

## Preliminary molecular relationships of Comoran day geckos (*Phelsuma*)

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**Abstract.** The genus *Phelsuma* contains 42 species of diurnal, mostly green-coloured geckos with a distribution encompassing Madagascar and the Comoro, Mascarene, Seychelles, Aldabra, and Andaman archipelagos in the Indian Ocean. In a first comprehensive assessment of their biogeography we sequenced a fragment of the 16S rRNA gene from representatives of most species. Because the short DNA sequences used were unable to provide an adequate resolution of most nodes, our results remain preliminary, but several biogeographic conclusions are possible: while *Phelsuma* from the Mascarenes and Seychelles, respectively, appear to be monophyletic radiations, the four Comoran taxa were arranged phylogenetically in three separate clades, and appear to have originated from at least two separate colonizations of this volcanic archipelago.

### Introduction

Day geckos of the genus *Phelsuma* are mainly confined to Madagascar and surrounding islands of the Comoros, Mascarene and Seychelles archipelagos, and Aldabra (Hallmann et al., 1997). A single species, *P. andamanense*, lives on the Andaman islands close to Myanmar. In contrast to most other geckos, *Phelsuma* species are generally diurnal. Most species are characterized by a bright green colour, although some species, such as *P. breviceps*, *P. masohoala* and *P. modesta*, are greyish. The status of *Rhoptropella ocellata*, a gecko species from the Namaqua Mountains in South Africa that had formerly been assigned to the genus *Phelsuma* is still controversially discussed (e.g., Russel, 1977; Röhl, 1998).

Due to their attractiveness to breeders, species of *Phelsuma* are important in the international reptile trade and are included in the Convention on the International Trade in Endangered Species CITES. Despite this general interest, their intrageneric phylogeny and classification is only insufficiently established. In a recent paper, Austin et al. (2004) provided a molecular analysis of relationships among 19 *Phelsuma* taxa, focusing on the relationships of the taxa from the Mascarene islands (Mauritius and Réunion). Based on sequences from

the mitochondrial 12S rRNA and cytochrome *b* genes and the nuclear *c-mos* gene, they provided evidence that these species and subspecies constitute an endemic radiation on the Mascarenes, and found *Rhoptropella* to be the sister group of the equally diurnal gecko genus *Lygodactylus* rather than *Phelsuma*.

The Comoro islands, located between Madagascar and Africa in the Mozambique channel, are a fully volcanic archipelago that has been colonized by overseas dispersal by a number of amphibian and reptile groups (Arnold, 2000; Vences et al., 2003). In the present paper, we provide new molecular data on *Phelsuma* relationships, based on mitochondrial sequences of the 16S rRNA gene. Our focus was to obtain a broad sampling of species and subspecies, and to analyse the relationships of the species from the Comoro islands in this preliminary screening of *Phelsuma* diversity.

### Materials and Methods

Tissue samples (muscle; fresh or preserved in ethanol) were available from 34 *Phelsuma* taxa. We included samples of eight other gecko species for hierarchical outgroup rooting (Figure 1, Table 1). DNA was extracted using the QiAmp tissue extraction kits (Qiagen). We amplified a ca. 560 bp fragment of the mitochondrial 16S rRNA via PCR. The primers and cycling procedures were as follows: 16SA (light chain; 5' - CGC CTG TTT ATC AAA AAC AT - 3') and 16SB (heavy chain; 5' - CCG GTC TGA ACT CAG ATC ACG T - 3'). Initial denaturation step: 90 s at 94°C; 33 cycles: denaturation 45 s at 94°C, primer annealing for 90 s at 55°C, extension for 90 s at 72°C.

PCR products were purified using the Qiaquick purification kit (Qiagen). We sequenced fragments on an ABI 377 automatic sequencer using standard protocols. Sequences were aligned automatically using Clustal X (Thompson et al., 1997). The 16S alignment was slightly refined by eye in the loop regions.

