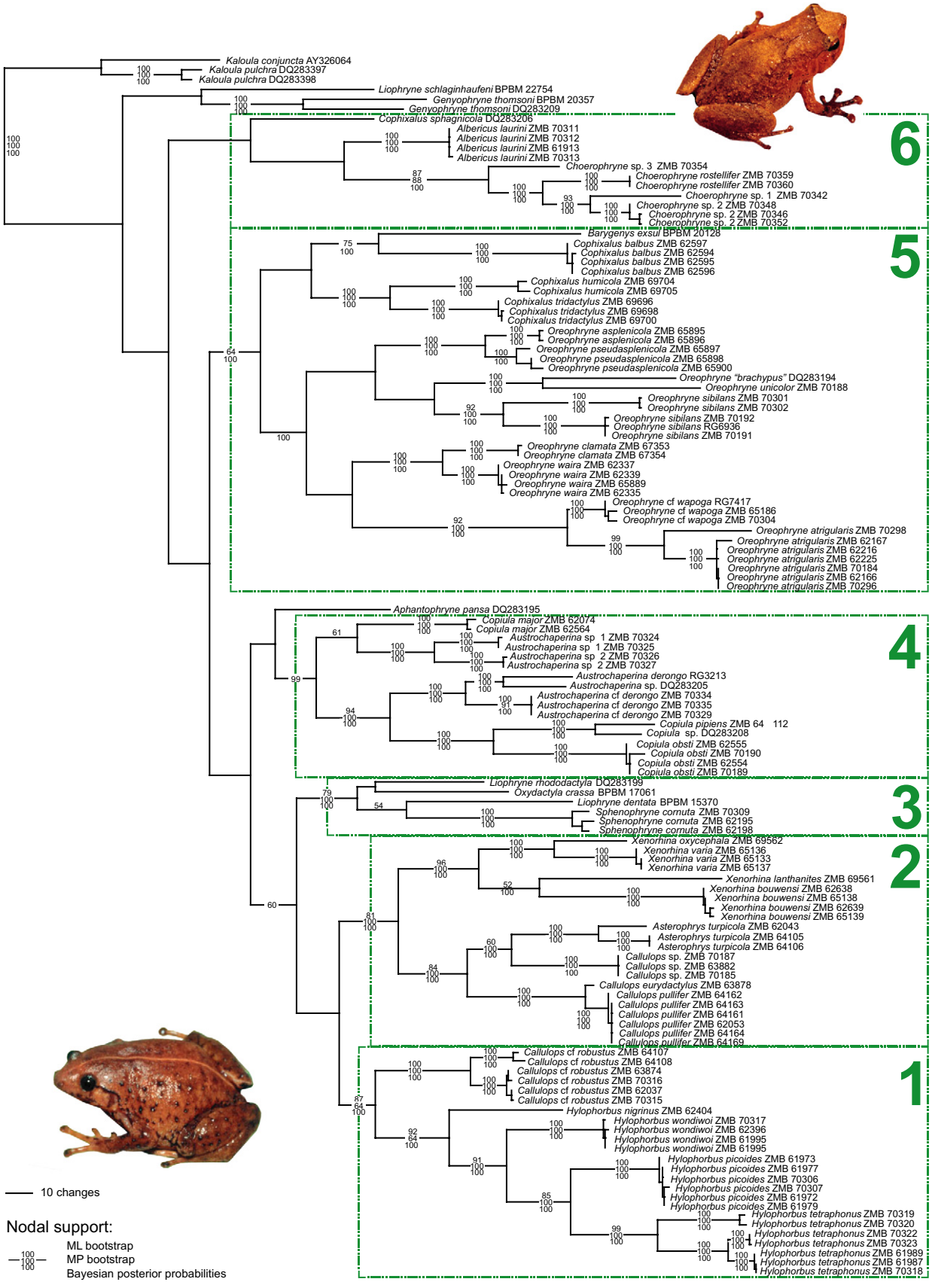


Fig. 1. Maximum likelihood phylogram based on concatenated 12S and 16S rDNA sequences. Numbers above branches indicate support of the shown topology by 500 ML bootstrap replicates, numbers on branches the support by 5000 MP bootstrap replicates, and numbers below branches the support by Bayesian posterior clade probabilities. Missing numbers indicate values <50. Boxes numbered 1 to 6 delimit clades that are concordantly revealed in the ML and BI tree.

to each other than either one was to the MP strict consensus cladogram (2005 equally parsimonious trees; 4086 steps; consistency index CI = 0.284; retention index RI = 0.714; rescaled consistency index RC = 0.203). Six clades were consistently recovered by the BI and ML analyses (Fig. 1, Nos. 1–6). Except for group four, these clades are also found in the MP strict consensus tree. The branching pattern within each of the six groups is concordant in all trees and receives substantial nodal support by means of bootstrapping or Bayesian posterior probabilities. Accordingly, *Hylophorbus* spp. and *Callulops* cf. *robustus* are sister taxa (group 1) closely related to group 2 (*Xenorhina* spp., *Asterophrys turpicola*, *Callulops* spp.) and group 3 (*L. rhododactyla*, *L. dentata*, *Oxydactyla crassa*, *S. cornuta*). *Apantophryne pansa* is the sister taxon of group 4 (*Copiula* spp., *Austrochaperina* spp.), together forming the sister taxon of the clade formed by the three former groups (1–3). In turn, group 5 (*Barygenys exsul*, *Cophixalus* spp., *Oreophryne* spp.) is shown as the sister taxon of the clade formed by groups 1 to 4. Group 6 (*Cophixalus sphagnicola*, *Albericus laurini*, *Choerophryne* spp.) is the sister taxon of all other groups (1–5) combined. In contrast to the well-resolved relationships within the aforementioned clades 1–6, the phylogenetic reconstructions produced by applying the different analytical methods differed with respect to the relationships between these groups. This inconsistency is accompanied by a generally low nodal support for the basal splits of the tree as depicted in Fig. 1. Some single species, such as *Aphantophryne pansa*, *Liophryne schlaginhaufeni*, and *Genyophryne* spp. are shown quite isolated at varying basal positions. Hence, their affinities remain dubious.

