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The influence of riverine barriers on phylogeographic patterns of Malagasy reed frogs (*Heterixalus*)

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ABSTRACT

We analyzed the influence of large rivers on the phylogeography of endemic widespread amphibians along Madagascar's east coast, using as models various species and species complexes of Malagasy reed frogs (*Heterixalus* spp.) that are specialized to either highland or lowland habitats. We assembled a dense sampling across the full species ranges and used mitochondrial (*cob*) as well as nuclear (*Rag-1*) DNA sequences to assess their phylogeographies. A multigene mtDNA phylogeny of each species was constructed to establish the relationships among the main phylogroups, in order to understand the geographical regions of clade origins and possible directions of historical range expansions. Distinct intraspecific lineages as diagnosed by mitochondrial haplotype clades were found in highlands and lowlands. Most geographical boundaries among these phylogroups did not coincide with rivers, indicating that the influence of rivers on the primary divergence of phylogroups is probably minor in these frogs. Nevertheless, we found evidence for the influence of one riverine barrier in the lowland species complex, where the most important genetic discontinuity (the border between *Heterixalus madagascariensis* and *H. alboguttatus*) coincides with the geographical position of the Mangoro River on Madagascar's central east coast. Analyses of the highland species *H. betsileo* revealed the existence of six deep haplotype lineages, separated in two major subpopulations which differ largely in altitudinal distribution and do not overlap with the geographical settings of rivers in the highlands. Furthermore, our analyses indicated that most of the major intraspecific lineages of reed frogs show signs of a rather recent population expansion.

1. Introduction

Large bodies of water are among the most obvious barriers to dispersal and hence to gene flow of terrestrial organisms, although occasional dispersal even over wide marine barriers is today acknowledged as having a major influence on biogeographical patterns (de Queiroz, 2005). Because of their geographically wide and temporally stable extension, large rivers clearly play a major role in delimiting the geographical distribution of species, as first noted by Wallace (1852) for Amazonian monkeys. While there are no doubts that rivers do limit the distributions of many species, their role in the actual process of species formation remains unclear: they might constitute the primary factor causing the isolation of populations and their divergence in allopatry, or they might simply be a barrier to secondary contact of expanding populations that previously diverged in refugia (Moritz et al., 2000; Vences et al., 2009). Several case studies have dealt with the influence of rivers on the distribution and genetic structure of species, especially in the Amazon where rivers are particularly wide and thus can be efficient barriers (Gascon et al., 1996, 1998, 2000; Lougheed et al., 1999; Patton et al., 2000; Hayes and Sewlal, 2004). As a general conclusion from these studies, the influence of rivers seems to be specific to taxa and particular geographical settings, but few groups of organisms appear to be as strictly limited by rivers as are primates. The strength of riverine barriers may decrease towards the headwaters where rivers are narrower, as shown in a number of vertebrate groups (e.g. Ayres and Clutton-Brock, 1992; Hayes and Sewlal, 2004).

Despite having no rivers as wide as the Amazon and its tributaries, Madagascar constitutes an ideal geographical setting for testing the influence of riverine barriers on diversification (Vences et al., 2009). This island can be interpreted as being geographically rather simple, with an eastern rainforest belt and a western dry forest belt separated by mountains. Rivers running either eastwards or westwards slice these biomes at regular intervals. The inter-river systems (IRS) are known to be centers of endemism

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for lemurs (Martin, 1972; Pastorini et al., 2003), although even the largest Malagasy rivers do not constitute absolute limits for all species of these primates (Goodman and Ganzhorn, 2004; Craul et al., 2007; Olivieri et al., 2007; Radespiel et al., 2008). For highly vagile taxa such as bats, rivers typically do not mark the geographical distributions of species (Weyeneth et al., 2011). In a dung beetle species, different phylogeographic lineages occur on each side of the Mangoro River in central eastern Madagascar (Knopp et al., 2011), although in general, rivers probably do not have a major influence on the diversification of dung beetles (Miraldo et al., 2011). Rivers have also been invoked as possible barriers to dispersal for Malagasy reptiles and amphibians (Yoder et al., 2005; Vieites et al., 2006; Boumans et al., 2007; Paquette et al., 2007).

Several alternative hypotheses have been proposed to explain the origin of the extraordinary species diversity of Madagascar's unique biota (e.g., Raxworthy and Nussbaum, 1995; Wilmé et al., 2006; Boumans et al., 2007; Wollenberg et al., 2008; reviewed by Vences et al., 2009), but whether any of these is prevalent remains unexplored. Large rivers, defined as >50 m wide at 20 km inland with probable barrier effects at least on lemurs in western Madagascar, are the Betsiboka and Tsiribihina, as well as a number of additional rivers in the north-west. In eastern Madagascar, the Mangoro and Mananara rivers have been emphasized as boundaries for species distribution in lemurs, anurans and reptiles (Goodman and Ganzhorn, 2004; Vieites et al., 2006; Boumans et al., 2007). However, convincing case studies based on dense sampling are scarce.

Most suitable for testing riverine barriers in Madagascar are species or species complexes that are widespread either along Madagascar's east or west coast, so that the effect of various rivers on their phylogeographic structure can be assessed simultaneously. Optimally, these are furthermore specialized to particular altitudinal levels allowing for a comparative assessment of the effect of river width on gene-flow disruption. One such suitable group consists of species of Malagasy reed frogs of the genus *Heterixalus* (Hyperoliidae). Contrasting with the high degree of microendemism in other groups of Malagasy anurans (e.g., Wollenberg et al., 2008, 2011), reed frogs are rather widespread in disturbed and secondary habitats but have relatively narrow elevational ranges (Glaw and Vences, 2007). Amphibians, in general, have low individual mobility which promotes genetically highly structured populations over short geographical distances, with the retention of signals of historical events that generated current species distributions (reviewed by Zeisset and Beebee, 2008). *Heterixalus*, relative to other Malagasy frogs, presumably are more vagile and comparatively good colonizers. Nevertheless, available data indicate that several species have genetically divergent haplotypes in geographically distant populations (Wollenberg et al., 2007).

All eleven of the currently recognized *Heterixalus* species are medium-sized treefrogs (snout-vent length 18–40 mm) of similar morphology, mainly distinguished by differences in color and pattern, which can also be variable within species, and by their advertisement calls (Blommers-Schlösser, 1982; Glaw and Vences, 1993, 2007; Wollenberg et al., 2007). These frogs are adapted to open areas outside forests and inhabit swamps, rice fields and forest edges, where they use lentic water bodies for reproduction (Blommers-Schlösser, 1982; Glaw and Vences, 2007; Gehring et al., 2010, 2011).

Although the monophyly of the Hyperoliidae and the inclusion of *Heterixalus* in this family have been supported already by morphological studies (Drewes, 1984; Channing, 1989), the inter-generic relationships of hyperoliids were disputed until molecular data became available. Analyses of mitochondrial and nuclear DNA sequences unambiguously support a sister group relationship of *Heterixalus* and the Seychellean monotypic genus *Tachycnemis*; the closest relatives of this clade are the African genera *Afrixalus* or *Hyperolius* (Richards and Moore, 1996; Vences et al., 2003a; Wollenberg et al., 2007). The diversification of *Heterixalus* in Madagascar occurred after a colonization event from Africa estimated at ca. 22 mya (Vences et al., 2003b), giving rise to the eleven species currently recognized.

In the present study we focus on two subclades of *Heterixalus*, based on the species-level phylogeny of *Heterixalus* by Wollenberg et al. (2007). These two subclades do not represent sister clades, and each is strongly supported by the previous study as monophyletic. The first of these comprises *H. betsileo* occurring in the submontane highlands of eastern Madagascar, and for completeness we also include some populations of its morphologically similar sister species *H. carbonei* from the west and north of the island (Vences et al., 2000; Glaw and Vences, 2007). The second clade contains three closely related species, *H. madagascariensis*, *H. alboguttatus* and *H. boettgeri*, which are confined to the eastern lowlands. These two groups are distributed roughly in parallel along the east coast at different altitudes, and only in some mid-altitude localities do species of the two clades occur in sympatry (e.g. *H. betsileo* and *H. alboguttatus* in Ranomafana). *H. madagascariensis*, *H. alboguttatus* and *H. boettgeri* occur allopatrically and are separated by very shallow genetic distances (only 0.38% 16S rRNA sequence divergence between *H. alboguttatus* and *H. boettgeri*; Wollenberg et al., 2007) and have similar advertisement calls. For the purpose of this study, the three taxa are therefore treated as a single species-level unit called the *H. madagascariensis* complex.

Herein we use Malagasy reed frogs as model species to test the influence of large rivers on the phylogeography of widespread amphibians distributed along Madagascar's east coast. We thoroughly sample the full species ranges and use mitochondrial as well as nuclear genes to assess their phylogeographies.

2. Material and methods

2.1. Geographical setting

Across much of Madagascar's eastern coastline, elevation increases rapidly from the sea westwards towards the central highlands (defined as the zone starting between 800 and 1000 m a.s.l.). Because of this geographical pattern, almost all rivers flow eastwards from the central highlands to the Indian Ocean over a comparatively short distance, dividing the lowland areas into several discrete subunits. None of these river systems form large meandering channels passing through large tracts of lowland habitat, and each can therefore be considered a potential geographic barrier to gene flow between populations on either side of the river. The only exception to this general topographic pattern of river systems is the Antainambalana River in the northeast, and the Canal de Pangalanes, which runs in parallel along the coast. The canal, constructed in the early 1900s, connects a chain of natural freshwater lakes and lagoons (e.g., Hance, 1958). Because of its recent origin, it is highly unlikely that this canal, through rafting of individuals along its current extent, has exerted any relevant influence on the phylogeographic structure of the target species.

2.2. Hypothesis testing

Given the geographical setting and the target species of this study, we define the following hypotheses and predictions to be tested using phylogeographic methods.

(i) If rivers exert a major influence on the phylogeographic structure of our target species, we expect that genetic discontinuities between populations are highest across river systems.
(ii) We also expect that phylogeographic structure would be more geographically concordant with river barriers in the lowland species, given that rivers are wider in the lowlands and thus their barrier effect is predicted to be stronger. In detail, this implies that phylogeographic subdivisions in the lowland species would be strictly arranged along a north–south axis, whereas east–west subdivisions might be possible in the highland species.
(iii) In the case of primary barrier divergence, phylogeographic lineages (or species) on each side of the barrier would be sister lineages; in the case of divergence after dispersal across the river, lineages on one side of the river may be nested among those on the other side.

As a basis for testing these predictions we used DNA sequences of fragments of one mitochondrial and one nuclear gene. A multigene mtDNA phylogeny of each species was used to reconstruct relationships among main phylogroups with maximum statistical support, in order to understand the geographical regions of clade origins and possible directions of historical range expansions. If rivers generate phylogeographic breaks within the studied species, we expect that genetic differences between populations on opposite sides of rivers are higher than between populations on the same river bank. We applied a variety of phylogeographic analyses aimed at elucidating the patterns of spatial genetic structure in *Heterixalus* in order to test whether rivers in eastern Madagascar are associated with significant genetic discontinuities in these frogs.