We applied a hierarchical likelihood ratio test for the goodness-of-fit of nested substitution models (for ingroup taxa only) using the program MODELTEST (Posada and Crandall, 1998). Due to the performance of multiple tests, we adjusted the significance level of rejection of the null

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hypothesis via the sequential Bonferroni correction to  $\alpha=0.01$  (Rice, 1989). We used the best-fitting model for subsequent phylogenetic analyses.

The data set was subjected to three different methods of phylogenetic reconstruction: (i) neighbor joining (NJ) (Saitou and Nei, 1987) using the specified substitution model; (ii) maximum parsimony with gaps treated as fifth character state; transitions and transversions were given equal weight; heuristic search with the TBR branch swapping algorithm; (iii) maximum likelihood (ML) analysis using the specified substitution model. All analyses were done with PAUP\* (Swofford, 2002). Robustness of NJ and MP tree topologies was tested by bootstrap analyses (Felsenstein, 1985), with 2000 replicates each (Hedges, 1992). Due to computational constraints, we used Quartet Puzzling (Strimmer and Von Haeseler, 1996) with 2000 permutations to infer reliability values for ML tree topologies. To gain confidence in Quartet Puzzling, we compared the resulting tree topology and the support values to those derived from 100 ML bootstrap replicates.

We statistically compared the resulting tree topology to alternative topologies (for description see below) applying the non-parametric likelihood ratio test of Shimodaira and Hasegawa (1999) with the following likelihood settings: HKY85 substitution model, ti/tv ratio = 2, empirical base frequencies; one-tailed SH test using 1000 RELL bootstrap replicates) as implemented in PAUP\* (Swofford, 2002).

## Results and Discussion

The alignment consisted of 580 bp for all taxa, with 311 variable and 228 parsimony informative sites. Empirical base frequencies were  $\pi_A=0.3846$ ,  $\pi_C=0.2645$ ,  $\pi_T=0.2247$  and  $\pi_G=0.1262$ . The strong anti-G bias indicates that no nuclear pseudocopies of the genes have been analysed (Zhang and Hewitt, 1996). Bases were homogeneously distributed among ingroup haplotypes ( $\chi^2$ -test:  $p=1.00$ ). The substitution model that fits our alignment best was the Tamura-Nei model with a proportion of invariable sites of  $I=0.2635$ , a gamma distribution shape parameter  $\alpha=0.4926$  and substitution rate  $_{[A-G]} = 3.3352$  and  $_{[C-T]} = 5.6206$  ( $-\ln L_{TN+I+G} = 4690.2271$ ).

Confirming the results of Austin et al. (2004), our tree (Fig. 1) indicates (1) that the genus *Phelsuma* is a monophyletic group, (2) that the Mascarene *Phelsuma* form a monophyletic radiation, and (3) that *Rhoptropella ocellata* may form a clade with *Lygodactylus* which together is the sister group of *Phelsuma*, although this latter result was neither sufficiently supported in our nor in the previously published analysis. Also the three included Seychellean *Phelsuma* form a monophyletic group in our tree, providing evidence that they constitute an endemic radiation as postulated by Radtkey (1996). As a surprising aspect, *P. andamanense* is positioned sister to *P. breviceps*, a morphologically rather unusual short-snouted, greyish species from Madagascar. This relationship remains to be tested with more extensive

data sets; morphologically, these two taxa bear no obvious similarities.