4. Discussion

4.1. Systematic implications

The genes used here do provide resolution at shallow, but not at deep branches. This could be due either to patterns of relatively fast molecular evolution, short fragment size, or because the Papuan microhylids qualify as a fast and star-like radiation. We believe that the latter is also true because the Australopapuan Microhylidae (= Asterophryinae) represent an ancient and isolated group that probably diverged from their Indo-Asian relatives about 67–84 Ma (Van Bocxlaer et al., 2006; Van der Meijden et al., 2007). Since then the complex geological history and changing patterns of land and sea (details in Keast, 1996; Hall, 2001; Metcalfe, 2001) may have possibly triggered a star-like radiation of microhylid frogs by isolating and reconnecting refugia (Menzies, 2006) as discussed for other New Guinean groups of organisms (Polhemus and Polhemus, 1998). However, deciphering the genealogical patterns of ancient radiations is particularly complicated and requires large data sets (Whitfield and Lockhart, 2007).

Despite the inability to resolve the deep branches, our study contributes to a better understanding of the Australopapuan Microhylidae.

Particularly controversial has been the partition into two subfamilies, Asterophryinae and Genyophryninae, suggested by Zweifel (1971, 1972) based on morphological characters (states of vertebral column, tongue, and maxillary). This partition was questioned by morphology-based workers (Savage, 1973; Burton, 1986), but also by molecular studies (Sumida et al., 2000; Frost et al., 2006). By showing that neither Genyophryninae sensu Zweifel (*Albericus*, *Austrochaperina*, *Aphantophryne*, *Choerophryne*, *Cophixalus*, *Copiula*, *Genyophryne*, *Liophryne*, *Oreophryne*, *Oxydactyla*, *Sphenophryne*) nor Asterophryinae sensu Zweifel (*Asterophrys*, *Barygenys*, *Callulops*, *Hylophorbus*, *Pherohapsis*, *Mantophryne*, *Xenorhina*) are monophyletic, we provide evidence in favour of Frost et al. (2006) suggestion to consider Genyophryninae a junior synonym of Asterophryinae.

By analogy to the subfamily level our results suggest that the recognition and delineation of several genera and/or the generic affiliation of various species is highly questionable. The new data presented here support the delimitation of six groups as phylogenetically stable units, which are not recognized by the morphology-based classification. While, traditionally, species with similar life styles are often grouped together, most of the six groups recognized herein contain species that display a variety of adaptations. A closer look at the character state evolution of morphological traits, which have been considered of diagnostic value, reveals a larger extent of homoplasy than so far expected. This homoplasy is the main reason for the mismatch between the morphology-based classification and the molecular trees. For example, Zweifel (1972, 2000) stated that a symphygnathine state of the maxillary is typical for the Asterophryinae (it should have been secondarily lost in *Hylophorbus*). In contrast, we assume that the symphygnathine state has evolved once only in the clade formed by *Asterophrys*, *Callulops*, and *Xenorhina* (group 2). It remains unclear whether the maxillary of *Hylophorbus* is secondarily eleutherognathine, as previously suggested (then apomorphic), or if *Callolups* cf. *robustus* has achieved the symphygnathine condition in parallel with group 2 (in which case *Hylophorbus* displays the plesiomorphic eleutherognathine condition) (Fig. 2, left). The situation is even more complicated with regard to the loss of clavicles and procoracoids, characters that have also been considered significant (Zweifel, 1972, 2000; Burton, 1986; Zweifel et al., 2003, 2005; Menzies, 2006).

In anurans, clavicles and/or procoracoids have been lost several times independently and the presence of a complete pectoral girdle (both structures present) is generally considered as the plesiomorphic trait (Duellman and Trueb, 1986; Burton, 1986) while re-acquisition of

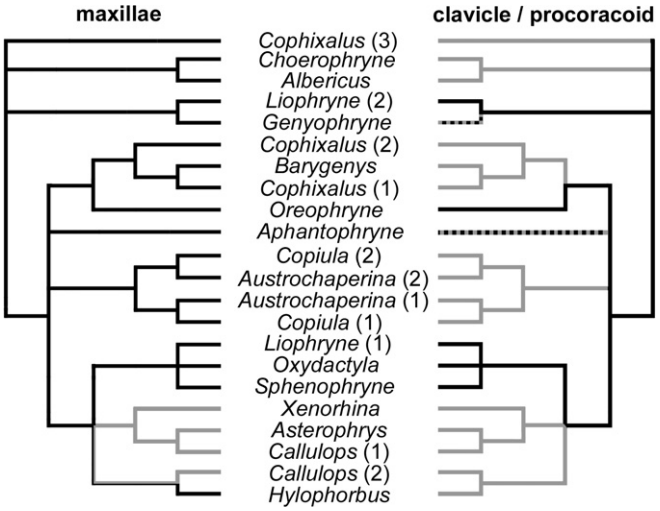


Fig. 2. Hypotheses of character evolution in three morphological features of the Australopapuan Microhylidae. Shown are only those phylogenetic splits that receive support from all three analytical methods (ML, BI, MP). Left: Hypothetical character state evolution of the maxillae (black = plesiomorphic, eleutherognathine; grey = apomorphic, symphygnathine). Right: Hypothetical character state evolution of clavicles and procoracoids (black = plesiomorphic, clavicles and procoracoids present; dashed = apomorphic, clavicles present, procoracoids absent; grey = apomorphic, clavicles and procoracoids absent). Data from Burton (1986), Zweifel (1972), and Menzies (2006).

structures lost previously is considered relatively unlikely (Burton, 1986). Given the postulate that presence of clavicles and procoracoids is the plesiomorphic condition in Australopapuan microhylids, our phylogeny suggests either that only the clavicles or both clavicles and procoracoids have been lost repeatedly (Fig. 2, right). Consequently, any attempt to define larger systematic groups by use of these characteristics is likely to be misled by homoplasy (Zweifel, 1972; Burton, 1986; Menzies, 2006). We assume that homoplasy in some morphological traits may result from parallel adaptations to similar life styles (which evolved in parallel themselves) in concert with design limitations (Wake, 1991).