2.3. Sample collection

Tissue samples were mainly collected by P.-S. Gehring, F. Ratsoavina, E. Rajeriarison, and François F. Randrianasolo in April and May 2009 and 2010 along an approximately 1000 km long north–south transect along Madagascar's east coast. Collection of *H. betsileo* samples from the central highlands benefited greatly by the collecting efforts of J. Randrianirina in February 2010. Additional tissue samples were obtained during fieldwork in Madagascar in the period from 2000–2010 by M. Vences, F. Glaw and D. R. Vieites (DRV). FGMV, FGZC and ZCMV refer to F. Glaw and M. Vences field numbers. PSG refers to field numbers of P.-S. Gehring.

Species identification in the field was mainly based on coloration patterns as shown in Glaw and Vences (2007) and Wollenberg et al. (2007), which were assessed although not individually noted in the field. However, species identification based only on chromatic characters can be misleading in these frogs and was therefore eventually ascertained through molecular analyses. Tissue samples were taken from frogs and preserved in 99% ethanol. Nearly all animals were immediately released after sampling. The tissue samples are deposited at the Zoological Institute of the Technical University of Braunschweig (Germany). Some selected voucher specimens were euthanized in a chlorobutanol solution, fixed in 90% ethanol and preserved in 70% ethanol. The specimens are held at the University of Antananarivo, Département de Biologie Animale, Madagascar (UADBA-FGMV, UADBA) and the Zoologische Staatssammlung München, Germany (ZSM). Collecting localities were selected *a priori* with the aim of covering the whole distribution at regular intervals, and of obtaining a representative sample size. Altogether we sampled a total of 385 individuals from 75 localities. Locality information was recorded with GPS receivers (see Supplementary material Tables S1–4).

Maps including an elevation layer of Madagascar (Supplementary material Fig. 2) were obtained using the program DIVA GIS (Version 7.3.0.1.; http://diva-gis.org).

2.4. DNA extraction, amplification and sequencing

Two molecular datasets were obtained for most of the samples and used for phylogeographic analyses: (i) a small but rather highly variable fragment of the mitochondrial cytochrome *b* gene (*cob*), and (ii) a fragment of the gene encoding the recombination activation protein (*Rag-1*), a single-copy nuclear marker widely used in resolving relationships among vertebrate species (Chiari et al., 2009) and known to be variable among and within *Heterixalus* species (Wollenberg et al., 2007).

Total genomic DNA was extracted from the tissue samples using proteinase K digestion (10 mg/ml concentration) followed by a standard salt-extraction protocol (Bruford et al., 1992). A fragment of ca. 550 base pairs (bp) of the cytochrome *b* gene (*cob*) was initially amplified using the primers Cytb-a and Cytb-c (Bossuyt and Milinkovitch, 2000). Species-specific primers were then designed from the obtained sequences (Table S5 in the online Supplementary material) and used thereafter. The obtained fragment, after discarding ambiguous nucleotides at the beginning and at the end, had a length of 316 bp (for details see the extended methods in Supplementary material).

We used *Rag-1* sequences of different *Heterixalus* species deposited in GenBank (Wollenberg et al., 2007) to design new genus-specific primers for this gene (Table S5), which amplified a fragment of 699 bp (for details see extended methods in Supplementary material).

To resolve the phylogenetic relationships between major mtDNA clades observed in the *cob* datasets with high statistical support, we amplified additional mitochondrial genes (*12S rRNA*, *16S rRNA*, *CO1* and *ND1*, as well as several intervening *tRNAs*) for two selected representatives of each major *cob* clade (2994 bp in total). PCR primers are listed in Table S5.

All obtained PCR products were purified by Exonuclease I and Shrimp Alkaline Phosphatase digestion and largely sequenced by Macrogen Inc. (Seoul, S. Korea, http://www.macrogen.com). Additional samples were sequenced using BigDye v3.1 cycle sequencing chemistry and run on a 3130xl genetic analyzer (Applied Biosystems). Chromatograms were quality checked by eye, and sequences were edited, where necessary, using CodonCode Aligner (v. 3.7.1, Codon Code Corporation). Newly determined sequences have been deposited in GenBank (accession numbers JQ346229–JQ346522 and JQ362960–JQ363616, details in Tables S1–4).

2.5. Haplotype networks and mtDNA phylogeny

The alignment of *cob* sequences was checked by hand and translated into amino acids; basic analyses of nucleotide variation were performed in MEGA version 5.05 (Tamura et al., 2011). The final alignment was imported into DNASP v5.10 (Librado and Rozas, 2009) and converted into Roehlfile format as needed for the software Network (see below).

In some intraspecific analyses a hierarchical tree format may be inappropriate for representing relationships among haplotypes because the period of time over which the samples have evolved is so short that ancestral and descendant haplotypes exist concurrently (Posada and Crandall, 2001; Kratysberg et al., 2004). In such instances, a haplotype network is more appropriate to illustrate relationships among the sampled haplotypes. We assessed the genetic structure through median-joining haplotype networks (Bandelt et al., 1999) using the program Network (version 4.6; http://fluxus-engineering.com). Post-processing calculation was done under the MP criterion ($\varepsilon = 10$) to search for the shortest tree. Networks

were manually redrawn and graphically processed in Corel DrawX3.

The *Rag-1* dataset was aligned in MEGA and then phased using the Phase algorithm (Stephens et al., 2001; Stephens and Scheet, 2005) implemented in the software DNASP (Librado and Rozas, 2009) in order to infer haplotypes (three runs under different starting seed numbers with 1000, 5000 and 10,000 iterations). We used haplotype reconstructions from the run with the highest average goodness of fit to the underlying coalescent model (no recombination, 10,000 iterations, other parameters set at default values). No indels or stop codons were detected in the nuclear haplotypes. The *Rag-1* gene datasets were checked for recombination in Datamonkey (Delport et al., 2010; Kosakovsky Pond and Frost, 2005; http://www.datamonkey.org) with GARD (Kosakovsky Pond et al., 2006), and in the recombination detection program RDP (Martin et al., 2010) where searches under RDP, Geneconv and Maxchi detected no recombination. Median-joining haplotype networks were constructed using the previously phased datasets.

Phylogenetic analyses of representatives of the major mtDNA phylogroups were performed using Bayesian inference (BI), maximum likelihood (ML), and maximum parsimony (MP). The complete mtDNA alignment consisted of 2994 bp in the *H. betsileo* dataset and of 2993 bp in the dataset of *H. madagascariensis*, *H. alboguttatus* and *H. boettgeri* (in the following summarized as the *H. madagascariensis* complex). Substitution models for ML and BI were determined by AIC in jModelTest 0.1.1 (Posada, 2008). The appropriate data partition scheme for BI analysis was determined by a comparison of Bayes factors of various alternative partition schemes according to the procedure outlined in Brandley et al. (2005). Partitioning schemes, selected models and Bayes factor comparisons are provided in Supplementary material Tables S6–S8. Analyses were performed in MrBayes 3.1.2. (Ronquist and Huelsenbeck, 2003) with two runs of 10 million generations (started on random trees) and four chains, sampling the Markov chains at intervals of 1000 generations.

MP analyses were conducted in PAUP* 4.0b10 (Swofford, 2002) and ML analyses in PhyML 3.0. (Guindon and Gascuel, 2003), respectively (for settings and detailed results of the ML and MP analyses, see Supplementary material).

In the phylogenetic analyses of *H. betsileo* we used 16 specimens of the closely related *H. carbonei* from two different localities: from the type locality Tsingy de Bemaraha ($n = 2$) and from Montagne d'Ambre ($n = 14$) as an outgroup. Sequences of a *H. betsileo* specimen from Vohiparara were used as an outgroup for analysis of the *H. madagascariensis* complex.

2.6. Sequence diversity indices and population genetic structure

All analyses were conducted separately for the *H. betsileo* and *H. madagascariensis* complex datasets. Haplotype (h_d), nucleotide (π) and sequence diversity (k) were estimated in DNASP 5.10 for each species and for each of the major mtDNA clades.

We assessed genetic structure using three approaches: first, we measured how the genetic variation is partitioned within the species complexes using *a priori* defined groups of populations delimited by the boundaries of major rivers within the AMOVA framework. Second, we used SAMOVA analyses to identify genetically homogeneous and maximally differentiated groups of populations in geographical space without *a priori* assumptions of previous population groupings. Finally, we identified geographic areas that are associated with a significant genetic change using BARRIER software. Afterwards we compared the results of the AMOVA with the SAMOVA and BARRIER analyses, in order to quantify how much genetic variation is partitioned by major rivers.

Analyses of molecular variance (AMOVA) were run in order to infer the hierarchical patterns of *cob* and *Rag-1* variation utilizing the program ARLEQUIN 3.5 (Excoffier and Lischer, 2010). AMOVAs were performed using pairwise differences and 10,000 random permutations; other settings were left as defaults. We performed hierarchical AMOVAs at three different levels to quantify how much variation is partitioned: (i) within populations, (ii) among populations, and (iii) among groups of populations that are delimited by the boundaries of major rivers.

For the *H. betsileo* populations, we assumed three different IRSs within the distribution area of this species (see Fig. S2 in Supplementary material), going from north to south: group I, populations north of the Mania River (the headwaters of the Tsiribihina River); group II, populations between the Mania and Mantsiatra Rivers (the headwaters of the Mangoky River); group III, populations south of the Mantsiatra River. To test for the influence of altitudinal differences between *H. betsileo* populations, we performed an AMOVA with two groups of mid-elevation populations: group I, northern populations (e.g. Andasibe, Vohidrazana), and group II southern populations (Ranomafana and Vohiparara) and group III including all highland populations.

For the *H. madagascariensis* complex (including *H. alboguttatus* and *boettgeri*) the following groups of populations, from north to south, were defined (see Fig. S2 in Supplementary material): group I, populations north of the Onibe River (excluding the population on the island of Nosy Boraha); group II, populations between the Onibe and Mangoro Rivers; group III, populations between Mangoro and Mananjary Rivers; group IV, populations between Mananjary and Mananara Rivers; group V, populations between Mananara and Mandrare Rivers.

Additionally, we examined the hierarchical genetic structure of *Rag-1* among and within major mtDNA clades for each species, to determine if nuclear variation and mtDNA variation are partitioned similarly.

