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RESEARCH ARTICLE

Restricted natural hybridization between two species of litter frogs on a threatened landscape in southwestern Brazilian Amazonia

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Abstract Natural hybridization between allopatric species following secondary contact has been poorly documented for Neotropical anurans inhabiting the Amazonian lowlands. We conducted a genetic survey across a contact zone between two species of litter frogs, *Allobates hodli* and *Allobates femoralis* (family Dendrobatidae), located on the left riverbank of the upper Madeira River, State of Rondônia, Brazil. We obtained tissue samples from 11 sampling sites on both riverbanks, covering approximately a 400 km long transect. We evaluated the genetic relationships between samples using haplotype networks and a distance-based phylogenetic tree obtained from a dataset of 16S rRNA mtDNA sequences. Estimates of genetic diversity, population structure, and the identification of sites where genetic admixture occurred were carried out by means of frequency-based methods and Bayesian inference on mtDNA and a set of four microsatellite loci, including samples collected throughout the study area. A reduced dataset including only microsatellite loci genotyped from samples on the left riverbank was applied in assignment tests for detecting levels of admixture at the contact zone and adjacent sampling sites, and for detecting and quantifying hybrid individuals. Our results suggest that genetic introgression between *A. hodli* and *A. femoralis* is restricted to the core area of the contact zone, where potential hybrids are less frequent than parental genotypes. Effects on the genetic variability of adjacent populations are only detected at sites located 1.5 km downstream and upstream of the core area, suggesting the existence of selection against hybrids, possibly mediated by postzygotic isolation mechanisms. The contact zone between *A. femoralis* and *A. hodli* is the first well delimited suture line between anuran species ever documented in the Brazilian Amazon. The settlement of two dams along the upper Madeira River poses an immediate threat to the gene flow and hybridization balance observed between the populations studied. Our results provided guidelines for a current monitoring program, aiming at the impacts of dams on this evolutionary system's dynamics.

Keywords Amazon · Madeira River · Hybrid zone · Genetic introgression · Dendrobatidae · *Allobates femoralis*

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Introduction

The succession of geological and climatic events occurring from Late Miocene to the present influenced current geographic distribution of animal species in the Amazonian lowlands (see Hoorn and Wesselingh 2010 for a recent review). Most speciation models considered for this region rely strongly on vicariance, and the retraction of past geological or ecological barriers is thought to have triggered the

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range expansion of many lineages that diverged in isolation, many of which reached secondary contact zones with other, closely related lineages (Haffer 1997; Moritz et al. 2000). Considering species that are distributed in primary rainforests not subject to seasonal flooding (i.e. intolerant to open habitats and floodplains), Amazonian rivers represent obvious boundaries to the geographic range expansion of lineages that evolved in allopatry. However, secondary contact zones are not always coincident with the current location of river channels, and suture lines are sometimes found on the same riverbank, often perpendicularly to river channels (Haffer 1997).

Several evolutionary outcomes can be expected from natural secondary contact between two lineages that diverged in allopatry, depending on the extent of neutral or adaptive differentiation accumulated between those lineages while isolated (Barton and Hewitt 1985; Coyne and Orr 2004; Allendorf and Luikart 2007). One is the formation of hybrid swarms, or populations predominantly constituted by hybrid individuals, originating from several generations of crosses between hybrid individuals or backcrosses between hybrids and parental populations (Seehausen 2004). A second outcome is expected when parental lineages diverged phenotypically and became adapted to distinct extremes of an environmental gradient, rendering a clinal or patchy contact zone, with frequency and direction of hybridization largely related to resource or habitat distribution, rendering a smooth gradient or a mosaic of parental and hybrid genotypes (e.g. Vorndran et al. 2002; Keller et al. 2008). The third possible outcome of secondary contact is the establishment of very narrow hybrid zones, dependent on the balance between selection against hybrid individuals and migration of parental genes from adjacent populations. These are frequently referred to as "tension zones", and can be characterized by the presence of parental genotypes within samples, and by geographically limited introgression of parental lineages or hybrid genotypes from the core area of the contact zone into the distribution of the second parental lineage (Barton and Hewitt 1985; Arnold et al. 1999; Jiggins and Mallet 2000). The local evolution of lineages following secondary contact is generally unpredictable as these models are density dependent, and selection regimes can change in time, for example, according to environmental conditions (Levin et al. 1996; Grant and Grant 1992). However, a few evolutionary trends can be presumed from the characterization of hybrid zones (such as the geographic replacement of parental populations by hybrid swarms, or the establishment of hybrid sinks reducing local genotypic variability), often with potential use for conservation planning (Seehausen et al. 2007; Dawe et al. 2009; Hird and Sullivan 2009).

