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Phylogeography of the arid-adapted Malagasy bullfrog, *Laliostoma labrosum*, influenced by past connectivity and habitat stability ☆

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ABSTRACT

The rainforest biome of eastern Madagascar is renowned for its extraordinary biodiversity and restricted distribution ranges of many species, whereas the arid western region of the island is relatively species poor. We provide insight into the biogeography of western Madagascar by analyzing a multilocus phylogeographic dataset assembled for an amphibian, the widespread Malagasy bullfrog, *Laliostoma labrosum*. We find no cryptic species in *L. labrosum* (maximum 1.1% pairwise genetic distance between individuals in the 16S rRNA gene) attributable to considerable gene flow at the regional level as shown by genetic admixture in both mtDNA and three nuclear loci, especially in central Madagascar. Low breeding site fidelity, viewed as an adaptation to the unreliability of standing pools of freshwater in dry and seasonal environments, and a ubiquitous distribution within its range may underlie overall low genetic differentiation. Moreover, reductions in population size associated with periods of high aridity in western Madagascar may have purged DNA variation in this species. The mtDNA gene tree revealed seven major phylogroups within this species, five of which show mostly non-overlapping distributions. The nested positions of the northern and central mtDNA phylogroups imply a southwestern origin for all extant mtDNA lineages in *L. labrosum*. The current phylogeography of this species and paleo-distributions of major mtDNA lineages suggest five potential refugia in northern, western and southwestern Madagascar, likely the result of Pleistocene range fragmentation during drier and cooler climates. Lineage sorting in mtDNA and nuclear loci highlighted a main phylogeographic break between populations north and south of the Sambirano region, suggesting a role of the coastal Sambirano rainforest as a barrier to gene flow. Paleo-species distribution models and dispersal networks suggest that the persistence of some refugial populations was mainly determined by high population connectivity through space and time.

1. Introduction

The persistence of habitats through time enables species to survive and also allows intraspecific lineages to persevere (Graham et al., 2006). For tropical species, there are several predicted consequences of past and present climate change. First, for many species, climatic fluctuations induced range shifts and demographic fluctuations. Second, areas of high historical habitat stability are the most species rich and also exhibit the highest levels of local endemism. At the intraspecific level, these areas typically retain ancestral lineages and may contain high levels of allelic diversity. On the other hand, areas of unstable habitat should contain fewer taxa and show shallow phylogeographic structure, except in areas where divergent lineages meet (Petit et al., 2003). Third, co-distributed organisms that share similar ecologies should

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also have experienced a similar history. These patterns have been found in the Australian wet tropics (Graham et al., 2006), Atlantic Forest of Brazil (Carnaval et al., 2009, 2014), Amazonian rainforest (Fouquet et al., 2012) and East Africa (Tolley et al., 2011).

Although many phylogeographic studies have augmented our knowledge of tropical biogeography and speciation, most such work has focused on the northern hemisphere (Beheregaray, 2008; Keppel et al., 2012), and within the tropics, the most prominent diversification models have been developed and tested based on taxa inhabiting rainforest or montane habitats (e.g., Fjeldså et al., 2012; Graham et al., 2006; Guarnizo and Cannatella, 2013; Moritz et al., 2000). Arid-adapted organisms can often be expected to show inverse patterns of distribution and diversification compared to those adapted to rainforest (e.g., Smith et al., 2012; Vargas-Ramírez et al., 2010) because suitable habitat and refugia for either of these groups constitute physiological dispersal barriers for the other.

One geographic area containing arid as well as humid biomes and particularly suited for comparatively testing hypotheses of diversification is Madagascar (Brown et al., 2014; Vences et al., 2009). The origins of the diverse and highly endemic biota of this island have puzzled biologists for centuries. It is now well established that most of Madagascar's endemic vertebrate clades arrived via overseas dispersal during the Cenozoic and subsequently diversified on the island (Samonds et al., 2013) where a central chain of mountains divides the rainforests of the east from the subhumid and arid biomes of the west (Fig. 1). Those clades that adapted to rainforest habitat are particularly species rich (Crottini et al., 2012a). The diversification of these humid-adapted clades was probably triggered by montane refugia (Raxworthy and Nussbaum, 1995), elevational heterogeneity (Wollenberg et al., 2008), ecogeographic barriers or ecotones (Yoder and Heckman, 2006), river catchment refugia (Wilmé et al., 2006), river barriers (Goodman and Ganzhorn, 2004), or a combination of these mechanisms (Brown et al., 2014). A striking characteristic of many animal and plant species of Madagascar is their restriction to very small distribution ranges which often appear to be smaller than 1000 km^2 and sometimes smaller than 300 km^2, a phenomenon named microendemism (Wilmé et al., 2006). Whether microendemic taxa are indeed more frequent in Madagascar than in other tropical areas, or have been more commonly noticed due to intensified research activities in this region is unresolved (Vences et al., 2009). Numerous molecular phylogenetic and phylogeographic studies have demonstrated that microendemism is a reality in Madagascar and extends to the intraspecific level. Many species are phylogeographically highly structured, with deep mitochondrial lineages restricted to small patches of habitat. This pattern has mainly been studied in rainforest species (e.g., Boumans et al., 2007; Kaffenberger et al., 2011; Miraldo et al., 2011; Wollenberg et al., 2008), and several case studies have also demonstrated substantial geographic structuring and small-scale endemism in the more arid regions of western Madagascar (e.g., Chan et al., 2012; Crottini et al., 2008; Olivieri et al., 2007; Olson et al., 2009; Raselimanana et al., 2009). Contrary to these findings, phylogeographic structure in amphibians inhabiting arid biomes is expected to be relatively low given that water bodies in such areas are typically unpredictable and temporary, and successful arid-adapted amphibians are hypothesized to be highly vagile (Chan and Zamudio, 2009; Rodríguez et al., 2015; Vences et al., 2002).

The Malagasy bullfrog, *Laliostoma labrosum*, is a monotypic species of the Mantellidae, a family endemic to Madagascar and the Comoro island of Mayotte. This stout, terrestrial species is morphologically adapted to a burrowing life in arid or seasonal environments (Vences and Glaw, 2003). *L. labrosum* has a generalized reproductive behavior that involves axillary amplexus and simultaneous breeding of many adult individuals in stagnant water bodies after heavy rains. Egg clutches are large (ca. 1500 eggs) and tadpoles are morphologically unspecialized (Schmidt et al., 2009) and thus probably omnivorous suspension feeders as most anuran larvae (Altig and McDiarmid, 1999). *L. labrosum* is distributed over most of western Madagascar and reaches the central highlands in the southern part of the island. This broad range traverses the main vegetation formations of this region, which include dry deciduous forest over much of the western and northern parts of the island and spiny bush in the south. It is also found in mesic forest and

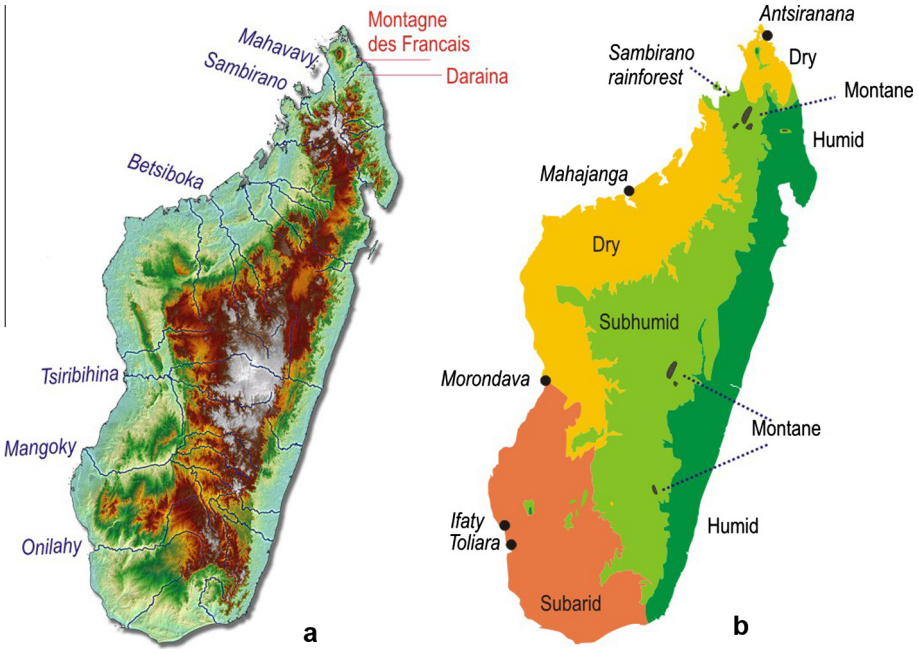

Fig. 1. The main rivers and topography of Madagascar (a), as well as the bioclimatic zones (b) and localities mentioned in the text. The bioclimatic zones are after Schatz (2000).

secondary vegetation in the Sambirano region in northwestern Madagascar and reaches Montagne des Français, Loky-Manambato and Daraina in the far north (Glaw and Vences, 2007; Rakotondravony, 2006; Vences and Glaw, 2000). Despite its large range, no intraspecific morphological or bioacoustic differentiation has been noted in *L. labrosum* (Vences and Glaw, 2003).