In the next step, we conducted a spatial analysis of molecular variance (SAMOVA) with Samova 1.0 (Dupanloup et al., 2002) which takes into consideration the geographical locations of sampling sites, and maximizes the proportion of genetic variance among K groups aiming to identify groups of populations that are geographically homogeneous and maximally differentiated in terms of the among-group component (F_{ct}) of the overall genetic variance (Dupanloup et al., 2002). Thereby the prior assumption of group composition required for AMOVA can be abandoned (Dupanloup et al., 2002). All possible grouping options were run in SAMOVA independently for the *cob* and *Rag-1* datasets of *H. betsileo* K {2,...,29} and the *H. madagascariensis* complex K {2,...,34} in order to determine the maximum value for F_{ct}, i.e. the proportion of total genetic variance representing differences among groups of populations, with 10,000 iterations from each of 100 random initial conditions.

To provide a geographic representation of genetic discontinuities between populations, we identified in a third step the geographic areas associated with significant genetic change using the methods of Manni et al. (2004) implemented in Barrier 2.2. With this software a geometric network connecting all the sampled populations is computed; then by calculating Monmonier's maximum-difference algorithm on a genetic distance matrix of the corresponding populations, boundaries can be identified where differences between pairs of populations are largest (Manni et al., 2004). These boundaries were calculated for the *H. betsileo* and *H. madagascariensis* complex datasets separately, for (i) the *cob* datasets only and (ii) for a combined dataset of *cob* and *Rag-1*.

The relationship between genetic differentiation (F_{st} distance matrices) among populations and their geographical distances (linear distance in km) was evaluated through a Mantel test with 10,000 permutations, as implemented in the software ARLEQUIN 3.1. Geographic distance matrices were obtained by converting

GPS locality data in the online software Geographic Distance Matrix Generator v.1.2.3 (Ersts, 2011). Mantel tests were conducted (i) on different datasets containing *cob* sequences and *Rag-1* of all populations and (ii) on *cob* sequences of grouped populations corresponding to major mtDNA clades, in order to avoid possible bias caused by long-term divergence among major clades. Long-term historical divergence between two or more groups of populations could confound the interpretation of isolation by distance (IBD) because the latter occurs on a much smaller time-scale (De Campos Telles and Diniz-Filho, 2005).

2.7. Demographic history

Evidence for population growth can be obtained from an examination of the distribution of pairwise differences among haplotypes, or mismatch distributions (Slatkin and Hudson, 1991) using ARLEQUIN 3.1. This analysis produces the index of raggedness (Harpending, 1994) and an estimate of the fit between the observed and expected mismatch (sum of squares deviations, SSD). The raggedness index is generally expected to assume a large value in relatively stable populations, which themselves exhibit a multimodal, or non-Poisson, distribution of haplotypic differences. Parameters for the spatial expansion model were estimated from the data assuming theta 1 (population size after expansion) as infinite. Goodness-of-fit of spatial expansion was assessed by calculating the significance of the raggedness index. We assumed no recombination and used 1000 simulations to estimate the probability of obtaining a raggedness index of less than the observed value. Raggedness indices were assumed to be smaller than 0.04 for expanding populations (Harpending, 1994). Generally, the raggedness index has been shown to be a powerful tool in quantifying population growth with limited sample sizes (Ramos-Onsins and Rozas, 2002).

Additional evidence for population expansion was obtained by the implementation of neutrality tests. Tajima's *D* (Tajima, 1989) and Fu's F_s (Fu, 1997) were calculated for the *cob* and the phased *Rag-1* dataset for each *Heterixalus* species separately using ARLEQUIN. Tajima's *D* was developed to check for selective neutrality but is widely used to test for population bottlenecks or rapid range expansions, which also cause departures from equilibrium (e.g. Althoff and Pellmyr, 2002; Toju and Sota, 2006). A significantly positive *D* is evidence of a population bottleneck or selection, whereas a significantly negative *D* is evidence of a recent range expansion (because of a large number of recent mutations relative to segregating sites assuming neutrality). A nonsignificant *D* is consistent with a population at drift-mutation equilibrium.

Negative F_s values reflect an excess of rare polymorphisms in a population, which is consistent with either positive selection or an increase in population size. Positive F_s values indicate an excess of intermediate-frequency alleles in a population and can result from balancing selection, population bottlenecks or the presence of population structure. We chose these tests because of their elevated statistical power in detecting significant changes in population size when using larger sample sizes (Simonsen et al., 1995; Ramos-Onsins and Rozas, 2002).

3. Results

DNA sequences of 316 bp of the mitochondrial *cob* gene were obtained from a total of 385 specimens (179 *H. betsileo*, 16 *H. carbonei*, and 190 specimens of the *H. madagascariensis* complex), and a fragment of 699 bp and 599 bp of the nuclear *Rag-1* gene from 169 specimens of *H. betsileo* and 140 specimens of the *H. madagascariensis* complex, respectively.

3.1. Mitochondrial DNA phylogeny and sequence diversity in H. betsileo

The *cob* dataset of the highland species *H. betsileo* used for network analyses incorporated 179 specimens with 62 unique haplotypes clustering in six major haplotype lineages (named HB1–HB6; Fig. 1). High values were found for mean haplotype diversity (h_d = 0.931; 76 segregating sites; 56 parsimony informative sites) and nucleotide diversity (π = 0.05; k = 14.87; see Table 1). We found seven different *cob* haplotypes (h_d = 0.742; 59 segregating sites; 56 parsimony informative sites) among the 16 sequenced individuals from three *H. carbonei* populations (See Supplementary material Table S10).

The full concatenated mtDNA dataset of *H. betsileo* contained 2994 total characters of which 275 were parsimony-informative (for details see extended results in Supplementary material). All phylogenetic analyses (MP, ML, BI) were consistent in placing samples from the northern Tsaratanana mountain range with *H. carbonei* from its western type locality Bemaraha rather than with *H. betsileo* (Fig. S1). No haplotype sharing was found between *H. betsileo* and *H. carbonei*. However, the three *H. carbonei* populations showed deep divergences from each other and require additional study. The 62 *H. betsileo* haplotypes clustered in four well supported mtDNA clades containing phylogroups HB1 + HB2, HB3, HB4 + HB5, and HB6, clearly correlating with their geographical distribution (Figs. 1B and C). Basal relationships among these clades, however, were not reliably and congruently resolved by the various analyses.

Lineage HB1 is restricted to the northernmost parts of the distribution of *H. betsileo.* HB2 occurs mostly at mid-elevations (600–1000 m a.s.l.; plus one locality, Tsinjoarivo, at 1300 m a.s.l.) of the northern central east. HB3 is mainly distributed in two localities of the southern central east. HB4 and HB5 comprise haplotypes from localities of the central highlands, roughly from Antsirabe to Ambohimahasoa (HB4) and Antananarivo to Ankaratra (HB5). HB6 includes all highland localities from the southern margin of the *H. betsileo* range, from Sahambavy in the north to the Andringitra mountain massif in the south. No haplotype sharing with the nearby populations of HB3 exists. The highest haplotype diversity with 16 different haplotypes (h_d = 0.912 ± 0.025) is in HB6, the second highest in HB1 + HB2 (h_d = 0.89 ± 0.081; Table 1). In most populations, haplotypes of only one major lineage were found, although occasional haplotype sharing of almost all major lineages does occur, usually in populations geographically adjacent to the main ranges of two or more of the major lineages. Exceptions are the populations in Ambohimahasoa and Tsarasaotra, which contained haplotypes from geographically distant lineages (Fig. 1C).

For a detailed overview of the genetic diversity of each population see Supplementary material Table S9.

3.2. Nuclear DNA sequence diversity in H. betsileo

Altogether 56 *Rag-1* haplotypes were identified in the 146 sequenced specimens of *H. betsileo* and *H. carbonei* (h_d = 0.7918; 47 segregating sites; Table 1). We included in the analyses of molecular diversity only those samples for which both sequences (*cob* and *Rag-1*) were available (explaining the lower sample sizes in Table 1). Samples of *H. carbonei* from two different localities (Tsingy de Bemaraha and Montagne d'Ambre) were included as well, given that haplotype sharing among the two species was observed. Forty *H. betsileo* and four *H. carbonei* individuals showed signs of heterozygosity in the *Rag-1* sequence and twelve *H. betsileo* were heterozygous in more than one nucleotide position, i.e. in 2 (n = 9) or 3 (n = 3) nucleotide positions. All heterozygotes were phased with high probability (above 0.95). Overall nucleotide variation