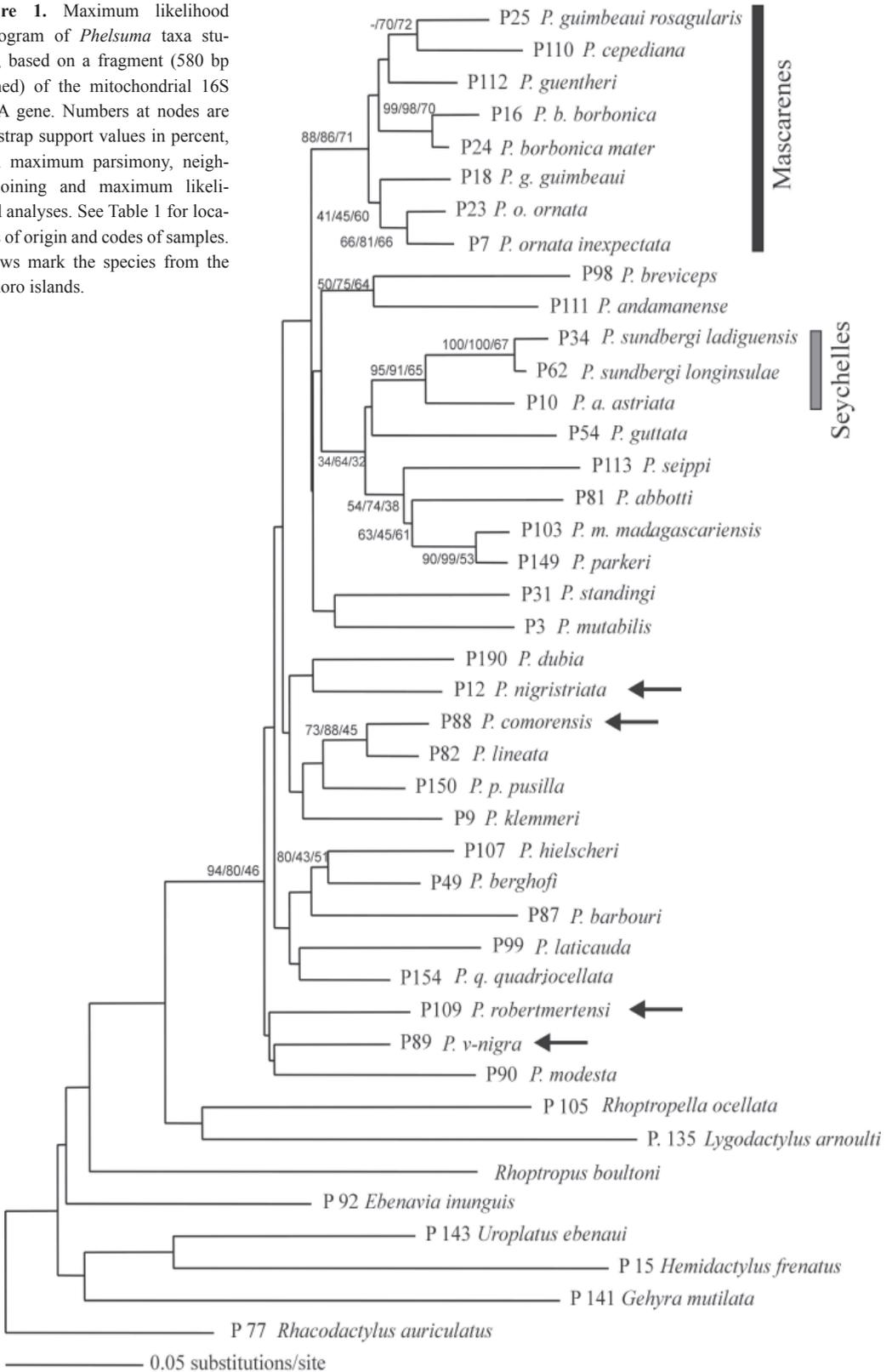
The phylogeny among Malagasy *Phelsuma* species was virtually unresolved in our data set, as it was in the tree of Austin et al. (2004) who included more nucleotides but less taxa in their analysis. With low support *P. hielscheri* is sister to *P. berghofi*, and *P. abbotti* appears related to *P. madagascariensis* and *P. parkeri*. The latter two species form a highly supported clade and show only one percent sequence divergence. This is among the lowest levels of sequence divergence among all studied *Phelsuma* species.

The four Comoran *Phelsuma* species are scattered among the Malagasy taxa, *P. comorensis* being sister to *P. lineata*, *P. nigristriata* being sister to *P. dubia*, and *P. robertmertensi* and *P. v-nigra* forming a clade with *P. modesta*. The latter two clades are poorly supported (Fig. 1), but several alternative hypotheses of monophyly of Comoran taxa (trees 1-3 in Table 2), proved to be significantly less likely than the best ML tree, providing evidence for non-monophyly of the Comoran taxa. Although relationships of *P. nigristriata* and *P. v-nigra* are not clarified, it seems probable that *P. comorensis* is closely related to the Malagasy *P. lineata*, and therefore colonized the Comoros independently from the other two species. This is further supported by the strong morphological and chromatic similarities between *P. comorensis* and *P. lineata*.