In this paper, we aim to expand the understanding of the biogeography of western Madagascar by examining the evolutionary history of this amphibian, a representative of a group known to be sensitive to climate change and, thus, appropriate for reconstructing past environmental changes (Carnaval and Bates, 2007). We take a multilocus approach to trace the history of *L. labrosum* and describe the current spatial pattern of genetic variation in this species. We use the ancestral distributions of mtDNA clades in *L. labrosum* as a surrogate for refugial areas and employ species distribution and paleoclimatological modeling to test if niche, climate, seasonal stability or dispersal networks can account for long-term persistence of populations. We interpret the pattern of genetic differentiation in this species in terms of diversification mechanisms proposed for Madagascar's biota (Vences et al., 2009).

2. Material and methods

2.1. Samples and molecular methods

Ethanol preserved tissues were collected from 124 *Laliostoma labrosum* individuals from 29 localities (Table 1) spanning the range of the species in Madagascar (see Table S1 for field numbers of samples). Two mtDNA fragments were PCR-amplified and sequenced for all individuals: a 550–555 bp fragment of the 16S rRNA gene used for barcoding in Malagasy amphibians (e.g. Vences et al., 2005) and a 625 bp fragment of cytochrome b (*cytb*). We amplified and sequenced two additional mtDNA genes for a subset of individuals representing major mtDNA clades as revealed by the concatenated 16S rRNA and *cytb* gene tree. These mtDNA genes included a 347 bp fragment of the 12S rRNA gene and a 662 bp section of the cytochrome oxidase subunit I gene (*cox1*), amplified and sequenced for 10 samples (Table S1). Three single-copy nuclear genes were PCR-amplified and sequenced for most individuals: a ~700 bp portion of the gene encoding brain-derived neurotrophic factor (*bdnf*), a ~400 bp fragment of the pro-opiomelanocortin gene (*pomc*) and a ~700 bp fragment of the recombination activating gene 1 (*rag1*). All nuclear genes were sequenced using both forward and reverse primers. Mitochondrial gene fragments were also sequenced for *Aglyptodactylus madagascariensis*, a representative of the sister group of *Laliostoma*, used as an outgroup. Oligonucleotides used for PCR amplification/sequencing and PCR conditions for each gene are given in Table S2. The PCR products were treated with ExoSAP and cycle-sequenced using dye-labeled terminators (Applied Biosystems) with the amplification primers, and analyzed on an ABI 3130 automated DNA sequencer (Applied Biosystems). Chromatographs were checked and sequences edited using CodonCode Aligner (v. 2.0.6, Codon Code Corporation). All sequences have been deposited in GenBank (KR337832–KR337871, KR337874–KR337877, KR337881, KR337884, KR337886–KR337888, KR337890–KR337978, KR348298–KR348365, KR479579–KR479864, KR809599–KR809707; details in Table S1).

Table 1
Sampling localities of *Laliostoma labrosum* ordered according to latitude. The number of frogs sampled from each locality (N), major mtDNA clade(s) as well as the number of unique haplotypes found in each locality (N_{haps}) are shown.

Site	Population	*N*	mtDNA clade	N_{haps}	Lat (*N*)	Long (*E*)
1	Antsiranana	3	N	3	−12.268	49.284
2	Ankarana	4	N	2	−12.934	49.127
3	Benavony	1	N	1	−13.700	48.483
4	Tsaratanana	1	W1	1	−14.538	48.749
5	Marosely	3	W1	3	−15.663	47.623
6	Ankarafantsika	5	W1	5	−16.667	46.950
7	Analamavo Forest	2	W1 (1), W2 (1)	2	−17.244	46.101
8	Ampasibe	2	W2 (1), W3 (1)	2	−18.532	44.593
9	Ampananira	1	W3	1	−18.773	44.370
10	Soatanana	1	W1	1	−18.778	44.470
11	Bemaraha	12	W1 (1), W2 (10), W3 (1)	12	−18.784	44.860
12	Ankavandra	6	W2	5	−18.772	45.292
13	Kirindy	17	W2 (1), W3 (16)	11	−20.067	44.657
14	Ankatrakatraka Forest	2	W3	2	−21.453	43.875
15	Beronto Forest	2	W3	1	−21.779	43.978
16	Antevankira Forest	3	W3	2	−21.946	44.046
17	Kapoky Forest	6	W3 (5), SC2 (1)	4	−22.044	45.125
18	Isalo	14	W3 (2), SC1 (12)	6	−22.421	45.274
19	Vohitsira	1	W3	1	−22.032	46.001
20	Ingaro	7	W3	5	−21.578	46.366
21	Ambalavao	3	W3	3	−21.830	46.930
22	Ihosy	2	SC1	1	−22.406	45.936
23	Ifaty	3	SC1 (1), SC2 (1), SW (1)	3	−23.150	43.617
24	Toliara	14	W3 (5), SC1 (5), SC2 (1), SW (3)	8	−23.350	43.667
25	Ejeda	5	SC1	3	−24.542	44.647
26	Tranomaro	2	SC1	2	−24.518	46.599
27	Andohahela	1	SC1	1	−24.817	46.610
28	Berenty	1	SC1	1	−24.983	46.283

2.2. Phasing and basic summary statistics

We aligned homologous nucleotide sequences using ClustalW implemented in MEGA 5.2 (Tamura et al., 2011). Polymorphic positions in the nuclear loci were carefully inspected to ensure correct and consistent identification of double peaks in heterozygotes. We used Phase version 2.1 (Stephens et al., 2001) to infer haplotypes at each nuclear locus for use in downstream haplotype-based analyses. At least four runs were performed for each locus with 1000 burn-in steps and 1000 iterations, allowing for intragenic recombination. We used a 0.6 output probability threshold for genotypes and haplotypes as this was shown to reduce the number of unresolved genotypes while only marginally increasing the number of false positives (Garrick et al., 2010). For *rag1*, we used a combination of experimental and computational methods to resolve the phase of segregating sites. After a preliminary run in Phase v2.1, we selected two individuals with unresolved haplotypes and PCR amplified them using high fidelity *Pfu* DNA polymerase (Promega). The amplification products were then cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen). At least 8 clones per amplification product were used for haplotype determination. Finally, we repeated the PHASE analyses using known haplotypes after preparing input files in SeqPhase (Flot, 2010). For the highly variable *pomc* gene fragment, preliminary Phase runs revealed that a substantial number (34 of 109) of haplotype reconstructions were uncertain ($P < 0.80$). We therefore discarded the first 125 bp of the alignment (which encompassed a highly variable fragment of the gene and a tri-allelic site) and repeated the Phase runs using the remaining 160 bp fragment for downstream haplotype-based analyses. Basic summary statistics including the numbers of segregating sites, haplotype diversity, nucleotide diversity, and mean number of nucleotide differences among haplotypes were calculated in DnaSP v5 (Librado and Rozas, 2009).

2.3. Phylogenetic methods

Mitochondrial gene fragments were concatenated providing a total of 1154 bp of mtDNA (16S rRNA and *cytb*) per individual for

all 124 *Laliostoma* samples and 2163 bp of mtDNA (12S and 16S rRNA, *cytb*, *cox1*) for 10 individuals. The two mtDNA datasets were analyzed separately under mixed-model Bayesian inference and discrete character evolution using maximum parsimony.

For the model-based analyses, we specified separate character sets for 12S rRNA, 16S rRNA and each codon position (cp) of the *cytb* and *cox1* gene fragments. PartitionFinder (Lanfear et al., 2012) was used to select the most appropriate partitioning scheme and nucleotide substitution models. We used the highest ranked scheme under the Bayesian information criterion. For the two-gene mtDNA dataset, a single partition that combined the 16S rRNA and the *cytb* gene fragments under a HKY + G nucleotide substitution model was selected. For the four-gene mtDNA dataset, the highest ranked scheme involved two partitions. The first partition combined 6 character sets (12S rRNA, 16S rRNA, 1st cp of *cox1*, 1st cp of *cytb*, 2nd cp of *cox1* and 2nd cp of *cytb*) under a K80 + I model of nucleotide evolution. The second partition included 2 character sets (3rd cp of *cox1* and 3rd cp of *cytb*) under a GTR + I model of nucleotide evolution. These partition schemes and models of nucleotide evolution were specified in MrBayes v. 3.2 (Ronquist et al., 2012) using a random starting tree, one cold and three heated chains (temperature at default = 0.2) for 50 million generations sampled every 1000 generations. Convergence of runs was checked in Tracer v1.5 (Rambaut and Drummond, 2007). We conservatively discarded half of the sampled generations as burn-in, summarizing 25,000 trees from the posterior distribution. The four-gene mtDNA dataset was also subjected to maximum parsimony analysis using the heuristic search option (100 random addition sequence replicates) and tree bisection reconnection branch-swapping in PAUP 4.0b10 (Swofford, 2003). Support for the resulting topology was assessed by examining a 50% majority-rule consensus tree after 1000 bootstrap pseudoreplicates.