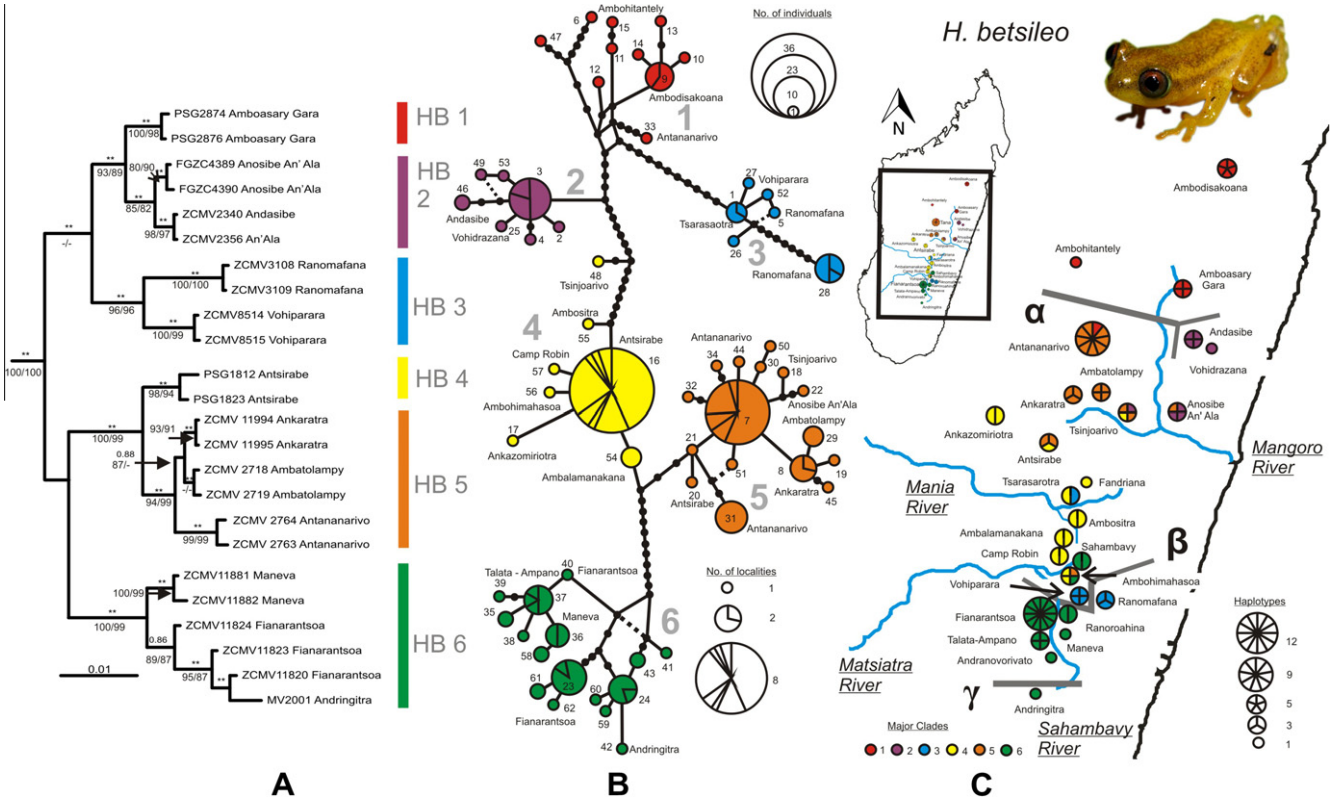

Fig. 1. Results of phylogenetic and phylogeographic analyses of the highland species *Heterixalus betsileo*. (A) Molecular phylogeny of major mtDNA clades within *H. betsileo* obtained by Bayesian inference under the best partitioning strategy, based on a total of 2994 base pairs of seven mitochondrial gene fragments. Node values indicate (above) Bayesian posterior probabilities (**>95; *>90%); (below) bootstrap values (>85%) of MP (first) and ML (second) analyses. The topology is equivalent in MP and ML analyses. Sequences of *H. carbonei* were used as outgroups (not shown). (B) Median-joining network of *H. betsileo cob* haplotypes. The consensus network of all the shortest trees is shown. Black dots are median vectors presumed unsampled or missing intermediates. Numbers denote haplotypes; colors denote the major mtDNA clades as identified by phylogenetic analyses; size is proportional to their frequencies. (C) Geographical location of the sampled populations and their haplotype composition, and results of the barrier analyses. Populations are colored according to the frequency of the haplotype clades identified by phylogenetic analyses. The first three genetic barriers α, β, and γ, identified by SAMOVA in the combined analyses of the *cob* and *Rag-1* datasets are represented by grey lines. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

(π = 0.02; k = 1.6) was lower than in the *cob* alignment for the same species.

Rag-1 haplotypes of *H. betsileo* were separated by a maximum of 11 mutational steps (from haplotypes no. 35 to 41), although most differed by one or two mutational steps (Fig. 2). Two haplotypes of *H. carbonei* from the Tsingy de Bemaraha were clearly separated from *H. betsileo*, but haplotype sharing occurred between *H. carbonei* from Montagne d'Ambre and *H. betsileo* (haplotype no. 1).

Although we detected only very shallow differentiation in the nuclear dataset, there was a low level of haplotype sharing among individuals corresponding to the northern (HB1, HB2, HB5) and southern (HB3, HB4, HB6) mitochondrial lineages of *H. betsileo*, with the exception of the widespread and most common haplotype 7 found across the entire distribution. The highest number of exclusive *Rag-1* haplotypes (n = 10) occurred in populations corresponding to the mtDNA lineage HB5 from central Madagascar (orange haplotypes), followed by the geographically proximate HB2 (n = 9) from mid-elevation localities. Generally, the *Rag-1* network showed a star-shaped pattern, pointing to a possible recent range expansion.

3.3. Genetic structure and differentiation of H. betsileo populations

We performed hierarchical AMOVAs on the mitochondrial *cob* and the nuclear *Rag-1* dataset at three different levels in order to examine the spatial partitioning of genetic variation: (i) within populations, (ii) among populations, and (iii) among groups of populations that are delimited by the boundaries of major rivers in the highlands. For these analyses we excluded the populations from mid-elevations.

A total of 38.9% of the genetic variation in *cob* was attributable to the grouping of populations delimited by the boundaries of major river systems (Table 3); more variation (49.1%) was explained if the *H. betsileo* populations were grouped according to altitude. Most of the variation (72.8% and 69.9% in IRSs and altitudinal analyses, respectively) in *Rag-1* was explained by the within-population variance component (Table 3). 13.4% of the variation in *Rag-1* could be accounted for by the distribution of major *cob* haplotypes (Table 2).

Two criteria were considered to select the number of groups in the SAMOVA analyses. First, the pattern of F_{CT} values was examined as a function of K. In particular, we checked the number of groups necessary for F_{CT} to reach a plateau. Secondly, we excluded configurations with one or more single-population groups, because this indicates that the group structure is disappearing. Due to the very low genetic structure in the nuclear dataset we restricted SAMOVA analyses to the *cob* datasets. The results of SAMOVA (Fig. 3; Table 4) revealed that sequences from 29 sampling sites of *H. betsileo* can be best placed in six groups (to avoid confusion we use "groups" equivalent to the term "populations" as used in SAMOVA); a further increase of K did not produce additional informative groups. The presence of six groups is indicated by an increase in F_{ct} followed by a slightly increasing plateau at six to ten groups. However, the assembly in six groups is in perfect accor-

Table 1
Molecular diversity in cytochrome *b* and *Rag-1* sequences for each *Heterixalus* species and for major mtDNA clades within species; population demographic statistics. H, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; *S*, number of segregating sites; *k*, sequence diversity; *D*, Tajima's *D* (Tajima, 1989); F_s, *Fu's* F_s (Fu, 1997); SSD, sum of squared deviations between the observed mismatch distribution and the distribution expected under a sudden demographic expansion model; r_{Harp}, raggedness index of the mismatch distribution as defined by Harpending (1994).

Species	mtDNA Lineage	*n*	H	*h* (±standard deviation)	*S*	*k*	*D*	F_s	*SSD*	r_{Harp}
cob molecular diversity										
H. betsileo	1	14	10	0.89 ± 0.08	24	4.97	−1.44	−2.535	0.008	0.02
H. betsileo	2	16	7	0.69 ± 0.124	8	1.525	−1.38	−2.621*	0.007	0.039
H. betsileo	3	14	6	0.79 ± 0.09	12	5.418	1.74	0.74	0.123	0.18
H. betsileo	4	45	7	0.32 ± 0.09	15	0.792	−2.40*	−2.972*	0.002	0.238
H. betsileo	5	49	16	0.77 ± 0.06	20	2.238	−2.14*	−5.225*	0.007	0.028
H. betsileo	6	38	16	0.91 ± 0.03	19	5.182	0.44	−2.58	0.025	0.031
	All	**179**	**62**	**0.93 ± 0.012**	**76**	**14.865**	**0.40**	**−12.906***	**0.011**	**0.013***
H. madagascariensis	1	24	6	0.5 ± 0.121	6	0.801	−1.52*	−2.663*	0.01	0.13
H. madagascariensis	2	12	5	0.803 ± 0.09	5	1.833	0.19	0.383	0.014	0.08
H. madagascariensis	3	7	5	0.857 ± 0.137	8	2.571	−1.44*	−1.017	0.21	0.22
H. madagascariensis	4	15	9	0.886 ± 0.069	16	3.229	−1.45*	−2.483*	0.002	0.01
	All	**61**	**25**	**0.904 ± 0.028**	**41**	**9.153**	**0.145**	**−2.929**	**0.024**	**0.038**
H. alboguttatus	1	31	14	0.927 ± 0.023	21	5.257	0.43	−1.145	0.048	0.075
H. alboguttatus	2	58	19	0.778 ± 0.054	26	2.682	−1.45*	−5.2*	0.02	0.058
H. alboguttatus	3	31	18	0.964 ± 0.02	23	3.087	−1.07	−12.0*	0.01	0.01
	All	**120**	**51**	**0.935 ± 0.017**	**60**	**6.879**	**−1.2**	**−24.87**	**0.016***	**0.018**
H. boettgeri	**All**	**9**	**6**	**0.889 ± 0.091**	**18**	**5.722**	**−0.66**	**0.295**	**0.256**	**0.162**
Rag-1 molecular diversity										
H. betsileo	1	13 (26)	8	0.78 ± 0.05	5	1.52	−0.34	−6.568*	0.006	0.09
H. betsileo	2	12 (24)	11	0.89 ± 0.04	8	1.68	−1.70*	−27.436*	0.014	0.1*
H. betsileo	3	11 (22)	6	0.73 ± (0.09)	3	1.09	0.12	−3.376*	0.023	0.156
H. betsileo	4	27 (54)	13	0.64 ± (0.07)	14	1.15	−1.92*	−25.632*	0.004	0.0532
H. betsileo	5	35 (70)	18	0.84 ± (0.04)	10	2.17	1.01	−15.178*	0.01	0.035
H. betsileo	6	60 (30)	8	0.3 ± (0.08)	8	0.36	−2.05	−21.244	0.004	0.32
	All	**153 (306)**	**50**	**0.762 (0.025)**	**34**	**1.604**	**−1.91***	**−27.665***	**0.003**	**0.03**
H. madagascariensis	1	27 (54)	7	0.532 ± 0.074	13	1.438	0.2	0.06	0.282*	0.125
H. madagascariensis	2	4 (8)	4	0.821 ± 0.101	3	1.107	0.18	−2.54*	0.043	0.271
H. madagascariensis	3	2 (4)	1	/	/	/	−0.04	−0.514	0.008	0.123
H. madagascariensis	4	10 (20)	4	0.647 ± 0.072	3	0.816	/	/	/	/
	All	**43 (86)**	**10**	**0.579 ± 0.053**	**16**	**1.22**	**−1.76***	**−3.227***	**0.001**	**0.05**
H. alboguttatus	1	11 (22)	3	0.329 ± 0.121	2	0.485	−0.156	−0.116	0.192	0.233
H. alboguttatus	2	38 (76)	2	0.366 ± 0.052	1	0.366	1.00	1.5	0.004	0.206
H. alboguttatus	3	20 (40)	3	0.512 ± 0.045	2	0.542	0.245	0.397	0.023	0.195
	All	**78 (156)**	**5**	**0.417 ± 0.039**	**4**	**0.47**	**−0.63**	**−1.339**	**0.005**	**0.155**
H. boettgeri	All	11 (22)	2	0.091 ± 0.081	1	0.091	−1.16	−0.956	0.0	0.677

* $P < 0.05$.

dance with the number of major mtDNA clades in the phylogenetic analyses. Moving from $K = 3$ to $K = 6$, different levels of SAMOVA closely reflect the geographic associations of haplotypes at different phylogenetic clade levels. With $K = 2$ the deep partition between the most southern clade HB6 and the clades HB4 and HB5 is recovered.