We discuss some taxonomic implications of our study for currently recognized genera. We refrain from any formal taxonomic decision, which is the task of a future revision that will have to reappraise morphological features in the light of a possibly parallel evolution of life styles and morphologies.

Albericus Burton and Zweifel, 1995 comprises 14 endemic species and was stated to be closely related to *Choerophryne* by Burton and Zweifel (1995). Menzies (2006) suggested that *Albericus* is part of a *Cophixalus*-group together with *Aphantophryne*, *Choerophryne* and *Copiula* and can be distinguished by the lack of clavicles and procoracoids. We included only one species, shown in a sister–group relationship with *Choerophryne*. In the MP and ML both genera form a monophyletic group with *C. sphagnicola* as their sister taxon. The phylogenetic position of this group is not well resolved, but the existence of a *Cophixalus*-group as delineated by Menzies (2006) is refuted.

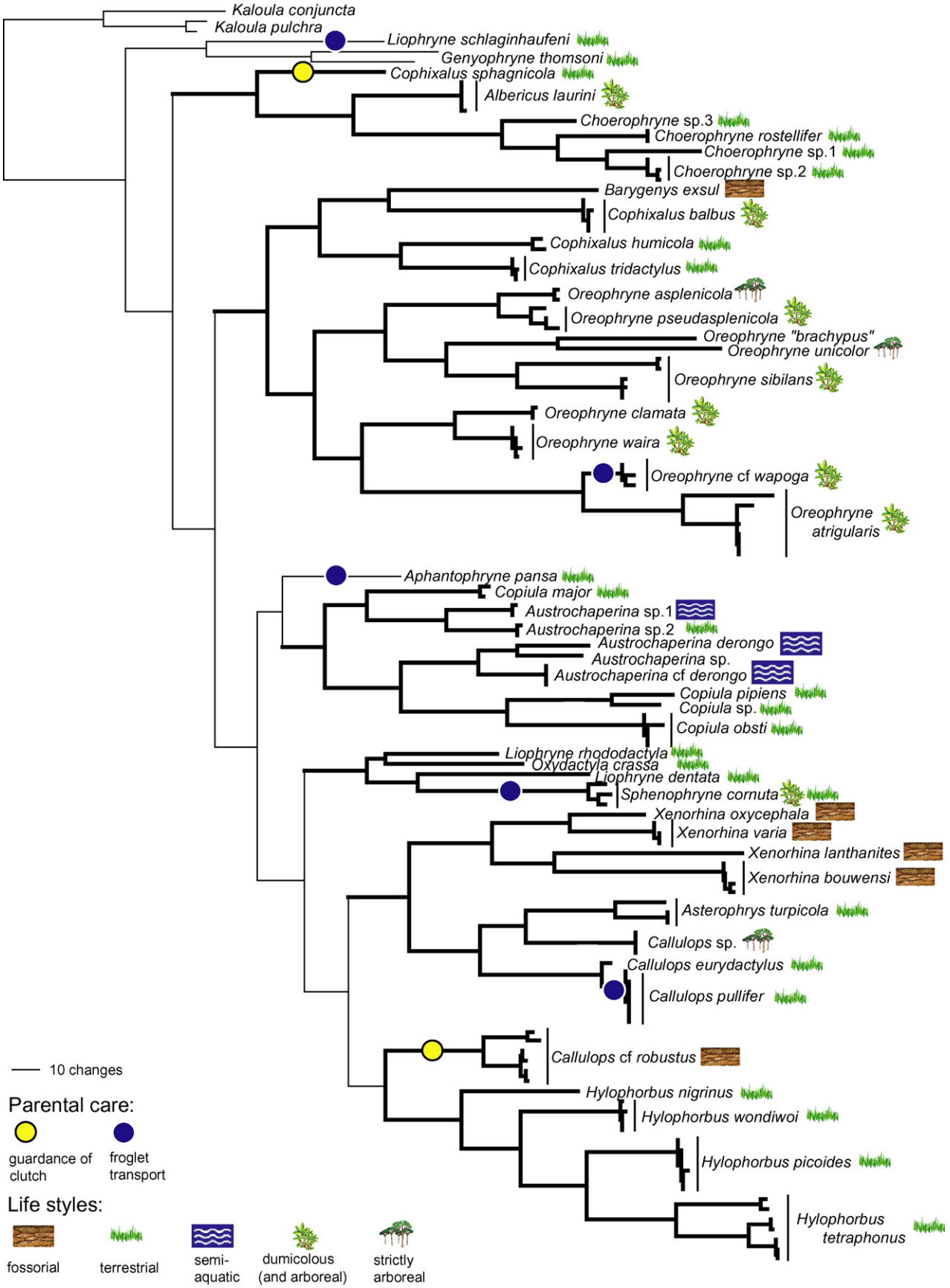
Aphantophryne Fry, 1917 comprises three species from eastern New Guinea. Here it is represented by *A. pansa*, which is inconsistently shown at various more basal positions. Its affinities remain unclear.