Independent from our study, Rocha et al. (in press) have recently published a phylogeny of Comoroan *Phelsuma* based on sequences the cytochrome b and 12S genes, with a more comprehensive sampling of Comoran populations but including fewer species. They found support for a Comoran clade containing *P. v-nigra*, *P. robertmertensi* and *P. pasteuri*, and, similar to our data, a strongly supported clade containing *P. comorensis* and *P. lineata*.

Close relationships among Comoran and Malagasy taxa are obvious for many organism groups and have recently even been demonstrated for frogs that apparently have been able to colonize Mayotte from Madagascar by overseas rafting (Vences et al., 2003). Our data provide a first indication that, within *Phelsuma*, at least two independent colonization events between Madagascar and the Comoros have occurred, and that the Comoran species of *Phelsuma* - unlike the Seychellean and Mascarene ones - are probably not a monophyletic radiation. More work is necessary to identify, among Malagasy *Phelsuma*, the sister species of each of the Comoran taxa, and to reconstruct the

**Figure 1.** Maximum likelihood phylogram of *Phelsuma* taxa studied, based on a fragment (580 bp aligned) of the mitochondrial 16S rRNA gene. Numbers at nodes are bootstrap support values in percent, from maximum parsimony, neighbor-joining and maximum likelihood analyses. See Table 1 for localities of origin and codes of samples. Arrows mark the species from the Comoro islands.



**Table 1.** Species sequences, voucher specimens (when preserved), localities and Genbank accession numbers for the analysed fragment of the 16S rRNA gene.

Species/Subspecies	Specimen no	Voucher specimen	Locality	Genbank accession
<i>P. astriata astriata</i>	P10	---	---	DQ270582
<i>P. abbotti chekei</i>	P81	---	---	DQ270565
<i>P. andamanense</i>	P111	---	---	DQ270559
<i>P. barbowi</i>	P87	UADBA FGMV 2000.86	Ankaratra, Madagascar	DQ270561
<i>P. borbonica borbonica</i>	P16	---	Le Brule, Reunion	DQ270554
<i>P. borbonica agalegae</i>	P24	---	Basse Vallee, Reunion	DQ270574
<i>P. berghofi</i>	P49	---	Somisiky, Madagascar (23°23'S, 47°32'O)	DQ270556
<i>P. breviceps</i>	P98	UADBA FGMV 2000.564	Arboretum Toliara, Madagascar	DQ270551
<i>P. cepediana</i>	P110	---	Mauritius (highlands)	DQ270563
<i>P. comorensis</i>	P88	ZSM 691/2000	Ivembeni, Grande Comore	DQ270552
<i>P. dubia</i>	P190	---	Comoros	DQ270550
<i>P. guentheri</i>	P112	---	---	DQ270581
<i>P. guimbeaui</i>	P18	---	Grande Case Noyale, Mauritius	DQ270557
<i>P. guimbeaui rosagularis</i>	P25	---	Gorges de la Riviere Noire, Mauritius	DQ270549
<i>P. guttata</i>	P54	---	---	DQ270575
<i>P. hielscheri</i>	P107	---	Morondava, Madagascar	DQ270555
<i>P. klemmeri</i>	P9	---	Type locality, Sambirano, Madagascar	DQ270566
<i>P. laticauda</i>	P99	ZSM 554/2000	Sambava, Madagascar	DQ270578
<i>P. lineata</i>	P82	ZSM 336/2000	Vohidrazana, Madagascar	DQ270570
<i>P. madagascariensis</i>	P103	ZSM 559/2000	Sambava, Madagascar	DQ270569
<i>P. modesta</i>	P90	ZSM 586/2000	Ifaty, Madagascar	DQ270572
<i>P. mutabilis</i>	P3	---	Beroroaha, Makay	DQ270573
<i>P. nigristriata</i>	P12	---	Mayotte	DQ270562
<i>P. parkeri</i>	P149	---	Pemba, Mozambique	DQ270576
<i>P. pusilla pusilla</i>	P150	---	---	DQ270579
<i>P. ornata inexpectata</i>	P7	---	Manapany les Bains/Reunion	DQ270577
<i>P. ornata ornata</i>	P 23	---	Grande Riviere Noire, Mauritius	DQ270567
<i>P. q. quadriocellata</i>	P154	---	Ranomafana, Madagascar	DQ270580
<i>P. robertmertensi</i>	P109	---	Mayotte	DQ270553
<i>P. seippi</i>	P113	---	---	DQ270560
<i>P. standingii</i>	P31	---	South of Isalo, Madagascar	DQ270564
<i>P. sundbergi longinulatae</i>	P62	---	Mahé, Seychelles	DQ270568
<i>P. sundbergi ladiguensis</i>	P34	---	La Digue, Seychelles	DQ270558
<i>P. v-nigra</i>	P89	ZSM 714/2000	north of Nioumamilima	DQ270571
<i>Ebenavia inunguis</i>	P92	ZSM 442/2000	Nosy Be, Madagascar	DQ270585
<i>Gehyra mutilata</i>	P141	UADBA FGMV 2000.407	Antananarivo, Madagascar	DQ270588
<i>Hemidactylus frenatus</i>	P15	---	Beroroaha/Makay, Madagascar	DQ270589
<i>Lygodactylus arnouliti</i>	P135	ZSM 394/2000	Ibity, Madagascar	DQ270587
<i>Rhacodactylus auriculatus</i>	P77	---	New Caledonia	DQ270584
<i>Rhoptropella ocellata</i>	P105	---	South Africa	DQ270583
<i>Uroplatus ebenau</i>	P143	---	Berara, Madagascar	DQ270586