In the Barrier analyses (for graphical representation see Fig. S3 in Supplementary material) we took into account the first five barriers of relevant genetic discontinuity (i) of the *cob* dataset only, (ii) of the *Rag-1* dataset only, and (iii) of the combined *cob* and *Rag-1* dataset of which the first three are represented in Fig. 1C as α, β, γ. The first barrier of the combined dataset (α in Fig. 1) separates the northern HB1 from the central highland HB5. In the *cob* analysis only this barrier is extended towards the east and separates additionally HB1 from HB2 around Andasibe and An'Ala. Furthermore this *cob* barrier also separates HB2 from the highland populations of HB5. The second barrier identified in the combined analysis (β in Fig. 1) is located between Ambohimahasoa and Fianarantsoa, thereby separating HB4 and HB6, and further eastwards separating Vohiparara (HB3) from Ranoroahina (HB6). Moreover, this barrier reflects the deep split in the phylogenetic tree between *H. betsileo* populations in Vohiparara and Ranomafana. In the *cob* dataset only, this barrier exceeds and separates the Ranomafana population additionally from populations belonging to the major mtDNA clades HB4 and HB6 from the central highlands. Thirdly, we recovered a barrier separating the most southern population located in the Andringitra massif from all other populations (γ in Fig. 1).

In summary, for *H. betsileo* one third of the genetic variation in the *cob* dataset could be explained by our *a priori* hypothesis of major river systems acting as potential barriers to gene flow. However, there was no support for IRS groupings in the SAMOVA and BARRIER analyses, which both revealed a clear altitudinal phylogeographic break.

Mantel tests on the mtDNA and nDNA datasets revealed significant values of isolation by distance for both genetic markers *cob* ($r = 0.427$; $P = 0.000$) and *Rag-1* ($r = 0.497$; $P = 0.000$). Mantel tests for each major *cob* lineage (see Supplementary material Table S10) revealed no signs of isolation by distance; all correlation coefficients were negative and below the significance threshold.

3.4. Demographics of H. betsileo populations

Considering *H. betsileo* as a single population in neutrality tests, values of Fu's F_s were highly negative and significant, whereas Tajima's *D* values were positive at a low and insignificant level (Table 1). Separate analyses for the six major mtDNA clades yielded negative F_s values in all but one of these (HB3), with statistical significance in three of them (HB2, HB4, HB5). In HB4 and HB5 significant negative *D* values were observed, indicating an excess of low frequency haplotypes, and hence, a significant deviation from neutrality.

In the full *Rag-1* dataset both D and F_s were significantly negative. All major mtDNA clades showed negative F_s values, almost all of them significant, except for HB6. Values of Tajima's *D* were positive but insignificant for HB3 and HB5.

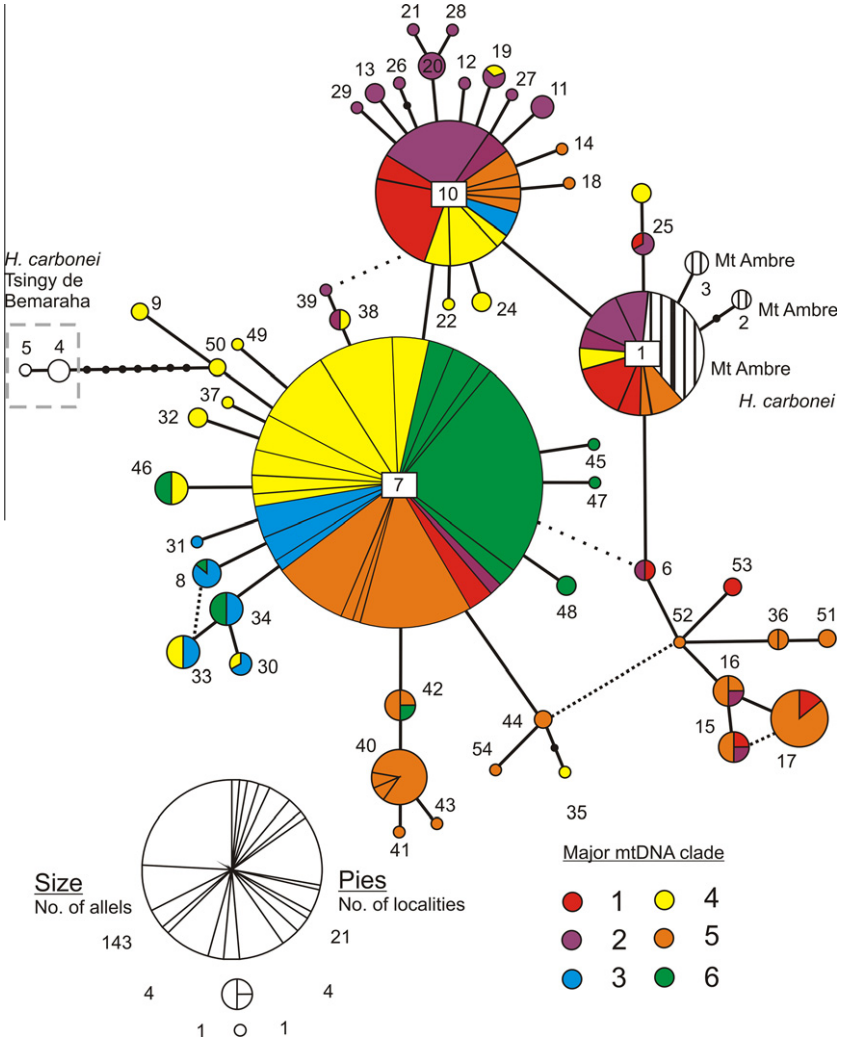

Fig. 2. Median-joining network of 50 *Rag-1* haplotypes in *Heterixalus betsileo* and four haplotypes in *H. carbonei*. The consensus network of all the shortest trees is shown. Black dots are unsampled or missing intermediates. Numbers denote haplotypes; colors denote the major mtDNA clades as identified by phylogenetic analyses; size is proportional to their frequencies. Dashed lines indicate alternative connections of equal statistical support. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

Table 3
Analysis of molecular variance (AMOVA) examining the partitioning of genetic variance for one mitochondrial (*cob*) and one nuclear (*Rag-1*) marker among and within populations of *Heterixalus betsileo*. The covariance components are indicated as either two asterisk meaning highly significant ($P < 0.01$), or one asterisk meaning significant ($P < 0.05$). *A priori* groupings were as follows: (A) three groups corresponding to the three identified Inter River systems (IRSs) I–II: (I) populations north of Mania River; (II) populations between Mania and Matsiatra River; and (III) populations south of Matsiatra River (B) three groups corresponding to groups of populations at different altitudes: (I) populations above 1000 m; (II) northern populations below 1000; (III) southern populations below 1000 m.

	(A) IRS	(B) Altitudinal
Grouping	IRS I–III	I–III
Cob		
Among groups	38.95**	49.06**
Among populations within groups	39.99**	33.94**
Within populations	21.07**	17.0**
Rag-1		
Among groups	11.96**	6.16**
Among populations within groups	15.25**	23.92**
Within populations	72.79**	69.92**

Non-significant raggedness indices ($r_{Harp} > 0.04$) in mismatch analyses supported population expansion in HB1, HB2, HB5, and HB6. For HB4, however, values of r_{Harp} were above the threshold of 0.04 but insignificant, in contrast with the highly significant results of the neutrality tests. Congruent with the neutrality tests, values r_{Harp} for HB3 were above 0.04 as well, but insignificant. Analyses of the *Rag-1* dataset containing all *H. betsileo* populations also supported a model of population expansion ($r_{Harp} = 0.03$, ns). As diagnosed by major mtDNA clades, only HB5 showed a considerable sign of expansion ($r_{Harp} = 0.035$, ns). In all other lineages the raggedness index was above the threshold of 0.04, although none were significant, which is in better agreement with a general trend of expansion than stable population sizes.

In summary, most of the major mtDNA clades of *H. betsileo* showed signs of population expansion in both studied markers, although not always at a significant level. Only in lineage HB3 did most values indicate a rather stable population.

3.5. Mitochondrial DNA phylogeny and sequence diversity in the H. madagascariensis complex

The *cob* dataset of the *H. madagascariensis* complex consisted of (i) 61 individuals corresponding to 25 haplotypes of *H. madagascariensis* ($h_d = 0.904$; 41 segregating sites; 29 parsimony informative sites), (ii) 120 specimens and 51 haplotypes of *H. alboguttatus*

Table 2
Analysis of molecular variance (AMOVA) examining the partitioning of genetic variation of one nuclear marker (*Rag-1*) within populations, among populations and among major mtDNA clades of each *Heterixalus* species separately (Halb = *H. alboguttatus*; Hbet = *H. betsileo*; Hboe = *H. boettgeri*; Hmad = *H. madagascariensis*) and of the *H. madagascariensis*-complex (*H. alboguttatus*, *boettgeri* and *madagascariensis* combined). The covariance components are indicated as either "**" highly significant ($P < 0.01$), "*" significant ($P < 0.05$).

	Halb	Hbet	Hboe	Hmad	Hmad-complex
Comparison between sampling localities					
Among populations	17.89**	27.54**	0	11.84*	21.93**
Within populations	82.11**	72.46**	100	88.16*	78.07**
Grouped in corresponding mtDNA clades					
Among major mtDNA clades	9.71**	13.44**	–	−2.83*	15.10**
Among populations within mtDNA clades	10.51**	15.78**	–	13.90*	8.73**
Within populations	79.75**	70.77**	–	88.93*	76.18**

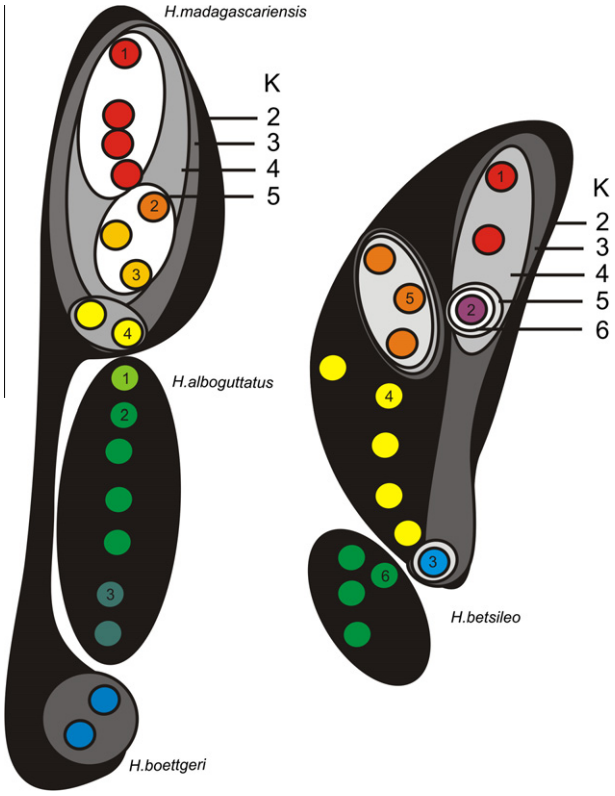

Fig. 3. Group structure as defined by a SAMOVA. On the left the results for the *Heterixalus madagascariensis* complex (*H. alboguttatus*, *H. boettgeri*, and *H. madagascariensis*); on the right those of *H. betsileo*. Ellipsoids identify population groups with pre-defined numbers of clusters (K). Circles indicate approximate geographical locations of populations; numbers and colors of circles denote the major mtDNA clades of each species. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

Table 4
SAMOVA-percentage of explained variation in species of *Heterixalus* corresponding to optimal grouping: *Heterixalus alboguttatus*, *H. boettgeri*, and *H. madagascariensis* (Habm), and *H. betsileo* (Hb) estimated among groups, among populations within groups, and within populations calculated for *cob*, where *K* is the number of groups.