2.4. Demographic analyses

Historical demography was assessed for major mtDNA clades W1, W2, W3 (western clades 1–3, see Section 3.2 for a definition of the mtDNA clades), combined W1 + W2 + W3 (termed W) and SC1 (southcentral 1). Each of these clades encompassed more than five sampled localities. For demographic analyses, we treated the localities as discrete demes in a metapopulation. Under a metapopulation model, if one gene copy is sampled per deme, then the ancestral process producing the sample is identical to the unstructured coalescent (Wakeley, 2004) and thus appropriate for demographic analyses. We constructed alignments for demographic analyses by randomly selecting a single mtDNA haplotype from each locality in which a given clade was present. Three summary statistics were used to detect departures from neutrality within the clades: Tajima's D, Fu's F_S and Ramos-Onsins & Rozas R_2. These statistics use different properties of the sequence data, i.e. the frequency spectrum of mutations (Tajima's D and R_2) or distribution of haplotypes in a sample (Fu's F), to draw conclusions on the processes that may have produced the pattern of variation seen in a sample. We used DnaSP for calculating these statistics and obtained confidence intervals by coalescent simulations conditioned on the number of segregating sites (1000 replicates). Mismatch distributions for each of the clades were computed in the R package pegas 0.5–1 (Paradis, 2010).

2.5. Population structure

For the mtDNA dataset, we conducted a spatial analysis of molecular variance in SAMOVA v1.0 using a matrix of pairwise differences between DNA haplotypes among sampling sites. This method uses a simulated annealing procedure that maximizes the proportion of total genetic variance due to differences between groups of populations (Dupanloup et al., 2002). We ran this program five times with the number of groups (K) set to 2–6; each analysis was subjected to 1000 iterations from 100 random initial conditions.

In a second step, we allocated the sampling sites into the SAMOVA-defined groups and assessed the extent to which they explain variation in the nuclear genes by conducting hierarchical analyses of molecular variance (AMOVAs) in Arlequin 3.5 (Excoffier and Lischer, 2010).

Overall differentiation among sampling sites was determined by calculating F_{ST} values in Arlequin. We next asked whether the major mtDNA clades determine genetic structure in the three nuclear markers by evaluating the proportion of autosomal variation among and within mtDNA clades in the AMOVA framework. We also calculated pairwise F_{ST} values for the nuclear markers among *L. labrosum* individuals grouped according to major mtDNA clades.

Population subdivision can also be identified by using allelic information from all loci simultaneously in the clustering algorithm implemented in Structure (Pritchard et al., 2000). We constructed an input matrix based on allelic states for three nuclear markers and mtDNA for each individual. For haploid mtDNA, haplotypes within major clades were collapsed and coded as the same allele. The default setting on the program expects two alleles per individual per locus; we coded the "second" mtDNA allele as missing data. Preliminary analyses showed that runs for $K > 10$ had low likelihood values, therefore in the final analysis we evaluated simulations for K values from 1 to 10, each simulation was repeated 20 times. We implemented the admixture model and independent allele frequencies among populations and used sampling locations as prior information for clustering. The MCMC consisted of 10^4 burn-in steps followed by 10^5 postburn-in iterations in each simulation. We inferred the most likely number of clusters in the data by assessing the likelihoods of the runs $L(K)$ and by examining ΔK values in Structure Harvester (Earl and vonHoldt, 2012) using the method of Evanno et al. (2005). CLUMPP v1.1.2 (Jakobsson and Rosenberg, 2007) was used to obtain mean estimates of cluster membership coefficients for each population. To visualize the results, we plotted the estimates of cluster membership coefficients for each population for the optimal K value in ArcGIS 10.2 (ESRI, 2014).

We checked for an association between genetic and geographic distances by conducting Mantel tests (1000 permutations) using pairwise F_{ST} distances (for mtDNA, *bdnf*, *pomc* and *rag1*) between populations and geographical distance matrices in Arlequin. We tested for an isolation by distance (IBD) effect at three spatial scales: using all samples, excluding sampling sites with mtDNA clade N (sites 1–3), and within the largest SAMOVA-defined cluster (sampling sites 4–17, 19–21). The latter two designs were used to test the effect of phylogeographic breaks on the IBD signal.

2.6. Ancestral distribution of mtDNA clades

The geographic locations of ancestors of the major mtDNA clades were estimated in Phylomapper v.1b1 (Lemmon and Lemmon, 2008). The input mtDNA genealogy consisted of BI of all non-redundant haplotypes in our two-gene mtDNA dataset (parameter settings were consistent with BI described above). Phylomapper requires that geographic coordinates are assigned to each haplotype. If several localities shared a haplotype, then we randomly selected one of these localities for analysis. The geographic coordinates of spatially coincident haplotypes were slightly shifted to avoid redundancy. Separate analyses were conducted for clades N, W1, W2, W3 and SC1 + SC2 (these clades were combined to increase the number of unique haplotypes in the analysis, see expanded tree in Fig. S1). Clade SW was omitted from the

analysis due to low sample size. Non-parametric rate-smoothing was applied to obtain an ultrametric genealogy used for optimizing the dispersal parameters and locations of ancestral nodes (1000 replicates). The ancestral locations and estimated point ages were imported into ArcMap 10.2.1 and interpolated into a continuous map according to Chan et al. (2011).

2.7. Modeling habitat stability and population connectivity

The current and past climate data (6 kya, 21 kya and 120 kya) comprised the original 19 standard Bioclim variables (30-arc second resolution, worldclim.org and Braconnot et al., 2007). These particular paleoclimates were provided because they represent the climatic extremes during the last 120 kya. We collected 61 unique occurrence records from literature and our own field work (Brown et al., 2014; Glaw and Vences, 2007); all records were georeferenced either based on GPS readings (own data and recent literature) or locality gazetteers (a small number of historical records mainly from Blommers-Schlösser and Blanc, 1991). Spatial data were rarefied at 20 km^2 to eliminate spatial clusters of localities (SDMtoolbox v1; Brown, 2014), which yielded 47 spatially independent occurrence localities used for modeling. Species distribution models (SDMs) were generated in MaxEnt v3.3.3k (Phillips et al., 2006). To optimize model performance, we tested different combinations of the five model feature class types and a range of regularization parameters (0.5–6; in 0.5 increments; Brown, 2014). Species distribution models were calibrated using spatial jackknifing ($k = 5$) and were evaluated based on omission rates (low being better) and AUC (high being better) of independent test data in SDMtoolbox v1. Spatial jackknifing (or geographically structured k-fold cross-validation) tests evaluation performance of spatially segregated, and independent, localities. Models were calibrated with all permutations of the five spatial groups using occurrence points and background data from four of the spatial groups and then evaluated with the withheld group.

To depict the spatial connectivity of phylogroups, we created dispersal networks among the major phylogenetic clades (Fig. 5A). We calculated least-cost corridors (LCC) among each locality in each shared clade and weighted them according to the method of Chan et al. (2011). Each individual weighted LCC was then summed to create the dispersal network of *L. labrosum*. A niche stability map was created by summing the current and three paleoSDMs. This map was inverted and used as the friction layer for each LCC calculation. This process was done in ArcGIS (ESRI, 2014) and using SDMtoolbox v1.

To assess the spatial correlations between (i) the dispersal network and the ancestral distributions, and (ii) the stability layers (climate seasonality and climate stability) and (iii) the niche stability layers, we measured Pearson product-moment correlation coefficients. Climate seasonality was calculated by standardizing the current Worldclim (Hijmans et al., 2005) variables BIO4 and BIO15 from 0 to 1 and summing both. Climate stability was obtained from Brown et al. (2014). To assess significance, the aforementioned raster layers were sampled at 10 km^2 and we used an unbiased correlation following the method of Dutilleul et al. (1993) in Spatial Analysis in Macroecology (Rangel et al., 2010).