Asterophrys Tschudi, 1838: One of two endemic species incorporated is shown as part of a larger monophylum comprising terrestrial species of *Callulops* (except for *Callulops* cf. *robustus*) and subterrestrial species of *Xenorhina*. An undescribed arboreal species assigned to *Callulops* by its skull morphology is in fact more closely related to *Asterophrys*. Consequently, the proposed sister–group relationship of *Xenorhina* (with the inclusion of *Xenobatrachus*) and *Asterophrys* as based on osteological characteristics (Zweifel, 1972; Fig. 6) is confirmed. The deep split among the sequences of *A. turpicola* (Fig. 3) separates two populations from the Wondiwoi Mts. on mainland New Guinea and Yapen Island, respectively. It remains to be clarified whether the Yapen population should be considered a separate species, for which the name *A. steini* should be applied, currently listed as a synonym of *A. turpicola*.

Austrochaperina Fry, 1912 contains various species from Australia, New Guinea, and New Britain. Hoskin (2004) provided a molecular phylogeny of Australian species based on 12S and 16S rDNA. This tree is widely consistent with the current classification of the Australian species, but in the absence of a larger geographical scale this work does not enlighten relationships with the New Guinean species. In each of the trees presented herein, *Austrochaperina* forms a monophyletic group with *Copiula major*, *C. pipiens*, and *C. obsti* to the exclusion of other species. Included within it is a sequence of '*Sphenophryne* sp.' obtained from GenBank here attributed to *Austrochaperina*. We find that populations of *A. derongo* from different localities (mainland New Guinea and Yapen, respectively) are separated by a relatively deep split (Fig. 3). Whether these populations should be considered conspecific remains to be clarified.

Barygenys Parker, 1936 is represented here by one of seven known species. Sumida et al. (2000) and Frost

Fig. 3. Distribution of parental care behaviour and life style forms among the New Guinean Microhylidae as mapped on the topology of the ML phylogram shown in Fig. 1. Bold lines indicate relationships consistently revealed in the BI and MP trees. Modes of parental care behaviour and life styles are mapped on the tree according to the data of Zweifel (1972, 1980, 2000), Günther (2001, 2002, 2006), Günther et al. (2001), Bickford (2002, 2004), Zweifel et al. (2005), Günther and Richards (2005), Günther and Knop (2006), and Menzies (2006).


Kaloula conjuncta
Kaloula pulchra
Liophryne schlaginhaufeni
Genyophryne thomsoni
Cophixalus sphagnicola
Albericus laurini
Choerophryne sp.3
Choerophryne rostellifer
Choerophryne sp.1
Choerophryne sp.2
Barygenys exsul
Cophixalus balbus
Cophixalus humicola
Cophixalus tridactylus
Oreophryne asplenicola
Oreophryne pseudasplenicola
Oreophryne "brachypus"
Oreophryne unicolor
Oreophryne sibilans
Oreophryne clamata
Oreophryne waira
Oreophryne cf wapoga
Oreophryne atrigularis
Aphantophryne pansa
Copiula major
Austrochaperina sp.1
Austrochaperina sp.2
Austrochaperina derongo
Austrochaperina sp.
Austrochaperina cf derongo
Copiula pipiens
Copiula sp.
Copiula obsti
Liophryne rhododactyla
Oxydactyla crassa
Liophryne dentata
Sphenophryne cornuta
Xenorhina oxycephala
Xenorhina varia
Xenorhina lanthanites
Xenorhina bouwensi
Asterophrys turpicola
Callulops sp.
Callulops eurydactylus
Callulops pullifer
Callulops cf robustus
Hylophorbus nigrinus
Hylophorbus wondiwoi
Hylophorbus picoides
Hylophorbus tetraphonus
10 changes
Parental care:
guardance of clutch
froglet transport
Life styles:
fossorial
terrestrial
semi-aquatic
dumicolous (and arboreal)
strictly arboreal

et al. (2006) suggested that it is more closely related to *Cophixalus*, which is confirmed by our trees. This result stands in conflict with the traditional placement of both taxa in different subfamilies, which reflects the very different external morphology of both taxa and their different life style (i.e. fossorial vs. arboreal).

Callulops Boulenger, 1888 encompasses 16 species from New Guinea, the Moluccas to the easternmost islands of the Louisiade Archipelago. As currently encompassed, this genus is, however, potentially polyphyletic. Species affiliated with *Callulops* do not form a monophyletic group. Some (*C.* spec., *C. eurydactylus*, *C. pullifer*) are more closely related with *Asterophrys*, while the *C. robustus* group forms the sister group of *Hylophorbus*. In addition, the type species, *C. fuscus*, occurs on Amboina and might not be closely related to the New Guinean species. Clarification of these aspects awaits future results.

Choerophryne Van Kampen, 1914 is endemic to New Guinea. Four species are currently affiliated with this genus; herein we present sequences of three still undescribed species that form a monophyletic group together with *C. rostellifer*. This group is consistently shown to form the sister group of *A. laurini*.

Cophixalus Boettger, 1892 is a species-rich genus that occurs on the Moluccas, New Guinea, and in northeastern Queensland. Three New Guinean species are included that form a group of closely related species together with the morphologically very different *B. exsul* (see above for the status of *Barygenys*). The relationships of this clade remain ambiguous, however. MP and ML trees suggest a sister–group relationship with *Oreophryne* whereas in the BI tree the relationships are not resolved. Another species of the genus, *C. sphagnicola*, is apparently not closely related to the former ones and occupies markedly different positions in each of the trees, which speaks for a relatively ancient separation of the lineage. In turn, this may indicate that *Cophixalus*, as currently defined, is not monophyletic.

Copiula Méhely, 1901: Three of eight species are included here and do not form a monophyletic group. Their affinities remain dubious. *C. major* is consistently shown outside a clade containing all *Austrochaperina* spp., but only distantly related to the other two species of the genus (*Copiula obsti* and *C. pipiens*); see under *Austrochaperina*.