routes of colonization between Madagascar and the various islands of the Comoro archipelago.

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## References

Austin, J.J., Arnold, E.N., Jones, C.G. (2004): Reconstructing an island

radiation using ancient and recent DNA: the extinct and living day geckos (*Phelsuma*) of the Mascarene islands. *Mol. Phylogenet. Evol.* **31**: 109-122.

Arnold, E.N. (2000): Using fossils and phylogenies to understand evolution of reptile communities on islands, In Rheinwald, G. (ed.): *Isolated Vertebrate Communities in the Tropics*. Proc. 4<sup>th</sup> Int. symp., Bonn: 309-323.

Felsenstein, J. (1985): Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**: 783-791.

Hallmann, G., Krüger, J., Trautmann, G. (1997): Faszinierende Taggeckos. Die Gattung *Phelsuma*. Natur und Tier Verlag: 229S.

**Table 2.** Alternative trees with monophyletic arrangement of the four *Phelsuma* species from the Comoro islands, and the statistical probability with which these were rejected in SH-tests.

	Tree topology (taxon names from Fig. 1 abbreviated)	P in SH-test
Comoroan taxa monophyletic on branch leading to <i>P. nigristriata</i>	((((((((((((P25_P_g_ro,P110_P_cep),((P16_P_b_bo,P24_P_b_ag),(P18_P_gimb,(P23_P_o_or,P7_P_o_ine))))),P112_P_gue),P31_P_stan),((((P34_P_s_la,P62_P_s_lo),P10_P_a_as),((P113_P_sei,(P81_P_abot,(P103_m_ma,P149_P_par))))),P54_P_gutt),P3_P_mutab)),(P98_P_brev,P111_P_and)),(((P190_P_dub,P107_P_hie),P154_P_q_q),(((P88_P_como,P12_P_nigr),P89_P_v_ni),P109_P_rob),P90_P_mode)),(P49_P_berg,P87_P_barb))),(((P82_P_line,P150_P_p_p),P9_P_klemm),P99_P_lati)),(Potu,Lygo)),R_boultoni),Ebenavia),(Uroplatus,Hemidact)),Gehyra),Rhacodact)	P = 0.003
Comoroan taxa monophyletic on branch leading to <i>P. comorensis</i>	((((((((((((P25_P_g_ro,P110_P_cep),((P16_P_b_bo,P24_P_b_ag),(P18_P_gimb,(P23_P_o_or,P7_P_o_ine))))),P112_P_gue),P31_P_stan),((((P34_P_s_la,P62_P_s_lo),P10_P_a_as),((P113_P_sei,(P81_P_abot,(P103_m_ma,P149_P_par))))),P54_P_gutt),P3_P_mutab)),(P98_P_brev,P111_P_and)),(((P190_P_dub,P107_P_hie),P154_P_q_q),P90_P_mode),P49_P_berg,P87_P_barb))),((((P88_P_como,P12_P_nigr),P89_P_v_ni),P109_P_rob),P82_P_line),P150_P_p_p),P9_P_klemm),P99_P_lati)),(Potu,Lygo)),R_boultoni),Ebenavia),(Uroplatus,Hemidact)),Gehyra),Rhacodact)	P = 0.000