	Habm ($K = 5$)	Hb ($K = 6$)
Among groups	97.03**	74.96**
Among populations within groups	1.49**	3.97**
Within populations	1.48**	21.07**

*Statistical significance $P < 0.05$.
** Statistical significance $P < 0.001$

($h_d = 0.935$; 60 segregating sites; 29 parsimony informative sites), and (iii) nine specimens and six haplotypes of *H. boettgeri* ($h_d = 0.889$; 18 segregating sites; six parsimony informative sites). Average nucleotide diversity was lower than in *H. betsileo*: *H. alboguttatus*: $\pi = 0.02$; $k = 6.88$; *H. boettgeri*: $\pi = 0.02$; $k = 5.72$; *H. madagascariensis*: $\pi = 0.03$; $k = 9.16$ (see Table 1).

The concatenated mtDNA dataset of the *H. madagascariensis* complex consisted of 2993 characters, of which 216 were parsimony-informative (for details see chapter extended results in Supplementary material). All phylogenetic analyses (MP, ML, BI) supported the same arrangement of main clades although not always with significant statistical support. All analyses placed *H. madagascariensis* with high support as the sister group of *H. alboguttatus* and *H. boettgeri* (Fig. 4A). Within this species four subclades were identified in congruence with the identified clusters in the network analyses (Fig. 4B). These haplotype clades showed clear geographic correlation following a north-to-south distribution. Clade HM1 represents the most northern sampling localities Sambava and Maroantsetra southwards to Ambodiriana (Fig. 4B). Haplotypes are differentiated by a maximum of five mutational steps, on average by two steps. HM2 was the sister group to HM1 and comprises haplotypes from Mahavelona and Tampolo, and exclusive haplotypes belonging to the island population of Nosy Boraha. HM3 from the central east coast was grouped with HM1 + HM2, although the relationships among these three clades were not strongly supported in the various analyses. A minimum of four *cob* substitutions separates this lineage from HM2, while it takes a minimum of 14 steps to connect HM3 with *H. madagascariensis* lineage HM4. Our analysis strongly groups HM1–HM3 to the exclusion of HM4. HM4 occurs in the most southern populations from Vatomandry and close to the Mangoro River. On average, values of genetic diversity were lower in northern populations than in HM4.

Heterixalus alboguttatus and *H. boettgeri* were sister groups. In the *H. boettgeri* network, haplotypes from St. Luce were the most divergent, separated by a minimum of nine mutational steps (Fig. 4C). In *H. alboguttatus*, three major mitochondrial clades were found (Fig. 4A). Based on the clustering in the network analyses (Fig. 4B) we summarized all haplotypes from the most northern sampling localities into group HA1, which in the phylogenetic analyses did not clearly resolve. HA2 included 19 *cob* haplotypes from an area roughly encompassing Mananjary in the north to Manombo north of the Mananara River. HA3 overlaps and occurs syntopically with HA2 in the localities of Manakara and Farafangana, whereas south of the Mananara river only HA3 is found. This lineage includes 18 different *cob* haplotypes. In general, genetic diversity indices of major *H. alboguttatus* haplotype clades were at comparable levels, HA1 showing a slightly higher nucleotide and sequence diversity (π; k), whereas haplotype diversity (h_d) was highest in HA3.

For a detailed overview on the genetic diversity of each population see Supplementary material Table S9.

3.6. Nuclear DNA sequence diversity in the madagascariensis complex

Overall variation in *Rag-1* sequences was very low in the *H. madagascariensis* complex. Only 10 different haplotypes out of

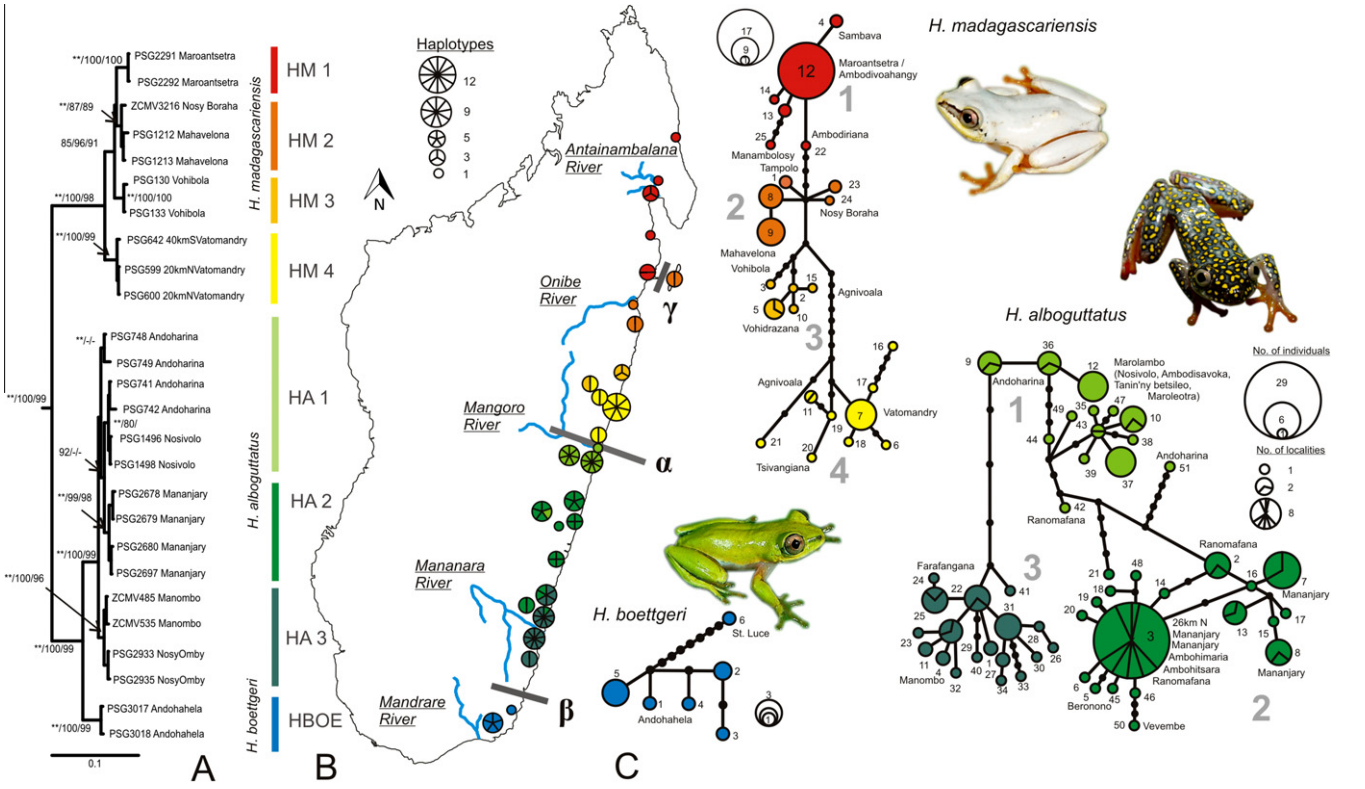

Fig. 4. Results of phylogenetic and phylogeographic analyses for the lowland species of the *Heterixalus madagascariensis* complex (*H. alboguttatus*, *H. boettgeri*, and *H. madagascariensis*). (A) Molecular phylogeny of major mtDNA clades within the different species obtained by Bayesian inference under the best partitioning strategy, based on a total of 2994 base pairs of seven mitochondrial gene fragments. Node values indicate Bayesian posterior probabilities (**>95; *>90%) followed by bootstrap values (>85%) of MP (second) and ML (third) analyses. The topology is equivalent in MP and ML analyses. Sequences of *H. betsileo* were used as an outgroup (not shown). (B) Geographical location of the sampled populations and their haplotype composition, and results of the barrier analyses. Populations are colored according to the frequency of the haplotype clades identified by phylogenetic analyses. The genetic barriers α, β, and γ are represented by grey lines; (C) Median-joining networks for *H. madagascariensis* (top), *H. alboguttatus* (right), and *H. boettgeri* (left) *cob* haplotypes. The consensus network of all the shortest trees is shown. Black dots are presumed unsampled or missing intermediates. Numbers denote haplotypes; colors denote the major mtDNA clades as identified by phylogenetic analyses; size is proportional to their frequencies. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

140 samples of *H. madagascariensis*, *H. alboguttatus*, and *H. boettgeri* from their entire ranges were identified (h_d = 0.477; 9 segregating sites; 5 parsimony informative sites; Table 1). Five *Rag-1* haplotypes (haplotypes no. 1, 5, 7, 9, 10) were exclusively found in populations of *H. madagascariensis* and two haplotypes (no 6 and 8) only in southern populations of *H. alboguttatus* (Fig. 5). Haplotypes 2 and 3 were most common and found in all three species and in almost all populations. Only subtle evidence for geographic structure was found, with a few exclusive haplotypes restricted to populations at the edge of the distribution area such as Sambava (no 1) and Nosy Boraha (no 10), but all of them are separated by just one or two substitutions from the more common haplotypes. Diversity indices (h_d, π; k) were overall highest in *H. madagascariensis* and without relevant differences among mtDNA lineages (Table 1). In *H. alboguttatus* values of diversity indices (h_d, π; k) were slightly higher in mtDNA lineage HA3 compared to HA1 and HA2. In *H. boettgeri* molecular diversity indices were lowest and no haplotype exclusive to this taxon was detected.

3.7. Genetic structure and differentiation in the H. madagascariensis complex

The variation in the *H. madagascariensis* complex *cob* dataset was almost entirely (96.5%) attributable to differences among species (i.e. the three deepest mitochondrial lineages within this species complex, Table 5). In contrast, most (74.0%) of the observed variation in *Rag-1* was associated with differences among individuals within populations. Population structure in *cob* also highly corresponded to riverine barriers in this species complex, explaining 78.6% and 73.2% of the variation in this gene in IRS1 (four rivers) and IRS2 (five rivers), respectively. *Rag-1* variation was

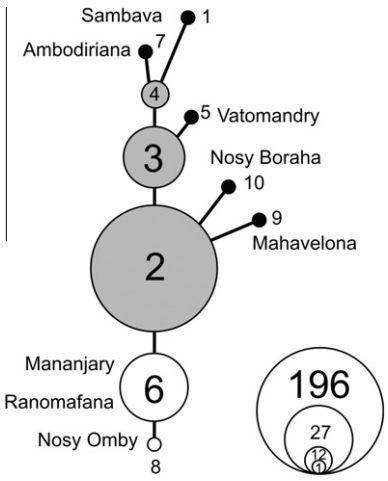

Fig. 5. Median-joining network of 10 *Rag-1* haplotypes of the *Heterixalus madagascariensis* complex (*H. alboguttatus*, *H. boettgeri*, and *H. madagascariensis*). The consensus network of all the shortest trees is shown. Numbers denote haplotypes; black haplotypes belong only to *H. madagascariensis*; white haplotypes were exclusively found in *H. alboguttatus*; grey haplotypes were shared by all three species; size is proportional to their frequencies.

explained by IRSs to a much lower extent (10.4% in IRS1 and 10.5% in IRS2). Major *cob* haplotypes (Table 2) determined 15.1% of the variation in *Rag-1*.