Genyophryne Boulenger, 1890 is a monotypic genus from Eastern New Guinea. In the MP and ML trees, it is close to *Liophryne schlaginhaufeni* while in the BI tree its affinities remain unresolved.

Hylophorbus Macleay, 1878 was removed from the synonymy of *Asterophrys* by Zweifel (1972). Herein, four of the eight currently known species are consistently shown to form the sister group of the *Callulops* cf. *robustus* group. As suggested by Zweifel (1972), *Hylophorbus* is not closely related to *Asterophrys*. A deep split separates the mainland populations of *H. tetraphonus* from the Yapen population. It remains to be clarified whether these populations should be considered conspecific.

Liophryne Boulenger, 1897 comprises six species from New Guinea and was removed from the synonymy of *Sphenophryne* by Zweifel (2000). Two species are included here. They do not form a monophyletic group. It remains questionable whether *Liophryne*, as currently defined, should be retained as a valid taxon.

Oreophryne Boettger, 1895 comprises 43 species from the Philippines, Sulawesi, the Lesser Sunda Islands, New Guinea, and New Britain. Here ten New Guinean species are included that form a monophyletic group closely related to *Cophixalus*. The sequence of "*O. brachypus*" was obtained from GenBank. Its identity is doubtful because the individual was collected on mainland New Guinea. This species, however, is known only from New Britain. The deep splits observed in *O. sibilans* and *O. atrigularis* correspond to spatial separation of possibly not conspecific populations.

Oxydactyla Van Kampen, 1913 is composed of five species from New Guinea and was removed from the synonymy of *Sphenophryne* by Zweifel (2000). The species included here, *O. crassa*, forms a clade with *L. rhododactyla*, *L. dentata* and *S. cornuta*, which fuels doubts about the validity of the genus as currently comprised.

Sphenophryne Peters and Doria, 1878 was partitioned into four genera by Zweifel (2000)—*Sphenophryne*, *Austrochaperina*, *Oxydactyla*, and *Liophryne* because of the diversity of adaptations and the great variety of lifestyles represented by these frogs. Menzies (2006) considered these genera to form a closely related group, which is recognized by the presence of an unreduced clavicle. Our trees suggest that this treatment is arbitrary and possibly misled by homoplasy. Because *Oxydactyla* and *Liophryne* are closely related to *Sphenophryne*, it remains to be tested whether they should remain in synonymy. *Austrochaperina* is clearly distinct.

Xenorhina Peters, 1863, here including *Xenobatrachus* as suggested by Frost et al. (2006), comprises 28 species from New Guinea and its satellite islands. It was removed from the synonymy of *Asterophrys* by Zweifel (1972). Four species analysed herein form the sister taxon of *Asterophrys* as suggested by Zweifel (1972).

In summary, we are convinced that further studies are needed with both a broader taxon sampling and a sufficient basis of sequences before we may achieve a more detailed understanding of the evolution and systematics of the Australopapuan Microhylidae.

4.2. Parental care evolution

New Guinean microhylid frogs generally deposit their eggs outside open water either in terrestrial or arboreal environments. This derived form of reproduction has

apparently evolved independently in the Asterophryinae. In addition, some species exhibit forms of parental care that may involve guarding of the clutch by the father and even subsequent transport of the hatchlings (Günther et al., 2001; Bickford, 2002, 2004; Günther, 2006). Parental (more specifically paternal) care has been reported from species of different genera while the reproductive strategies of most species remain unknown. Mapping the known cases of paternal care behaviours onto the phylogeny confirms the assumption, already implied by the current taxonomic treatment of the relevant species, that different forms of paternal care have probably evolved several times independently within the Asterophryinae. Froglet transport has been documented in five species (Günther et al., 2001; Bickford, 2002, 2004; Günther, 2006). These species belong to five different groups as defined here and are almost evenly distributed across the tree (Fig. 3). Even if we take into account that parental care behaviour may have remained undetected in further species, this result suggests that these behaviours have, at least in part, evolved in parallel, similar to other morphological and behavioural traits (Fig. 2). In turn, the repeated evolution of paternal care suggests that this behaviour is highly beneficial providing increased fitness.

In general it is known that organisms dynamically adjust their investment into parental care according to the number of offspring in their brood, past investment, genetic relatedness, and alternative mating opportunities, all of which affect the value of current offspring relative to potential future offspring. It may therefore be very difficult to identify the factors that determine the costs and benefits of parental care behaviour (Clutton-Brock, 1991). In addition, it was shown for fish (and may also hold true for frogs) that it is often the male that provides parental care in species with external fertilization (Ridley, 1978; Gross and Shine, 1979), not because the male obtains greater benefits from this care, but probably because it pays fewer costs (Clutton-Brock, 1991).

Bickford (2004) hypothesized that microhabitat-specific selection pressures are causing the evolution and maintenance of parental care in the New Guinean Microhylidae. In fact, this statement describes only the proximate dimension of this phenomenon. However, the parallel evolution of paternal care strategies in the different clades suggests that intrinsic (i.e. evolutionary) factors may also play a role. Zweifel (1972) argued that the evolution of parental care behaviour in these frogs is driven by the initial increase of parental investment involved with the acquisition of a direct development. This hypothesis accounts for the ultimate or evolutionary dimension of parental care evolution. Accordingly, a direct larval development requires large reserves of yolk delivered with the egg, which in turn leads to considerably decreased clutch sizes compared to species that possess free-swimming tadpoles but increased size of each single egg. A sheltered development within a hidden and potentially guarded nest and other, more elaborate, forms of parental care help to avoid the risky period of free larval life, and thus compensate for the low fecundity that is unavoidably connected with this reproductive strategy.