3. Results

3.1. Nucleotide variation and haplotype determination

We obtained 16S rRNA and *cytb* gene fragments (1154 bp) from 124 frogs. 12S rRNA and *cox1* gene fragments were sequenced for a subset of 10 individuals representing major mtDNA clades in *Laliostoma*. Sequences of *bdnf* and *pomc* were obtained from 109 frogs, and 119 individuals were successfully sequenced for *rag1*. Translation of the mtDNA (203 and 220 amino acids for *cytb* and *cox1*, respectively) and nuclear protein-coding genes (213, 52 and 206 amino acids for *bdnf*, *pomc* and *rag1*, respectively) yielded peptide sequences without stop codons or frame shifts. Basic summaries of the nucleotide variation in each of the 4 mitochondrial and 3 nuclear gene fragments are given in Table 2. Out of the four mtDNA fragments, the highest number of segregating sites was found in *cytb* (93). Nucleotide variation in the nuclear genes was similar (12–19 segregating sites per gene).

Phase results were consistent across runs for all loci. In *bdnf*, only one nucleotide position in one genotype had a phase call of low probability (0.57), all other phase calls and haplotype reconstructions were well-resolved ($P \geqslant 0.95$). After cloning, phase calls for heterozygous nucleotide positions in *rag1* were resolved at $P > 0.8$ in all but six individuals (5% of the dataset). Phase calls for heterozygous nucleotide positions in the short *pomc* fragment were resolved at $P > 0.8$ in all except 9 individuals (8% of the dataset). We considered this an acceptable level of error and retained the phase-uncertain individuals because omitting them could lead to a bias in estimates of population genetic parameters (Garrick et al., 2010).

3.2. Gene trees and haplotype networks

The 2-gene and 4-gene mtDNA datasets contained 232 and 386 variable nucleotide positions, with 101 and 74 parsimony informative sites, respectively. The relationships among the major clades inferred from both datasets were the same (Figs. 2 and S1), with similar and generally high posterior probabilities at major nodes. Three main clades are apparent: a north (N)/west clade (W), a southcentral clade (SC) and a southwestern clade (SW). Mean uncorrected pairwise distances (*p*-distances, Table S3) among these clades were 0.2–0.9% (16S) and 4.5–6.5% (cytb). The western and southcentral clades can be further differentiated into 3 (W1-3) and 2 (SC1-2) subclades, respectively. All of these phylogroups roughly correspond to geographic positions along the north–south axis of Madagascar (Fig. 3). The SW phylogroup forms the sister clade to all other mtDNA lineages but is geographically restricted to the vicinities of Toliara and Ifaty. The next split in the genealogy

Table 2
Summary statistics calculated for 4 mitochondrial gene fragments and 3 nuclear loci in *Laliostoma labrosum* and a comparison of uncorrected *p*-distances to the outgroup, *Aglyptodactylus madagascariensis*. Values for *n*, *S*, π, and *h* were calculated without sequences from the outgroup.

Gene	n_{ind}	*n*	*S*	π	*h*	*p*-$dist_{max}$	*p*-$dist_{Am}$
mtDNA:							
12S rRNA	10	347	2	0.0029	3	0.006	0.065
16S rRNA	124	545	23	0.0037	24	0.011	0.110
cytb	124	609	93	0.0270	67	0.069	0.209
cox1	10	662	41	0.0215	9	0.034	0.212
nuclear DNA:							
bdnf	109	640	12	0.0014	15	0.008	0.018
pomc[a]	109	160	14	0.0038	19	0.025	na
rag1	119	618	19	0.0019	20	0.010	0.062[b]

n_{ind} – Number of individuals sequenced.
n – Number of nucleotides (after exclusion of indels in 12S and 16S rRNA).
S – Number of segregating nucleotide sites.
π – Nucleotide diversity.
h – Number of haplotypes.
p-$dist_{max}$ – The largest p-distance found within *L. labrosum*.
p-$dist_{Am}$ – Uncorrected mean p-distance between *A. madagascariensis* and *L. labrosum*.
[a] The original *pomc* alignment contained 285 nucleotide positions with 36 segregating sites.
[b] A total of 484 bp was used for the comparison between *Laliostoma* and *Aglyptodactylus*.

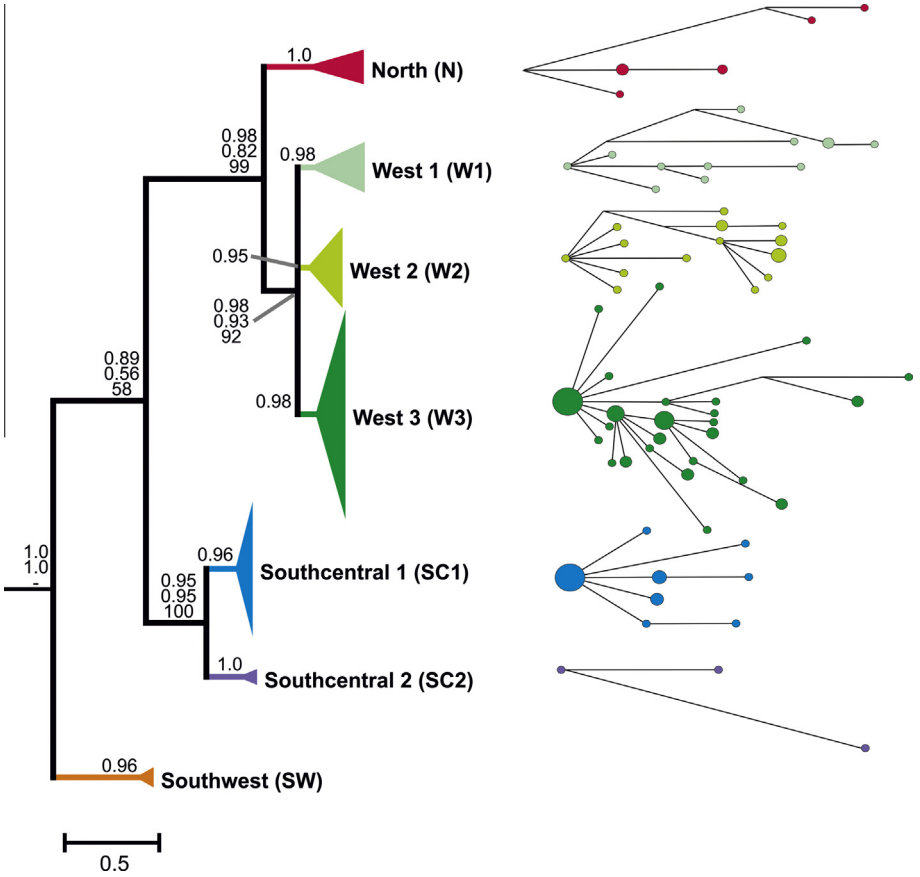

Fig. 2. Majority-rule consensus tree based on a posterior of 25,000 trees from a mixed-model Bayesian analysis of concatenated mitochondrial sequences (16S rRNA and *cytb* genes: 2-gene dataset) from all *Laliostoma labrosum* samples. Similar haplotypes belonging to each clade are collapsed; see Fig. S1 for a fully expanded tree. A larger mtDNA fragment (12S and 16S rRNA, *cytb* and *cox1* genes) was used to confirm relationships among major mtDNA clades using a mixed-model Bayesian approach and a maximum parsimony analysis (see text for details). Posterior probabilities for nodes based on the two-gene dataset are given above branches. If three numbers are present, then the top two numbers refer to posterior probabilities from the Bayesian analyses of the two-gene and four-gene datasets, and the third refers to bootstrap support in the maximum parsimony analysis using the four-gene dataset. Outgroup (*Aglyptodactylus madagascariensis*) is not shown. Median-joining networks including all haplotypes in each major clade are shown to the right of the tree.

is moderately supported only in the 2-gene Bayesian analysis, therefore the sister group relationship between SC and N/W is uncertain. MtDNA variation within both of the SC phylogroups is low (Figs. 2 and S1). In contrast, the N and W clades contain considerable mtDNA variation and are geographically structured.

Haplotype networks for the nuclear genes revealed one to three high-frequency haplotypes and between 13 and 19 low-frequency variants that differ by one or two substitutions from the former (Fig. 4). Subtle geographical lineage sorting was apparent in *bdnf* and *rag1*: the northernmost sampling sites (numbers 1–3) contained unique alleles in *bdnf* and a subset of the *rag1* alleles present in western and southern Madagascar.

3.3. Historical demography and distribution of ancestral mtDNA haplotypes

Mismatch distributions for major mtDNA clades in *L. labrosum* are shown in Fig. 3; we rejected demographic stability in favor of demographic expansion in all three neutrality tests for clade W (encompassing subclades W1, W2 and W3, Table 3), but not for clade SC1. Likewise, we inferred expansion in subclade W3 in two of three tests. Ancestral geographical distributions for major mtDNA clades as inferred by Phylomapper (Fig. 5B) are located in extreme northwestern (clade N), northcentral (W1), central (W2, W3) and southwestern (SC1 + SC2) Madagascar. Notably, none of the ancestral distributions overlapped.