Moreover, in a separate analysis we compared the genetic variation between the *H. madagascariensis* and *H. alboguttatus* populations in close proximity to the Mangoro River (northern bank: Vatomandry and Tsivangiana vs. southern bank: Ambodisavoka, Andoharina, Bac Sahlehy, Nosivolo and Tanin' ny Betsileo). The differences in *cob* among populations across the river was very high (98.1%), suggesting that gene flow between *H. madagascariensis* and *H. alboguttatus* is almost absent across the Mangoro River.

The SAMOVA revealed that the *cob* dataset of the *H. madagascariensis* complex can be best partitioned into five groups, as a further increase of *K* did not produce additional informative clusters as suggested by a very shallow increase in F_{ct} followed by a plateau at five to ten groups. These five groups correspond to species boundaries plus the major mtDNA clades identified in *H. madagascariensis*. The highest geographic discontinuity separated *H. alboguttatus* and *H. madagascariensis* populations located north and south of the Mangoro River. For $K = 3$, *H. boettgeri* was separated from its closely related sister species *H. alboguttatus*. At $K = 4$ and $K = 5$, different levels of SAMOVA closely reflect the geographic associations of haplotypes at different phylogeographic populations in *H. madagascariensis*, separating HM4 from other *H. madagascariensis* populations (Fig. 3, Tables 3 and 4; Table 6).

The Barrier analyses (for graphical representation see Fig. S4 in Supplementary material) were conducted on (i) the *cob* dataset only, (ii) the *Rag-1* dataset only, and (iii) the combined *cob* and *Rag-1* datasets (Fig. 4B). The first barrier recovered by both datasets (α in Fig. 4B) separates *H. alboguttatus* and *H. madagascariensis* between the population of Vatomandry (*H. madagascariensis*) and Nosivolo (*H. alboguttatus*). Further barriers reflect differences between the two molecular markers: in *cob* a barrier strictly separates *H. madagascariensis* and *H. alboguttatus*; the combined analysis also groups the populations from Andoharina, Ambodisavoka and Bac Sahlehy with *H. madagascariensis*, most probably due to the very shallow differences and haplotype sharing in *Rag-1*. The second barrier was equally supported by both analyses and separates *H. boettgeri* populations from *H. alboguttatus* (β in Fig. 4B). The split between *H. madagascariensis* populations of mainland Madagascar and the island of Nosy Boraha was the third barrier supported by both datasets (γ in Fig. 4B). Additional barriers to gene flow were supported by only the *cob* data or the combined dataset (see Supplementary material Fig. S4).

In summary, our *a priori* AMOVA settings dividing the *H. madagascariensis* complex into groups delimited by major river systems explained a considerable proportion of the variation in the *cob* dataset (~75%). Moreover, there was strong support of these *a priori* groupings in the SAMOVA and BARRIER analyses, which both revealed differentiation of populations on opposite sides of the Mangoro River and its influence on the genetic structure of the lowland species. In *H. madagascariensis* the SAMOVA also suggested the Onibe River as a potentially important barrier to gene flow in the northern region. Due to little variation and considerable haplotype sharing between populations, analyses with *Rag-1* did not give consistent results.

Mantel tests were performed separately for the three species of the *H. madagascariensis* complex. For *H. alboguttatus* significant IBD values were detected in both datasets: *cob*, $r = 0.462$; $P < 0.001$; *Rag-1*, $r = 0.387$; $P < 0.001$. Geographic and genetic distances were also positively correlated within all of the major mtDNA lineages of this species, although only lineage HA2 attained statistical significance ($r = 0.325$; $P = 0.031$). In *H. madagascariensis* the positive correlation between *cob* variation and geographic distance was insignificant ($r = 0.19$; $P = 0.067$); no indication of IBD was found in the *Rag-1* dataset ($r = -0.25$; $P = 0.9$). IBD was not detected for any of the major *cob* lineages. Likewise, no significant IBD was found in *H. boettgeri* (*cob* and *Rag-1*).

3.8. Demography of the H. madagascariensis complex

Fu's F_s statistic was negative but insignificant ($P > 0.02$) for *H. madagascariensis* and *H. alboguttatus* in the *cob* dataset, while it was positive but insignificant for *H. boettgeri* (Table 1). Repeating the analyses for each major mtDNA lineage of *H. madagascariensis* and *H. alboguttatus* separately, HA1 and HA3, and HM1 and HM4 showed signs of population expansion evidenced by significant negative F_s values. This observation was in agreement with significantly negative values of Tajima's *D* in HA1, HM1 and HM4 (and a non-significant negative value in HA3).

In the *Rag-1* dataset Tajima's *D* and Fu's F_s were both significantly negative ($P < 0.05$) in *H. madagascariensis* and negative but insignificant in *H. alboguttatus* and *H. boettgeri* (Table 1). IBD was not detected in any of the major mtDNA clades with the exception of lineage HM2 for which a significant negative F_s value was observed.

Non-significant raggedness indices ($r_{Harp)} < 0.04$) for *cob* mismatch analyses in *H. madagascariensis* and *H. alboguttatus* were consistent with population expansion (Table 1). Of the major mtDNA clades within the species, only HA3 and HM4 showed some evidence for population expansion (Table 1). All other clades showed values above the threshold, although none of them at a significant level, suggesting stable populations. Likewise, raggedness indices exceeded the 0.04 threshold for *Rag-1* in all species, but were non-significant in all cases.

Altogether, populations of *H. alboguttatus* showed the most distinct signs of population expansion in the *cob* dataset, especially in the southernmost populations of the species. Evidence of expansion in *H. madagascariensis* was supported only for lineage HM3 by both neutrality tests and mismatch analyses. Results for *H. boettgeri* were inconclusive. Analyses of the nuclear dataset were largely uninformative.

Table 5
Analysis of molecular variance (AMOVA) examining the partitioning of genetic variation for one mitochondrial (*cob*) and one nuclear (*Rag-1*) marker among and within populations of different lowland *Heterixalus* species. The covariance components are indicated as either "**" highly significant ($P < 0.01$), "*" significant ($P < 0.05$). *A priori* groupings were as follows: (A) three groups corresponding to the three lowland species *H. alboguttatus*, *boettgeri*, and *madagascariensis*; (B) four groups corresponding to the Inter River Systems (IRS) I–IV: (I) populations north of the Onibe River (excluding the Nosy Boraha population); (II) populations between the Onibe and Mangoro Rivers; (III) populations between Mangoro and Mananara Rivers; (IV) populations between Mananara and Mandrare Rivers; (C) five groups corresponding to the Inter River Systems (IRS) I–V (I) populations north of the Onibe River (excluding the Nosy Boraha population); (II) populations between the Onibe and Mangoro Rivers; (III) populations between Mangoro and Mananjary Rivers; (IV) populations between Mananjary and Mananara Rivers; (V) populations between Mananara and Mandrare Rivers.

	(A) Species level	(B) IRS 1	(C) IRS 2
Grouping	(1) *H. madagascariensis* (2) *H. alboguttatus* (3) *H. boettgeri*	IRS I–IV	IRS I–V
Cob			
Among groups	96.51**	78.63**	73.24**
Among populations within groups	2.16**	19.56**	24.59**
Within populations	1.33**	1.81**	2.17**
Rag-1			
Among groups	11.03**	10.39**	10.48**
Among populations within groups	14.92**	14.05**	12.84**
Within populations	74.05**	75.56**	76.68**

Table 6
Results from SAMOVA analysis with different pre-defined numbers of hierarchical groups. Fixation indices and associated *P* values are reported. $^{*}P < 0.01$.

Species	*K*	F_{CT}	F_{SC}	F_{ST}
H. betsileo	2	0.455*	0.689*	0.831*
	3	0.602*	0.53*	0.813*
	4	0.672*	0.437*	0.813*
	5	0.706*	0.287*	0.79*
	6	0.75*	0.158*	0.789*
	7	0.75*	0.119*	0.787*
H. albo/boe/mad	2	0.956*	0.714*	0.988*
	3	0.965*	0.622*	0.987*
	4	0.969*	0.546*	0.986*
	5	0.97*	0.5*	0.985*
	6	0.97*	0.488*	0.985*

4. Discussion

The four species of Malagasy reed frogs studied herein are characterized by remarkable genetic structure in mitochondrial DNA, approximately corresponding to category I of Avise (2000): deep mitochondrial gene trees with an allopatric distribution of major lineages, and with private haplotypes in populations. Only at a few localities did divergent *cob* haplotypes of different major mtDNA clades co-occur. Altogether, the data suggest that riverine barriers vary in how they affect the genetic structure of *Heterixalus* lineages along the east coast of Madagascar.

4.1. Some but not all rivers maintain genetic differentiation in the lowlands

Our initial hypothesis predicted that genetic variation within lowland species is strongly subdivided by rivers, while in the highland species less subdivision should occur and genetic structure should not be correlated with river systems.

The mtDNA genealogies of the lowland species were characterized by two types of discontinuities in the geographical distribution of genetic diversity: (a) deep phylogeographic breaks among units that currently are considered different species (*H. alboguttatus*, *H. boettgeri*, and *H. madagascariensis*), and (b) phylogeographic discontinuities at the intra-specific level along the east coast. Together, these suggest a complex evolutionary scenario. One main result, however, agrees completely with the prediction that the most important genetic discontinuity exists at the geographical position of the Mangoro River, the largest river on Madagascar's east coast. This river separates populations of *H. madagascariensis* and *H. alboguttatus*. These two species are distinguished by divergent mtDNA and color pattern, and our dense sampling close to the northern and southern river banks did not reveal any admixture or introgression among these lineages. At least in this case, the Mangoro River constitutes a clear barrier to dispersal and gene flow, agreeing with predictions (i) and (ii). Dispersal and colonization patterns of *H. madagascariensis* and *H. alboguttatus* with respect to the Mangoro River give a mixed signal. The phylogenetic tree of *H. madagascariensis* revealed a general trend of south to north structuring of major clades, suggesting that this species originated in the river basin and from there dispersed northwards, in agreement with a general trend of higher genetic diversity (h_d and π) in southern populations. On the contrary, *H. alboguttatus* shows a trend of dispersal from more southern refugia northwards towards the Mangoro River, with clades in general being more shallow than in *H. madagascariensis*. In the demographic analyses, populations of *H. alboguttatus* showed the strongest signs of population expansion. However, the intraspecific phylogeny of *H. alboguttatus* was not well-resolved, and therefore our hypotheses on the origin of the species and the geographical direction of population expansions are tentative.