A correlation between increased egg size, decreased clutch size and presence of parental care has also been recognized in other amphibians (Nussbaum and Schultz, 1989; Crump, 1995), in fishes (Gross and Sargent, 1985; Sargent et al., 1987), and even invertebrates (Köhler et al., 2004). Most authors tried to explain the increase in egg size as a phenomenon that results from the presence of parental care (as reviewed by Kolm and Ahnesjö, 2005). However, it was demonstrated that neither in amphibians (Nussbaum, 1987; Nussbaum and Schultz, 1989) nor in fish is such a causal correlation likely to exist (Sargent et al., 1987; Kolm and Ahnesjö, 2005). By contrast, in amphibians the evolution of large eggs typically precedes the evolution of parental care, rather than the reverse (Summers et al., 2006).

We hypothesize that the presence of direct development is the factor that causes the evolution of parental care. Hence, if we want to understand why Australopapuan microhylids exhibit parental care, we have to ask why they possess a direct larval development. A possible answer to this question was already given by Zweifel (1972), who argued that the scarcity of freshwater bodies suitable for spawning in most of New Guinea explains this phenomenon.

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Appendix A

See Table A.1.

Table A.1
Material used in the study

Taxon name	Locality	Field No.	Inventory No.	GenBank Accession No.	
				16S	12S
Outgroup taxa					
Dasypops schirchi Miranda-Ribeiro, 1924	Brazil: Reserva do Vale	—	CFBH-T 71	DQ283095	
Calluella guttulata Blyth, 1856	Vietnam: Gia Lai Province	—	FMNH 252955	DQ283144	
Kalophrynus pleurostigma Tschudi, 1838	Malaysia: Sabah	—	FMNH 230844	DQ283146	
Phrynomantis bifasciatus Smith, 1847	pet trade	—	—	DQ283154	
Breviceps mossambicus Peters, 1854	Tanzania: Morogoro	—	RdS 903	DQ283155	
Gastrophryne olivacea Hallowell, 1856	USA: Arizona, Santa Cruz Co.	—	ATH 476	DQ283268	
Microhyla heymonsi Vogt, 1911	Vietnam: Ha Giang, Yen Minh	—	AMNH A163850	DQ283382	
Ctenophryne geayi Mocquard, 1904	Guyana: Berbice River	—	AMNH A166444	DQ283383	
Kaloula pulchra Gray, 1831	Vietnam: Ha Tinh Province, Huong Son	—	AMCC 106697	DQ283397	
	pet trade	—	—	DQ283398	
Kaloula conjuncta Peters, 1863	Philippines: Negros	RMB2252	CMNH	AY326064	
Elachistocleis ovalis Schneider, 1799	Guyana: Berbice River	—	AMNH A141136	DQ283405	
Platypelis grandis Boulenger, 1889	Madagascar: Antsiranana	—	AMNH A167214	DQ283410	
Stumpffia cf. *psologlossa* Boettger, 1881	Madagascar: Antsiranana	—	AMNH A167359	DQ283411	
Microhyla sp.	pet trade	—	—	DQ283422	
Gastrophryne elegans Boulenger, 1882	Belize: Cockscomb Basin	RdS726	—	DQ283426	
Callulina kisiwamsitu Sá, Loader and Channing, 2004	Tanzania: West Usumbara Mts.	RdS936	—	DQ283429	
Dyscophus guineti Grandidier, 1875	pet trade	—	—	DQ283434	
Hamptophryne boliviana Parker, 1927	Peru	—	—	DQ283438	
Ingroup taxa					
Albericus laurini Günther, 2000	WNG: Wondiwoi Mts.	RG6988	ZMB 61913	EU100104	EU100220
	WNG: Wondiwoi Mts.	RG7689	ZMB 70311	EU100105	EU100221
	WNG: Wondiwoi Mts.	RG7690	ZMB 70312	EU100106	EU100222
	WNG: Wondiwoi Mts.	RG7723	ZMB 70313	EU100107	EU100223
Aphantophryne pansa Fry, 1917	PNG: Bulolo	—	ABTC 49605	DQ283195	
Asterophrys turpicola Schlegel, 1837	WNG: Wondiwoi Mts.	RG7020	ZMB 62043	EU100108	EU100224
	WNG: Yapen Isl.	RG7442	ZMB 64105	EU100109	EU100225
	WNG: Yapen Isl.	RG7495	ZMB 64106	EU100110	EU100226
Austrochaperina sp.	PNG: Namosado	—	AMS R122221	DQ283205	
Austrochaperina sp. 1	WNG: Wondiwoi Mts.	RG7606	ZMB 70324	EU100111	EU100227
	WNG: Wondiwoi Mts.	RG7714	ZMB 70325	EU100112	EU100228
Austrochaperina sp. 2	WNG: Wondiwoi Mts.	RG7697	ZMB 70326	EU100117	EU100233
	WNG: Wondiwoi Mts.	RG7698	ZMB 70327	EU100118	EU100234
Austrochaperina derongo Zweifel, 2000	WNG: Wapoga camp	SR3213	—	EU100113	EU100229
Austrochaperina cf. *derongo* Zweifel, 2000	WNG: Yapen Isl.	RG7458	ZMB 70334	EU100114	EU100230
	WNG: Yapen Isl.	RG7512	ZMB 70335	EU100115	EU100231
	WNG: Yapen Isl.	RG7755	ZMB 70329	EU100116	EU100232
Barygenys exsul Zweifel, 1963	PNG: Rossel Isl.	FK10338	BPBM 20128	EU100119	EU100235
Callulops spec.	WNG: Wondiwoi Mts.	RG7309	ZMB 63882	EU100121	EU100237
	WNG: Wondiwoi Mts.	RG7682	ZMB 70187	EU100122	EU100238
	WNG: Wondiwoi Mts.	RG7717	ZMB 70185	EU100123	EU100239
Callulops eurydactylus Zweifel, 1972	WNG: Fakfak Mts.	RG7112	ZMB 63878	EU100120	EU100236
Callulops pullifer Günther, 2006	WNG: Wondiwoi Mts.	RG6939	ZMB 62053	EU100124	EU100240
	WNG: Wondiwoi Mts.	RG7603	ZMB 64161	EU100125	EU100241
	WNG: Wondiwoi Mts.	RG7604	ZMB 64162	EU100126	EU100242
	WNG: Wondiwoi Mts.	RG7605	ZMB 64163	EU100127	EU100243
	WNG: Wondiwoi Mts.	RG7626	ZMB 64164	EU100128	EU100244
	WNG: Wondiwoi Mts.	RG7687	ZMB 64169	EU100129	EU100245
Callulops robustus Boulenger, 1898	WNG: Wondiwoi Mts.	RG7032	ZMB 62037	EU100131	EU100247
	WNG: Wondiwoi Mts.	RG7705	ZMB 70315	EU100134	EU100250
	WNG: Wondiwoi Mts.	RG7706	ZMB 70316	EU100135	EU100251
	WNG: Wondiwoi Mts.	RG6949	ZMB 63874	EU100130	EU100246
	WNG: Biak Isl.	RG7369	ZMB 64107	EU100132	EU100248
	WNG: Biak Isl.	RG 7370	ZMB 64108	EU100133	EU100249
Choerophryne sp. 1	WNG: Yapen Isl.	RG7804	ZMB 70342	EU100136	EU100252
Choerophryne sp. 2	WNG: Yapen Isl.	RG7401	ZMB 70346	EU100139	EU100255
	WNG: Yapen Isl.	RG7407	ZMB 70348	EU100140	EU100256
	WNG: Yapen Isl.	RG7466	ZMB 70352	EU100141	EU100257
Choerophryne sp. 3	WNG: Yapen Isl.	RG7490	ZMB 70354	EU100142	EU100258