3.4. Population structure

The SAMOVA analysis suggested three distinct genetic groups in the mtDNA (Fig. 3) because the percentage (~62–64%) of genetic variation explained by among-group variation reached a plateau at $K = 3$ (Table S4). This assemblage consisted of a northern group (sampling sites 1–3), a widespread central group (sampling sites 4–17 and 19–21) and a southern group (sampling sites 18, 22–28). The spatial pattern in mtDNA differentiation explained only 10% of the variation in *rag1* and null for *pomc*, but contributed substantially (45%) to variation in *bdnf* (Table S5).

Overall F_{ST} across populations (Table S6) was high in mtDNA and *bdnf* but relatively low in *pomc* and *rag1*. A similar pattern was observed for differentiation in nuclear markers and mtDNA if individuals were grouped according to major mtDNA clade, with the exception of a nonsignificant value for *pomc* (Table S6). Closer inspection of pairwise F_{ST} values for nuclear genes showed that differentiation was driven mostly by the N and SC1 phylogroups (Table S7).

Mean $L(K)$ and ΔK in the Structure analyses peaked at $K = 3$ (Fig. S2). There was a clear geographical pattern in the distribution of the clusters (Fig. 6), with a northern (red[1] in Fig. 6), western (green) and southeastern cluster (blue). However, substantial

[1] For interpretation of color in Fig. 6, the reader is referred to the web version of this article.

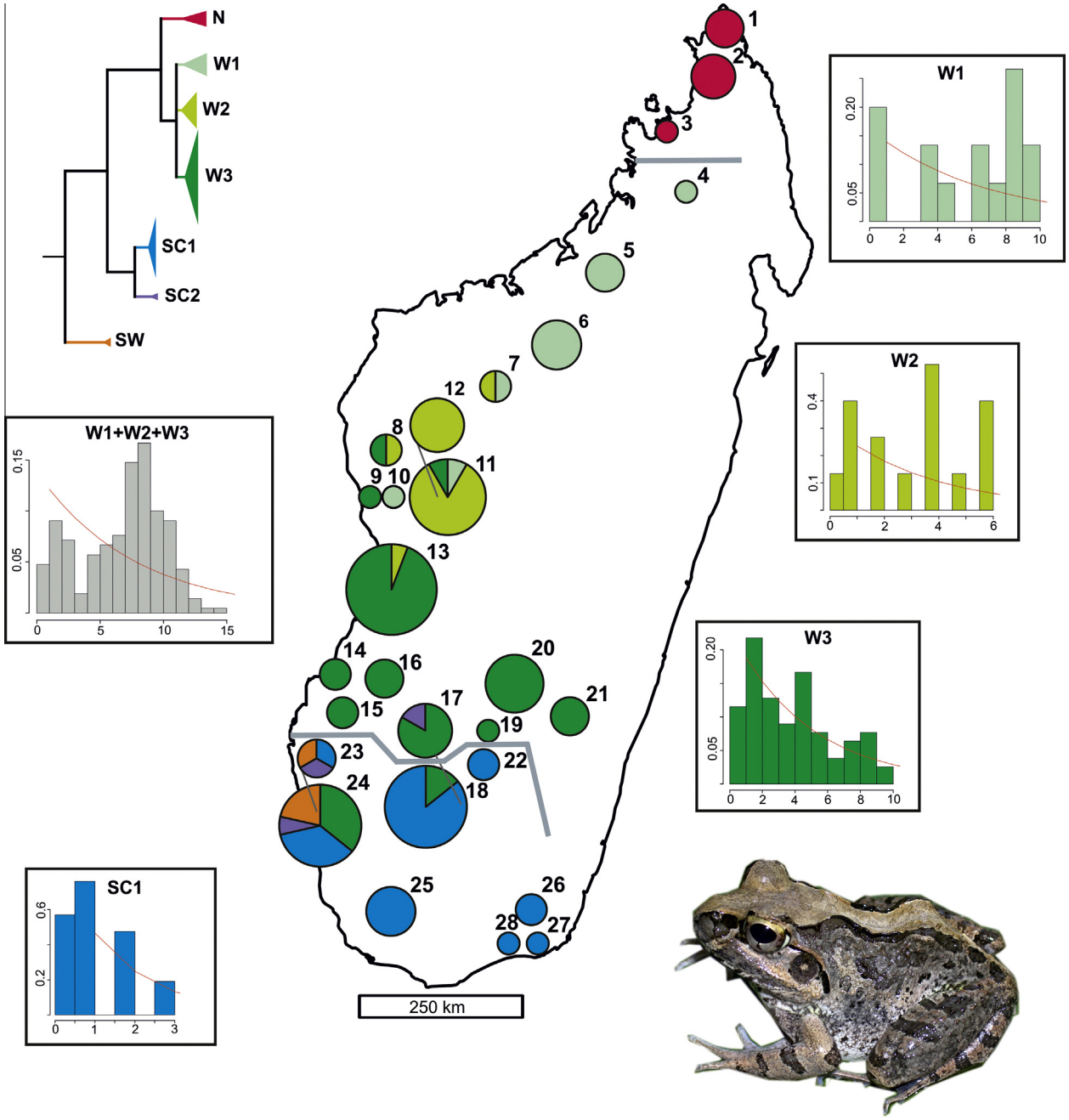

Fig. 3. Map of Madagascar showing sampling localities of *Laliostoma labrosum*; different colors denote major mtDNA clades (inset and Fig. 1). The diameters of the pie charts are proportional to sample size. Numbers refer to populations sampled in this study (see Table 1 for precise locality information). Thick grey lines traversing the island represent genetic barriers determined by a spatial analysis of molecular variance (SAMOVA). Mismatch distributions for each clade with number of sampled localities over five are shown. Red curves show the expected distribution for a demographically stable population; number of nucleotide substitutions between haplotypes and haplotype frequencies on x and y axes, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

admixture was also evident, with several sampling sites having about equal proportions of all three clusters (e.g. sites 5 and 6).

There was a moderately strong association between genetic and geographical distances in mtDNA at three spatial scales: all sampling sites ($r = 0.5278$, $P < 0.001$), excluding sampling sites with clade N ($r = 0.3988$, $P < 0.001$) and within the largest SAMOVA defined cluster, roughly equivalent to sampling sites with clade W ($r = 0.3988$, $P < 0.001$). For nuclear markers, IBD was detected in *bdnf* ($r = 0.5473$, $P = 0.012$) and *rag1* ($r = 0.5382$, $P = 0.003$) but not *pomc* ($r = 0.0128$, $P = 0.42$) at the largest scale including all samples, but was nonexistent (*bdnf*, $r = -0.016$, $P = 0.503$; *rag1*, $r = 0.0099$, $P = 0.412$) if the northernmost sampling sites (mtDNA clade N) were excluded from analyses.

3.5. SDM, habitat stability and population connectivity

Species distribution models for current time, 6 kya, 21 kya and 120 kya (Fig. 5F–I; Fig. 5C shows niche stability through time) depict high habitat suitability, continuous in space and time, along the western and southern coastal lowlands of Madagascar, and in south central Madagascar. Moreover, an isolated patch of suitable habitat has been present in the northernmost part of the island. The spatial correlations between the dispersal network/ancestral distributions, stability layers and SDM are shown in Table 4. Not surprisingly, the dispersal network (Fig. 5A) was correlated with both current and SDM stability layers (as the SDM stability layer was used to calculate the dispersal network). In contrast there

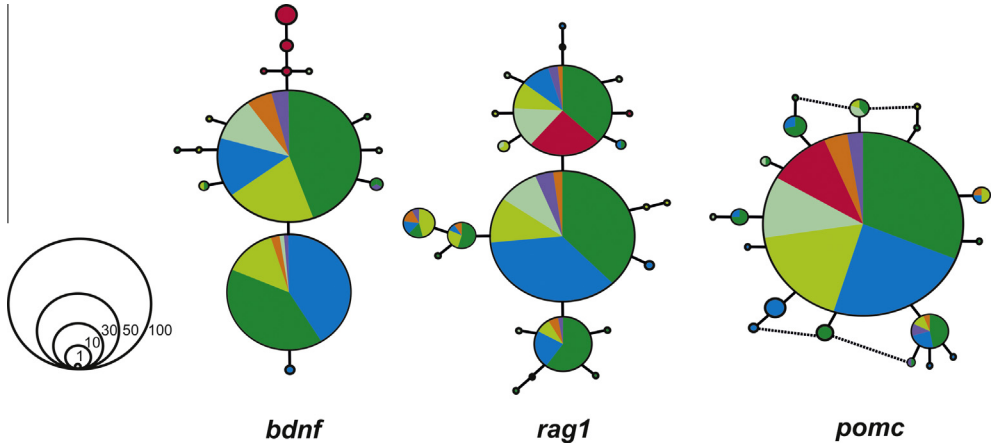

Fig. 4. Statistical parsimony networks of DNA variation in three nuclear genes in *Laliostoma labrosum*. Sizes of circles are proportional to number of sequences (scale located at left-hand side of figure). Color coding denotes the major mtDNA clades (as in Figs. 2 and 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Neutrality tests for major mtDNA clades in *Laliostoma labrosum* with at least five sampled populations (*N*). The empirical distributions of Tajima's *D*, Fu's F_S and Ramos-Onsins and Rozas R_2 were drawn from coalescent simulations based on the number of segregating sites (*S*). The clade W incorporates all populations from subclades W1, W2, and W3. Asterisks mark significant values at $P < 0.01$.