In its most restrictive interpretation, the riverine-barrier hypothesis predicts that rivers are absolute barriers to migration and gene flow. Depending on the size of the river and the ability of taxa to cross water bodies, this may be a reasonable hypothesis (e.g. Díaz-Muñoz, 2011; Li et al., 2009; Jalil et al., 2008). However, most studies have shown that rivers are only relative barriers that reduce but do not eliminate gene flow and migration (e.g. Gascon et al., 2000; Pastorini et al., 2003; Knopp et al., 2011). If the Mangoro River had caused an initial vicariant event leading to allopatric speciation in lowland *Heterixalus* species, population expansion away from the river or stable populations are expected. In this respect, two alternative hypotheses can be proposed:

(i) If our phylogenetic inference of a basal position of southern populations within *H. alboguttatus* is correct, we then suggest that the two species have diverged in two discrete refugia – *H. madagascariensis* at the northern edge of the Mangoro River basin, and *H. alboguttatus* in south-eastern Madagascar. If the wet forest region has become fragmented during dry and cool geological periods, e.g. at glacial maxima (Ray and Adams, 2001), the forest refugia may have persisted along river basins, as predicted by the watershed hypothesis by Wilmé et al. (2006). Population expansion of *H. alboguttatus* northwards stopped at the Mangoro. In this scenario, the river maintained the discreteness of both lineages because the altitudinal range (<1000 m a.s.l.) of the lowland species did not allow large numbers of migrants to reach and cross the river at its headwaters where it is narrower. It remains unclarified, however, why *H. madagascariensis* diversified on the northern part of the Mangoro basin but did not cross the river with subsequent dispersion southwards while *H. alboguttatus* was still restricted to a more southern refugium.

(ii) The alternative hypothesis would assume that contrary to our phylogenetic tree, the most basal divergences within *H. alboguttatus* occurred close to the Mangoro River (as is the case in *H. madagascariensis*), with subsequent dispersal towards the southern part of its range. This hypothesis is testable by a more extensive sampling of mitochondrial genes, or full mtDNA genomes, in the phylogenetic analysis, and would then indeed suggest a primary divergence of these two species caused by the Mangoro River barrier.

Independent from the past influence of the Mangoro on the divergence of *H. alboguttatus* and *H. madagascariensis*, at present the river clearly acts as an efficient barrier impeding admixture of these two species. Even if at regular intervals individuals manage to cross the river and hybridize, their genes would quickly dilute in the gene pool of the dense populations of the closely related resident species. It would be highly informative to understand the fitness of putative hybrids and population densities along the river in order to assess whether tension zone dynamics reinforce the barrier function of the river. Tension zones result when interspecific crosses produce unfit hybrids and are expected to settle along environmental barriers associated with a reduced population density (Barton and Hewitt, 1985). Knopp et al. (2011) showed that Malagasy dung beetles (*Nanos binotatus*) inhabiting the Mangoro drainage are only slightly differentiated at upstream localities on opposite sides of the river, but they do not share haplotypes on either riverbank close to the estuary, a pattern congruent with our results.

The distribution of the northern species *H. madagascariensis* is divided by two other larger river systems, the Onibe and the Antainambalana. The distribution of the HM1 lineage across the Antai-

nambalana River did not show any signs of restricted gene flow. On the other hand, SAMOVA indicated a genetic discontinuity between HM1 and HM2 principally coinciding with the Onibe River. On the southern banks of the river, we found the northern most distribution of HM2 haplotypes, but unfortunately no samples are available from its northern banks, with a sampling gap of roughly 80 km to the next sites (where HM1 was present).

The Mananara River on the southeast coast of Madagascar bisects the distribution of *H. alboguttatus* and is another potential barrier to dispersal and gene flow (Vences et al., 2009), as has been shown for several lemur species (Goodman and Ganzhorn, 2004) and also for several reptiles (Boumans et al., 2007). We found haplotypes of the HA3 clade both south and north of the Mananara River, without any sign of restricted gene flow. Nevertheless, the geographic location of the Mananara coincides with the southern distribution limit of HA2, whereas HA3 occurs both north and south of this river.

We found no indication that the divergence between *H. alboguttatus* and *H. boettgeri* coincides with any river system in southern Madagascar. Unfortunately, we could not identify the exact contact zone among these two species, which are separated by a sampling gap of roughly 100 km.

4.2. Genetic structure in highland frogs is not influenced by rivers

Six distinct mitochondrial clades were identified in the highland species *Heterixalus betsileo*. Clades HB1, HB2 and HB3 formed a monophyletic group mostly from mid-elevation localities below 1000 m a.s.l., ranging from the northernmost populations around Lac Alaotra to populations in the southern central east in the Ranomafana area. Clades HB4, HB5 and HB6 form a poorly supported monophyletic group from central and southern highland areas above 1000 m a.s.l. Haplotypes belonging to the six major lineages largely occur allopatrically but can co-occur in contact-zone populations such as Anosibe An'Ala, Tsinjoarivo, or Ambohimahasoa, providing some evidence for long-distance dispersal. This allopatric distribution of main lineages causes a positive isolation-by-distance (IBD) signal in the species, but no IBD was detected within the mtDNA-diagnosed lineages. The geographic distribution of the major mtDNA clades and the areas containing significant genetic discontinuities do not coincide with major river systems intersecting the range of *H. betsileo* (Fig. 1). For instance, haplotypes no. 2 and no. 16, and lineages HB2, HB4, and HB5, are found on both sides of the Mangoro River (Fig. 1).

On the contrary, the most important phylogeographic breaks (barriers α and β) were found between populations at different altitudes, such as HB1 and HB2 from mid-elevations vs. HB5 from the central highlands, and within HB3 between Ranomafana (mid-elevation) and Vohiparara and other highland localities (Fig. 1).

4.3. The lack of concordance between mitochondrial and nuclear markers

We detected discrepancies in the spatial patterns of genetic variation in *cob* vs. *Rag-1*. Geographically structured variation in *Rag-1* haplotypes was nearly absent in the *H. madagascariensis* complex, although according to current taxonomy this complex contains three distinct species, and in fact no mitochondrial-haplotype sharing was observed among these. More extensive geographical structure was found in the *Rag-1* data of the highland species, but also here the differentiation was low.

In continuously distributed species, phylogeographic discontinuities in nonrecombining units such as mtDNA may arise if individual dispersal distances and population sizes are low (Irwin, 2002; Kuo and Avise, 2005). Disagreement between the genealogies of unlinked loci may also be an indication that observed phylogeographic breaks are not caused by longstanding historical barriers (Kuo and Avise, 2005). Similarly, in lowland *Heterixalus* species, genetic discontinuities may represent recent expansion events rather than long term barriers to gene flow.

Malagasy reed frogs are locally abundant, and their wide geographic distributions and ability to live in anthropogenic habitats suggest at least moderate dispersal abilities, but unfortunately nothing is known about individual dispersal distances or sex-biased dispersal, which may also cause discrepancies between mtDNA and nuclear gene trees (Lampert et al., 2003; Canestrelli et al., 2007). However, sex-biased dispersal alone is unlikely to explain the observed geographic pattern in genetic variability because it implies geographical asymmetry in gene-flow over a vast area. Therefore, the low level of variation and weak population structure in the nuclear gene may be best explained by the slower substitution rate and/or retention of ancestral polymorphism in populations caused by a larger effective population size and twice the number of dispersing individuals in nuclear genes as compared to the matrilinear mitochondrial locus (Hare, 2001).

5. Conclusions

Generally, the structure of the gene networks and the deep divergences in the mtDNA gene trees of *Heterixalus* species suggest that a complex array of factors has led to the observed phylogeographic patterns. The riverine barrier effect is obviously not the only driver of population differentiation and diversification in these frogs. Nevertheless, the predicted stronger influence of rivers in the lowland species was confirmed by the observed mitochondrial subdivision within the *H. madagascariensis* complex. The Mangoro River has sustained the discreteness of *H. madagascariensis* and *H. alboguttatus* found on opposite riverbanks but probably did not cause the primary divergence between these two lineages. In the northern species *H. madagascariensis*, the Onibe River probably contributes to population differentiation by curbing gene flow between populations located north and south of the river. The origin of the two sister species *H. alboguttatus* and *H. boettgeri* may be explained by allopatric diversification within a northern and southern refuge, respectively, leading to the formation of the two discrete lineages.

The positions of rivers in central Madagascar and the phylogeographies of highland species were not coincident. The observation that *H. betsileo* populations are considerably structured in both molecular markers (to a lower degree in the nDNA) suggests that probably other geophysical features have influenced their histories since these reedfrogs are thought to be rather good dispersers and are not bound to primary rainforest habitats.

Nevertheless, one explanation for this pattern may be found in historical expansions and contractions of forest cover during past glaciation events causing the fragmentation of suitable habitat for these anurans. Since reed frogs largely avoid closed primary rainforest, an expansion of forest cover may have limited their distributions to several isolated refugia probably restricted to swamp and wetland areas. For example, the major mtDNA lineage HB3 from Ranomafana has persisted in this general area without any signs of recent or historical population expansion. Given its ecological setting, this population has reached the western border of the eastern rainforest belt without any chance for further expansion. The expansion of the rainforest after drier periods may have reduced the suitable habitats of these frogs, and therefore possible refugia were restricted to large swamp areas, such as in the region around Antananarivo and Fianarantsoa, where the highest haplotype diversity occurs. Nevertheless, we cannot rule out the possibility of frequent unintended anthropogenic translocation of reedfrogs (or their clutches) in these important trading centers as the origin for the observed genetic diversity.

Our phylogeographic analyses indicate a rather recent population expansion in most major mtDNA lineages of *H. alboguttatus*, *H. betsileo* and *H. madagascariensis*. One could argue that this pattern may be influenced by the transformation of primary habitats caused by human activities, especially through the establishment of extensive areas of cultivated ricefields. The available information suggests that most of the forests disappeared due to slash-and burn agriculture less than 150 years ago (Burney et al., 2003; Green and Sussman, 1990). This activity may have recently facilitated the population expansion of reed frogs, but the deep divergences observed in our phylogenetic analyses point to much older processes that probably reach back to the Plio-Pleistocene climatic vagaries. Our results suggest a rather complex and dynamic biogeographic history of reed frogs in which riverine barriers most probably only played a minor though not negligible role.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.05.018.

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