Table A.1 (*continued*)

Taxon name	Locality	Field No.	Inventory No.	GenBank Accession No.	
				16S	12S
Choerophryne rostellifer Wandolleck, 1911	WNG: Wondiwoi Mts.	RG7611	ZMB 70359	EU100137	EU100253
	WNG: Wondiwoi Mts.	RG7612	ZMB 70360	EU100138	EU100254
Choerophryne sp.	PNG: Mt. Menawa		ABTC 47720	DQ283207	
Cophixalus balbus Günther, 2003	WNG: Yapen Isl.	RG7434	ZMB 62594	EU100143	EU100259
	WNG: Yapen Isl.	RG7435	ZMB 62595	EU100144	EU100260
	WNG: Yapen Isl.	RG7487	ZMB 62596	EU100145	EU100261
	WNG: Yapen Isl.	RG7502	ZMB 62597	EU100146	EU100262
Cophixalus humicola Günther, 2006	WNG: Yapen Isl.	RG7492	ZMB 69704	EU100147	EU100263
	WNG: Yapen Isl.	RG7494	ZMB 69705	EU100148	EU100264
Cophixalus sphagnicola Zweifel and Allison, 1982	PNG: Wau		ABTC 47881	DQ283206	
Cophixalus tridactylus Günther, 2006	WNG: Wondiwoi Mts.	RG7615	ZMB 69696	EU100149	EU100265
	WNG: Wondiwoi Mts.	RG7631	ZMB 69698	EU100150	EU100266
	WNG: Wondiwoi Mts.	RG7726	ZMB 69700	EU100151	EU100267
Copiula major Günther, 2002	WNG: Wondiwoi Mts.	RG6687	ZMB 62074	EU100152	EU100268
	WNG: Wondiwoi Mts.	RG7337	ZMB 62564	EU100153	EU100269
Copiula obsti Günther, 2002	WNG: Yapen Isl.	RG7340	ZMB 62555	EU100154	EU100270
	WNG: Yapen Isl.	RG7341	ZMB 62554	EU100155	EU100271
	WNG: Yapen Isl.	RG7633	ZMB 70189	EU100156	EU100272
	WNG: Yapen Isl.	RG7696	ZMB 70190	EU100157	EU100273
Copiula pipiens Burton and Stocks, 1986	WNG: Yapen Isl.	RG7486	ZMB 64112	EU100158	EU100274
Copiula sp.	PNG: Sinyarge	—	AMS R124417	DQ283208	
Genyophryne thomsoni Boulenger, 1890	PNG: Sudest Isl.	FK9507	BPBM 20357	EU100159	EU100275
	PNG: Bulolo	—	ABTC 49624	DQ283209	
Hylophorbus nigrinus Günther, 2001	WNG: Yapen Isl.	RG7385	ZMB 62404	EU100160	EU100276
Hylophorbus picoides Günther, 2001	WNG: Wondiwoi Mts.	RG6871	ZMB 61972	EU100162	EU100278
	WNG: Wondiwoi Mts.	RG6943	ZMB 61979	EU100163	EU100279
	WNG: Wondiwoi Mts.	RG7024	ZMB 61977	EU100164	EU100280
	WNG: Wondiwoi Mts.	RG7638	ZMB 70306	EU100165	EU100281
	WNG: Wondiwoi Mts.	RG7721	ZMB 70307	EU100166	EU100282
		RG 6690	ZMB 61973	EU100161	EU100277
Hylophorbus wondiwoi Günther, 2001	WNG: Wondiwoi Mts.	RG6753	ZMB 61995	EU100174	EU100290
	WNG: Wondiwoi Mts.	RG6762	ZMB 61995	EU100175	EU100291
	WNG: Wondiwoi Mts.	RG7336	ZMB 62396	EU100176	EU100292
	WNG: Wondiwoi Mts.	RG7707	ZMB 70317	EU100177	EU100293
Hylophorbus tetraphonus Günther, 2001	WNG: Wondiwoi	RG6956	ZMB 61987	EU100167	EU100283
	WNG: Wondiwoi Mts.	RG6958	ZMB 61989	EU100168	EU100284
	WNG: Wondiwoi Mts.	RG7610	ZMB 70318	EU100171	EU100287
	WNG: Yapen Isl.	RG7459	ZMB 70319	EU100169	EU100285
	WNG: Yapen Isl.	RG7476	ZMB 70320	EU100170	EU100286
	WNG: Nabire-Mapia road	RG7739	ZMB 70322	EU100172	EU100288
	WNG: Nabire-Mapia road	RG7740	ZMB 70323	EU100173	EU100289
Liophryne dentata Tyler and Menzies, 1971	PNG: Cloudy Mts.	FK5201	BPBM 15370	EU100178	EU100294
Liophryne schlaginhaufeni Wandolleck, 1911	PNG: West Sepik Prov.	FK11771	BPBM 22754	EU100179	EU100295
Liophryne rhododactyla Boulenger, 1897	PNG: Bulolo	—	ABTC 49566	DQ283199	
Oreophryne asplenicola Günther, 2003	WNG: Yapen Isl.	RG7760	ZMB 65895	EU100180	EU100296
	WNG: Yapen Isl.	RG7761	ZMB 65896	EU100181	EU100297
Oreophryne atrigularis Günther, Richards and Iskandar, 2001	WNG: Wondiwoi Mts.	RG6608	ZMB 62216	EU100182	EU100298
	WNG: Wondiwoi Mts.	RG6772	ZMB 62225	EU100183	EU100299
	WNG: Wondiwoi Mts.	RG6944	ZMB 62166	EU100184	EU100300
	WNG: Wondiwoi Mts.	RG7023	ZMB 62167	EU100185	EU100301
	WNG: Wondiwoi Mts.	RG7607	ZMB 70296	EU100186	EU100302
	WNG: Wondiwoi Mts.	RG7609	ZMB 70194	EU100187	EU100303
	WNG: Nabire-Mapia road	RG7744	ZMB 70298	EU100188	EU100304
Oreophryne brachypus Werner, 1898	PNG: 8 km NNE Amelei	—	AMS R129618	DQ283194	
Oreophryne clamata Günther, 2003	WNG: Wondiwoi Mts.	RG7693	ZMB 67353	EU100189	EU100305
	WNG: Wondiwoi Mts.	RG7694	ZMB 67354	EU100190	EU100306
Oreophryne pseudasplenicola Günther, 2003	WNG: Yapen Isl.	RG7772	ZMB 65897	EU100191	EU100307
	WNG: Yapen Isl.	RG7793	ZMB 65898	EU100192	EU100308
	WNG: Yapen Isl.	RG7795	ZMB 65900	EU100193	EU100309
Oreophryne sibilans Günther, 2003	WNG: Wondiwoi Mts.	RG6936	ZMB	EU100194	EU100310
	WNG: Wondiwoi Mts.	RG7719	ZMB 70191	EU100197	EU100313
	WNG: Wondiwoi Mts.	RG7720	ZMB 70192	EU100198	EU100314
	WNG: Yapen Isl.	RG7456	ZMB 70301	EU100195	EU100311