Clade	*N*	*S*	Tajima's *D*	F_S	R_2
W1	6	21	−0.346	0.019	0.148
W2	5	16	−0.398	−1.121	0.182
W3	13	41	−1.263	−7.410*	0.082*
W	21	68	−1.437*	−13.337*	0.067*
SC1	7	8	−1.434	−1.217	0.182

was little correlation of climate stability and climate seasonality with the dispersal network. The ancestral distributions of mtDNA clades (i.e., Phylomapper results) were also correlated with the current SDM and niche stability (and were not based on SDMs). Notably, we detected a strong and highly significant correlation between areas of high potential ancestral distribution and areas of high spatial connectivity among lineages. The ancestral areas identified by Phylomapper were also significantly correlated to areas of high annual climate seasonality.

4. Discussion

4.1. Low genetic differentiation across the range of Laliostoma labrosum

Compared to other mantellids, or tropical frogs in general, *L. labrosum* shows overall low mtDNA divergence across populations. In the 16S rRNA gene the maximum differentiation among *L. labrosum* clades equalled 0.9%, and the maximum differentiation among individuals was 1.1%. Much higher intraspecific divergence was found for two rainforest mantellid assemblages separated by ca. 250 km (mean of 3.8% in 16S rRNA for 37 species; Pabijan et al., 2012). Likewise, intraspecific differentiation in *cox1* is nearly twice as high in other mantellids (Perl et al., 2014). The low mtDNA sequence divergence between *L. labrosum* populations, even at sites separated by >1400 km, is surprising considering that most widespread amphibian species comprise geographically separated and often divergent genetic lineages (Vences and Wake, 2007). Although low differentiation at this scale is rare in amphibians, it is present in some terrestrial species and has been attributed to frequent movement of individuals among breeding sites, severe population bottlenecks or recent expansion from glacial refugia (e.g. Burns et al., 2007; Makowsky et al., 2009; Vásquez et al., 2013; Vences et al., 2002). Several hypotheses could explain the low level of mtDNA differentiation in *L. labrosum*: (i) range expansion after a species-wide bottleneck, (ii) selection or (iii) high levels of gene flow among populations. We find it unlikely that this species originated recently because it is substantially divergent from other mantellid frogs (11% divergence in 16S rRNA to its closest relative, Table 2), and together with *Aglyptodactylus*, forms one of the oldest clades in the mantellid phylogeny (Glaw et al., 2006). Although a recent species-wide bottleneck is inconsistent with the finding of seven major mtDNA clades in *L. labrosum* (one of which shows further extensive subdivision and evidence for demographic expansion) we cannot exclude that an ancient (pre-Pleistocene) bottleneck may be responsible for the shallow differentiation observed in contemporary populations of this species. Low divergence among populations due to positive selection removing neutral genetic variation is incompatible with the high number of mtDNA haplotypes, including many unique to specific sampling sites. Under a recent selective sweep we would expect a single haplotype predominating in most populations, or a star-like mtDNA gene tree if the sweep took place in the past.

The alternative explanation of relatively high vagility homogenizing genetic variation, at least at the regional level, is in line with the substantial admixture in both mtDNA and nuclear markers observed in some populations of *L. labrosum*. For instance, sampling sites in southwestern Madagascar (e.g. localities 23 and 24) contain individuals with three (SW, SC and W) of the four major mtDNA clades found throughout the range of the species (Fig. 3), while some sites (e.g. localities 5, 6 and 12) in central Madagascar show extensive admixture of the 3 genetic clusters identified by Structure (Fig. 6). The low divergence in both genomes may be the consequence of gene flow via movement of individuals among breeding sites. The small pools of standing water that this species uses as breeding sites are transient elements of the landscape and are thus unreliable in terms of annual breeding success. High rates of movement and low site fidelity may be advantageous for anurans living in seasonal and arid environments (Chan and Zamudio, 2009). Moreover, periodical heavy flooding has the potential to displace tadpoles of this species over considerable distances, which may also lead to population admixture. Elevated levels of dispersal and gene flow among populations have also been suspected for Malagasy microhylids of the genus *Scaphiophryne*, which are, much like *L. labrosum*, fossorial species that breed synchronously after heavy rains in temporary standing

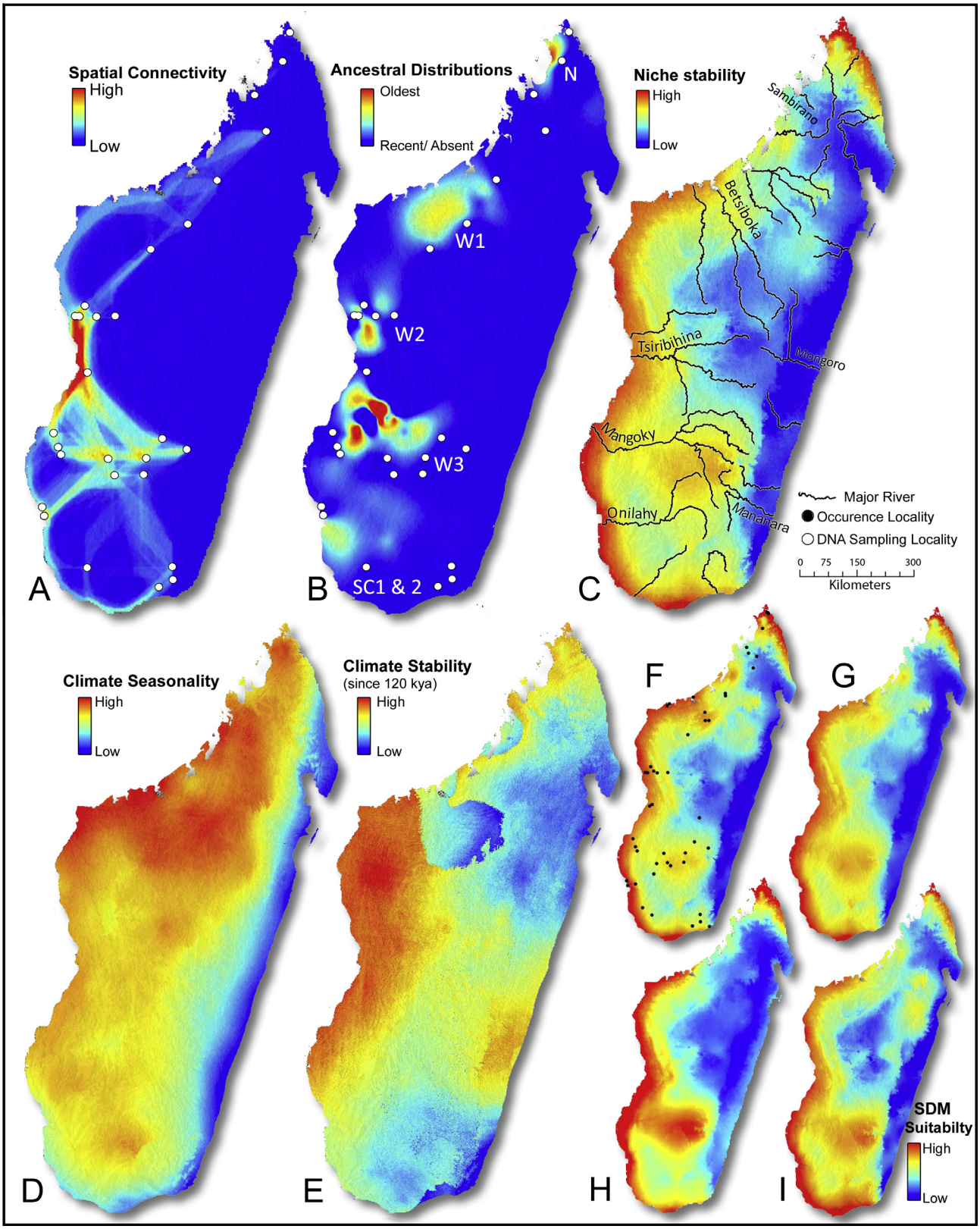

Fig. 5. Spatial genetic patterns and hypotheses of climatic stability. (A) Dispersal network of *Laliostoma labrosum* depicting the connectivity of clades through suitable habitat. Warmer colors depict areas of highest predicted connectivity through time. (B) Relative age and areas of ancestral distributions of mtDNA clades as inferred by Phylomapper. Warmer colors depict key ancestral areas. (C) Stability of the niche of *L. labrosum* since 120 kya based on a species distribution model. (D) Contemporary climate seasonality. Warmer colors depict areas of higher climate seasonality. (E) Climate Stability. Warmer colors depict areas of higher climatic stability (since 120 kya). (F–I) Species distribution models of *L. labrosum*. Warmer colors depict areas of higher predicted suitability ((F) Current time; (G) 6 kya; (H) 21 kya; (I) 120 kya).