Comoroan taxa monophyletic on branch leading to <i>P. v-nigra</i>	((((((((((((P25_P_g_ro,P110_P_cep),((P16_P_b_bo,P24_P_b_ag),(P18_P_gimb,(P23_P_o_or,P7_P_o_ine))))),P112_P_gue),P31_P_stan),((((P34_P_s_la,P62_P_s_lo),P10_P_a_as),((P113_P_sei,(P81_P_abot,(P103_m_ma,P149_P_par))))),P54_P_gutt),P3_P_mutab)),(P98_P_brev,P111_P_and)),(((P190_P_dub,P107_P_hie),P154_P_q_q),P90_P_mode),P49_P_berg,P87_P_barb))),((((P88_P_como,P12_P_nigr),P89_P_v_ni),P109_P_rob),P9_P_klemm),P82_P_line),P150_P_p_p),P99_P_lati)),(Potu,Lygo_sp)),R_boultoni),Ebenavia),(Uroplatus,Hemidact)),Gehyra),Rhacodact)	P = 0.002

- Hedges, S.B. (1992): The number of replications needed for accurate estimation of the bootstrap P value in phylogenetic studies. *Mol. Biol. Evol.* **9**: 366-369.
- Posada, D., Crandall, K.A. (1998): MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.
- Radtkey, R.R. (1996): Adaptive radiation of day-geckos (*Phelsuma*) in the Seychelles archipelago: a phylogenetic analysis. *Evolution* **50**: 604-623.
- Rice, W.R. (1989): Analyzing tables of statistical tests. *Evolution* **43**: 223-225.
- Rocha, S., Posada, D., Carretero, M.A., Harris, D.J. (in press): Phylogenetic affinities of Comoroan and East African day geckos (genus *Phelsuma*): multiple natural colonisations, introductions and island radiations. *Molecular Phylogenetics and Evolution*.
- Röll, B. (1999): Biochemical and morphological aspects of the relationship of the Namaqua day gecko to *Phelsuma* und *Rhotropus* (Reptilia, Gekkonidae). *Zoology* **102**: 50-60.
- Russel, A. P. (1977): The genera *Rhotropus* and *Phelsuma* (Reptilia: Gekkonidae) in southern Africa: a case of convergence and a reconsideration of the biogeography of *Phelsuma*. *Zool. Afr.* **12**: 393-408.
- Saitou, N., Nei, M. (1987): The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.
- Shimodaira, H., Hasegawa, M. (1999): Multiple comparison of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* **16**: 1114-1116.
- Strimmer, K., von Haeseler, A. (1996): Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* **13**: 964-969.
- Swofford, D.L. (2002): Paup\*: Phylogenetic analysis using parsimony (and other methods), version 4.0b10, Sinauer Associates, Sunderland/Massachusetts.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**: 4876-4882.
- Vences, M., Vieites, D.R., Glaw, F., Brinkmann, H., Kosuch, J., Veith, M., Meyer, A. (2003): Multiple overseas dispersal in amphibians. *Proc. Roy. Soc. London B* **270**: 2435-2442.
- Zhang, D.-X., Hewitt, G.M. (1996): Nuclear integrations: challenges for mitochondrial DNA markers. *Trends Ecol. Evol.* **11**: 247-251.