(*continued on next page*)

Table A.1 (*continued*)

Taxon name	Locality	Field No.	Inventory No.	GenBank Accession No.	
				16S	12S
WNG: Yapen Isl.	RG7477	ZMB 70302	EU100196	EU100312	
Oreophryne unicolor Günther, 2003	WNG: Wondiwoi Mts.	RG7700	ZMB 70188	EU100199	EU100315
Oreophryne waira Günther, 2003	WNG: Yapen Isl.	RG7478	ZMB 65882	EU100200	EU100316
	WNG: Yapen Isl.	RG7748	ZMB 62337	EU100201	EU100317
	WNG: Yapen Isl.	RG7770	ZMB 62339	EU100202	EU100318
	WNG: Yapen Isl.	RG7771	ZMB 62335	EU100203	EU100319
Oreophryne cf. *wapoga* Günther, Richards and	WNG: Yapen Isl.	RG7416	ZMB 65186	EU100204	EU100320
Iskandar, 2001	WNG: Yapen Isl.	RG7417	ZMB	EU100205	EU100321
	WNG: Yapen Isl.	RG7759	ZMB 70304	EU100206	EU100322
Oxydactyla crassa Zweifel, 1956	PNG: Mt. Simpson	FK7402	BPBM 17061	EU100207	EU100323
Sphenophryne cornuta Peters and Doria, 1878	WNG: Wondiwoi Mts.	RG6941	ZMB 62195	EU100208	EU100324
	WNG: Wondiwoi Mts.	RG7030	ZMB 62198	EU100209	EU100325
	WNG: Wondiwoi Mts.	RG7703	ZMB 70309	EU100210	EU100326
Xenorhina bouwensi De Witte, 1930	WNG: Wondiwoi Mts.	RG7312	ZMB 62639	EU100211	EU100327
	WNG: Wondiwoi Mts.	RG7338	ZMB 62638	EU100212	EU100328
	WNG: Wondiwoi Mts.	RG7621	ZMB 65138	EU100213	EU100329
	WNG: Wondiwoi Mts.	RG7622	ZMB 65139	EU100214	EU100330
Xenorhina lanthanites Günther and Knop, 2006	WNG: Yapen Isl.	RG7791	ZMB 69561	EU100215	EU100331
Xenorhina oxycephala Schlegel, 1858	WNG: Wondiwoi Mts.	RG7620	ZMB 69562	EU100216	EU100332
Xenorhina varia Günther and Richards, 2005	WNG: Yapen Isl.	RG7753	ZMB 65133	EU100217	EU100333
	WNG: Yapen Isl.	RG7778	ZMB 65136	EU100218	EU100334
	WNG: Yapen Isl.	RG7779	ZMB 65137	EU100219	EU100335

Abbreviations: WNG, Western New Guinea; PNG, Papua New Guinea.

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