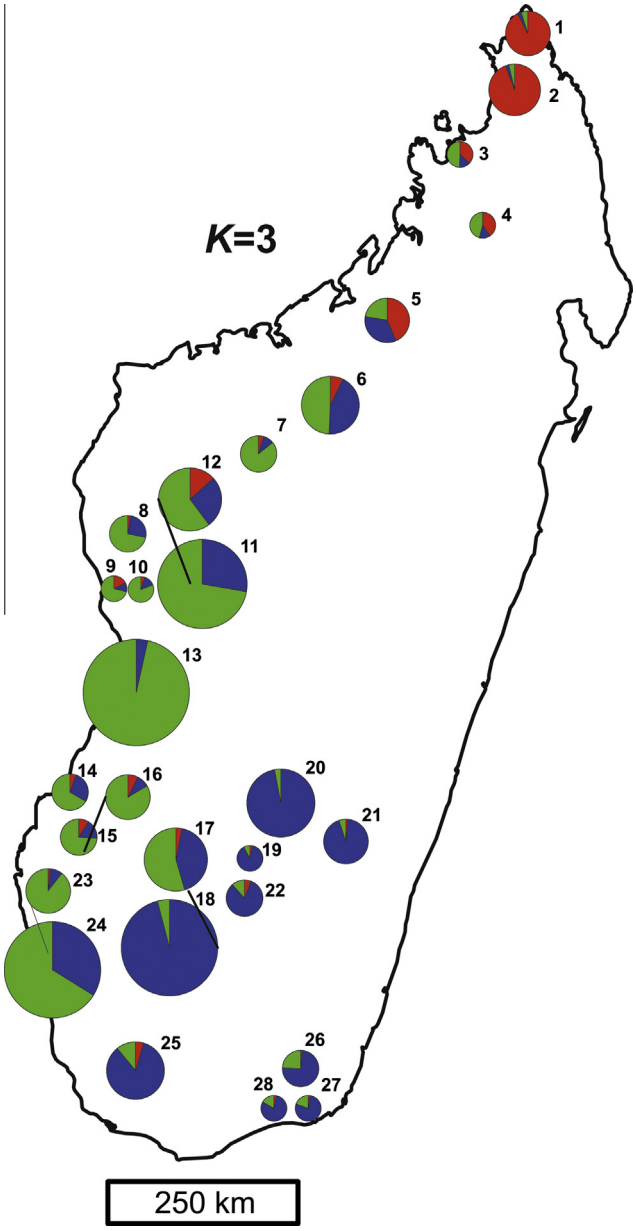

Fig. 6. Population subdivision in *Laliostoma labrosum* delimited by Structure based on three nuclear loci and mtDNA at $K = 3$. Mean estimates of membership coefficients for each population were based on 20 replicate analyses.

Table 4
Tests for spatial correlations (R^2 and P values) between ancestral distributions of mtDNA clades (Phylomapper results)/dispersal networks and current species distribution model, niche stability through time, climate stability and seasonal stability.

Pearson's R	Ancestral distribution	Dispersal network
Current SDM	0.344 (0.005)	0.72 (0.007)
SDM stability	0.336 (0.007)	0.517 (0.004)
Climate stability	0.205 (0.12)	0.29 (0.139)
Seasonal stability	0.326 (0.047)	0.195 (0.268)
Ancestral distribution	*	0.588 (0.0001)

water (Crottini et al., 2008; Vences et al., 2002). However, direct estimates of post-metamorphic dispersal are unavailable for any of these species; thus, the contributions of dispersal stages other than breeding migrations, e.g. juvenile movement, could also underlie low genetic differentiation in this species (Funk et al., 2005). *L. labrosum* is a rather large-sized species compared to other mantellids (males up to 48 mm snout-vent length, females up to 80 mm), and range size as well as genetic population divergence have been found to be size-correlated in this family of frogs (Pabijan et al., 2012; Wollenberg et al., 2011). Moreover, *L. labrosum* exhibits at least five of seven traits (large body size, adult habitat independent of standing water, aquatic oviposition site, large clutch size, exotrophic development) that are hallmarks of the optimal range-expansion phenotype inferred to promote dispersal and range expansion in toads (Van Bocxlaer et al., 2010).

4.2. Multiple regional refugia and demographic expansion

Despite overall low mtDNA divergence, we discerned 7 major mtDNA phylogroups within *L. labrosum*. Excluding clades SW and SC2 with restricted distributions in the southwest, all other major mtDNA clades (N, W1, W2, W3 and SC1) occur in distinct regions along the north–south axis of Madagascar (Fig. 3). The ancestral distributions of these clades (Fig. 5B) are completely allopatric. The initial splits in the mtDNA gene tree occurred between the SW clade with a restricted current distribution, followed by a second dichotomy involving clades in southern and southcentral Madagascar (SC1, SC2), vs. northern and western clades (N, W). The nested positions of the clades imply a southwestern origin for all extant mtDNA lineages of *L. labrosum* and subsequent vicariant formation of lineages distributed further north.

Regional range expansion can be inferred for some mtDNA phylogroups based on the geographic structure observed in widespread clades (N, W, and SC1) and their spatial overlap in the west-central and southwestern parts of the island. Further support comes from demographic expansion drawn from neutrality tests in the W clade. If high population connectivity inferred from generally low levels of divergence holds at least at the regional scale, then the redistribution of genetic variation from two or more refugial populations (Petit et al., 2003) could have contributed to the elevated levels of genetic diversity in both genomes observed in central and southwestern Madagascar (e.g. sites 5, 6, 12, 23, 24).

Variation in mtDNA in *L. labrosum* is consistent with the existence of regional phylogroups with genetic discontinuities at their peripheries, tempered by the expansion (leading to spatial overlap) of some of these groups. Each regional population in *L. labrosum* seems to have had an idiosyncratic history. For instance, southwestern Madagascar (e.g. sites 23, 24) has retained several old mtDNA lineages that could be attributed to the continuous persistence of a large population of *L. labrosum* in this area, while southcentral Madagascar lacks old lineages but harbors clade W3, which has undergone recent spatial and demographic expansion. The current pattern in genetic variation, the ancestral distributions of the mtDNA clades and overall shallow divergence in mtDNA suggest that cycles of fragmentation and allopatric divergence during the Pleistocene may have triggered intraspecific differentiation in this species. Paleoecological data show that Quaternary climate change in Madagascar involved cooler and drier glacial periods and warmer and moister interglacials, including a last glacial maximum in phase with conditions in the northern hemisphere (Burney, 1996; Burney et al., 1997, 2004; Gasse and Van Campo, 1998).

The genetic subdivisions in this species are evocative of the "refugia within refugia" pattern of the Apennine, Iberian and Balkan peninsulas (Canestrelli et al., 2012; Gómez and Lunt, 2007; Pabijan et al., 2015). This model involves the establishment of multiple independent centres of differentiation on a small geographic scale during adverse climatic conditions. Cyclical contractions and expansions of divergent lineages from spatially proximate refugia may produce a complex pattern of admixture (Godinho et al., 2008), as detected for *L. labrosum* in central and southwestern Madagascar. Local persistence of divergent lineages

was also prominent in the history of arid and subarid southern Australia (reviewed in Byrne, 2008). Interestingly, Byrne (2008) concluded that increased aridity in the Pleistocene, causing the expansion of sandy deserts, forced most water-dependent species into multiple mesic refugia along the peripheries of inhospitable terrain. Others have postulated mesic refugia for dry tropical regions of Africa (Arctander et al., 1999; Nichol, 1999) and South America (Carnaval and Bates, 2007). Our results suggest that drought-intolerant species in western Madagascar could have been confined to a number of mesic refugia during periods of increased aridity in the Quaternary.

The ancestral distributions of the mtDNA clades, and thus the potential Quaternary refugia for *L. labrosum*, roughly coincide with several low-elevation river catchments thought to have sustained drought-sensitive taxa through the most arid periods of the Quaternary by the buffering effect of riverine forests (Wilmé et al., 2006). Land in between major rivers is deemed to have been inhospitable for most taxa; hence, populations in different catchments would have differentiated in allopatry (Wilmé et al., 2006). Due to its emphasis on recent climatic events, this hypothesis may be particularly well-suited to explain intraspecific differentiation, i.e. at the phylogeographic level, and especially in arid seasonal western Madagascar, where the effect of past climate changes on forest vegetation might have been more severe. This may explain why several other studies focusing on interspecific divergences and including eastern Madagascan taxa found an inadequate match between current distributions and Wilmé's areas of endemism (Brown et al., 2014; Gehring et al., 2012; but see Pearson and Raxworthy, 2009). In *L. labrosum*, the ancestral area of clade N coincides with the northern bank of the Mahavavy River, that of W1 with the Betsiboka River, those of W2 and W3 are located north and south of the Tsiribihina River, and that of SC1 + SC2 is on the southern bank of the Onilahy River. Paleoecological data support the existence of at least one of these putative refugia for *L. labrosum*. Pollen spectra from Anjohibe Cave in northwestern Madagascar, situated within the ancestral distribution of clade W1, indicate that an ecosystem similar to the present-day palm savannah has persisted for at least the last 40 kyr (Burney et al., 1997). This type of grassland is currently inhabited by *L. labrosum*.

4.3. Phylogeographic breaks and isolation-by-distance

We found a well-delimited phylogeographic break separating the three northernmost sampling sites (containing mtDNA of clade N) from all central and southern populations. This break is evident in mtDNA and two nuclear genes (private alleles in *bdnf*, lineage sorting in *rag1*) and coincides with a swathe of monsoon rainforest known as the Sambirano region and currently extending roughly 30–50 km to the north and south of the Sambirano River (Fig. 1b). This biogeographic barrier receives considerable rainfall, shows numerous floristic similarities to the eastern rainforests, has more pronounced topographic relief than surrounding regions, and divides the drier areas of western Madagascar, dominated by deciduous forest, into two sections, north and south of the Sambirano. Concordant phylogeographic breaks in a number of vertebrates roughly coincide with the boundaries of the Sambirano region and include frogs (Crottini et al., 2012b; Rodríguez et al., 2015; Vences and Glaw, 2002), reptiles (Boumans et al., 2007; Lemme et al., 2013; Münchenberg et al., 2008; Nagy et al., 2007; Orozco-Terwengel et al., 2008; Vences et al., 2014) and lemurs (Guschanski et al., 2007; Pastorini et al., 2003). Moreover, this region constitutes the only gap in terms of low SDM stability and suitability for *L. labrosum* in otherwise continuously high values along the entire west coast of Madagascar (Fig. 5C and F–I). We suggest that the Sambirano region is an effective wet forest barrier for *L. labrosum*, and the genetically differentiated lineage that today occurs north of this region may have arisen through a founder event during more arid climatic phases of the Plio-Pleistocene (as invoked e.g., by Wilmé et al., 2006). Alternatively, the emergence of the Sambirano forest in the Late Miocene or Pliocene (Wells, 2003) may have fragmented a previously continuous range of *L. labrosum* and isolated two groups that today are genetically distinct. We lack a reliable time tree for the *Laliostoma* phylogroups, and refrain from applying molecular clock methods due to the lack of primary calibration points within laliostomines. Mean *cytb* divergence among clades is 0.041 (range: 0.010–0.065; Table S3). An evolutionary rate of 1% divergence per lineage per million years (the midpoint of the range estimated across vertebrates; Gillooly et al., 2005) implies a time scale of 0.5–3.25 my, suggesting that diversification of major *Laliostoma* mtDNA clades occurred in the Pleistocene to Late Pliocene.

Currently, *L. labrosum* can be found in deforested fragments of the Sambirano region, e.g. near rice fields, and sometimes penetrating into lowland rainforest nearby such degraded areas (Glaw and Vences, 2007). This begs the question if land-use changes associated with the expansion of agriculture in this area may have led to secondary contact and hybridization between the distinct phylogeographic lineages to the north and south of this region.

Under a model of isolation-by-distance (IBD), allele frequencies gradually change with geographic location such that spatially close sampling locations are genetically more similar than distant ones. Thus, detection of IBD implies limited dispersal. We detected an IBD effect within *L. labrosum* in three of the four genetic markers if all sampling sites were used in the analyses. However, IBD disappeared in the nuclear markers and weakened in mtDNA if the three northernmost populations (populations 1–3) were excluded from the analysis. Meirmans (2012) showed that an abrupt change in allele frequencies associated with a phylogeographic break can give a misleading signal of IBD (a false positive). We attribute the IBD signal in nuclear markers to the major phylogeographic break within *L. labrosum* located in northern Madagascar (see below). To explore the relationship between genetic and geographic distances, we tested for IBD within the largest SAMOVA-defined group, roughly corresponding to sampling sites with mtDNA of clade NC. This design specifically excluded the phylogeographic discontinuities in the north and south associated with clades N and SC, respectively. The IBD effect was still relatively strong and significant, implying limited dispersal of mtDNA at the regional scale, although we cannot completely disregard the influence of minor phylogeographic breaks within clade NC.

4.4. Habitat stability and population connectivity through time

In tropical rainforest, species diversity and genetic diversity have both been correlated to habitat stability over the course of Quaternary climate change (Carnaval and Moritz, 2008; Carnaval et al., 2009; Graham et al., 2006). In *L. labrosum*, niche stability is also correlated to potential ancestral distribution, but our current and past SDMs encompass large parts of the coastal lowlands of western Madagascar and suggest that the ecological conditions of this region have remained suitable through time for this species. Based on SDM modeling alone, the potentially suitable habitat for *L. labrosum* may not have changed much in the last 120 kya, with little indication of climate induced range contractions to spatially restricted refugia (with the exception of the extreme northern part of the island). Interestingly, the phylogeographic pattern of this species shows allopatric lineage diversification and expansion, suggesting a prominent role of vicariant events. We also observed a strong correlation between the ancestral distributions of mtDNA clades and areas of high spatial connectivity among lineages, despite the small area of the latter. The dispersal network

provides a better, although imperfect, match to the ancestral distribution of mtDNA clades. The results suggest that the population-level processes accounted for by the dispersal network seem to exert a greater influence on the persistence of this species than habitat determinants alone.

Our results suggest that a key factor defining potential refugia for *L. labrosum* is high population connectivity. One possible mechanism could involve breeding site availability as a limiting factor in arid environments. For pond breeding amphibians, population persistence heavily relies on population connectivity determined by both breeding site configuration at the landscape level and by the dispersal ability of the species (reviewed in Cushman, 2006). The climatic vagaries of the Quaternary in Madagascar included cooler and drier periods, especially at the LGM (Burney et al., 2004; Gasse and Van Campo, 1998). During these times, breeding sites for species dependent on standing pools of freshwater were probably scarcer than they are today, but amphibians such as *L. labrosum* often rely on temporary ponds, which might seasonally arise also in extremely arid areas such as southwestern Madagascar, far from any permanent river or lake. Future analyses of past potential distribution areas of arid-adapted frogs such as *L. labrosum* need to take into account additional spatial factors that might facilitate such temporary waters, such as permeability of substrate.

4.5. Conclusions

L. labrosum shows relatively low genetic differentiation in both mtDNA and nuclear loci across a distribution spanning the entire western arid zone of Madagascar. Low overall divergence is accompanied by admixture of distinct phylogroups in both genomes in central and south central Madagascar, and recent demographic expansion of at least one mtDNA clade. This pattern may be caused by high regional gene flow due to the frequent movement of adults or larvae among breeding sites. Despite relatively low genetic differentiation, we observed seven major mtDNA phylogroups in *L. labrosum*. Their geographical distributions and nested positions in the mtDNA genealogy imply a southwestern origin for all extant mtDNA lineages. The current phylogeographic pattern and paleo-distributions of major mtDNA phylogroups suggest that geographic barriers such as moist forest of the Sambirano region and vicariance during intervals of drier and cooler climate in the Quaternary (Burney, 1996; Burney et al., 1997, 2004; Gasse and Van Campo, 1998) were important in the history of this species. Five potential refugia were identified in northern, western and southwestern Madagascar based on the pattern of spatial genetic variation, consistent with a multiple refugia model (Gómez and Lunt, 2007) that may be representative of arid and subarid regions worldwide (Byrne, 2008). We present evidence that population connectivity through space and time (modeled by dispersal networks) was a major determinant of the persistence of some of these populations. This insight offers a complementary approach to purely genetic or habitat stability models of population persistence by incorporating population processes at the landscape level.

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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.2015.05.018.

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