Hybrid zones or suture lines between closely related anuran species are frequently found along limited transects of their peripheral geographic distribution (Barton and Hewitt 1985; Jiggins and Mallet 2000; Hofman and Szymura 2007; Wells 2007; Lemmon et al. 2007; Vogel and Johnson 2008; Moritz et al. 2009). However, the occurrence of contact zones and the description of areas of possible genetic introgression between divergent lineages of Amazonian lowland anurans have been poorly documented in the literature (e.g. point records are briefly mentioned in Brown and Twomey 2009; Simões et al. 2010).

In early 2005, a narrow and well-delimited contact zone between two species of Amazonian frogs of the genus Allobates (Family Dendrobatidae) was discovered on the left riverbank of the upper Madeira River (Simões et al. 2008). The contact zone coincides with the boundary between two geomorphological units, evidenced on the channel of the river by a group of large rapids, locally known as Cachoeira do Jirau. At the time, the two species were thought to represent distinct morphotypes of the widespread brilliant-thighed poison frog, *Allobates femoralis* (Simões et al. 2008; Amézquita et al. 2009). Recently, summing information on the geographic distribution, mtDNA molecular phylogeny, and available evidence on morphological and acoustic differentiation, one of the former morphotypes was described as a new species, which has a restricted geographic distribution, being parapatric to, and highly divergent from the *A. femoralis* populations inhabiting the upper Madeira River basin (Simões et al. 2010).

Despite its recognition for at least 5 years, the contact zone between *A. hodli* and *A. femoralis* has not been subject to detailed studies aiming at its characterization and current evolutionary dynamics. As the two species are not each other's sister clades (Simões et al. 2010), and have been distinct lineages for at least 2.5 million years (and most probably for around 4.5 million years—Santos et al. 2009), the presence of mtDNA markers typical of one of the lineages within the genome of the other can be unambiguously attributed to genetic introgression rather than to incomplete lineage sorting from a recent polymorphic common ancestor.

In this study, we conduct a genetic characterization of the contact zone between *A. femoralis* and the recently described *Allobates hodli*, evaluating the occurrence of hybridization between these two species. Additionally, we evaluate how secondary contact affects the local distribution of genetic variability in comparison to nearby populations of both species using mtDNA and microsatellite markers. Current development policies are ubiquitous along this segment of the Madeira River (Clemons 2007), including the construction of two large hydroelectric power plants at the level of Santo Antônio and Jirau rapids, and associated dams (Fig. 1), roads, power-lines, construction sites, and villages to be used in resettlement plans for local human populations.

Our main goal is to provide a first insight into the natural patterns of genetic structure among these model species. This information can be used as a valuable guideline for monitoring programs aiming at accessing the impacts of contemporary environmental changes resulting from such policies. In that sense, our results can point out specific locations where sampling sites should be established in order to monitor shifts in genetic diversity parameters and hybridization dynamics in both species, after the settlement of the two power plants.

Methods

Study area

The study area comprises terra-firme (not seasonally flooded) forests along a ≈400 km segment of the upper Madeira River, in southwestern Brazilian Amazon, from the village of Fortaleza do Abunã, in Rondônia, to the vicinities of the city of Humaitá, State of Amazonas (Fig. 1). Along this segment, the Madeira River is generally entrenched, 1 km wide in average, flowing fast through a system of successive rafts and rapids. Expressive areas of floodplains occur adjacent to the river channel only downstream of Porto Velho, corresponding to areas included in the municipality of Humaitá (DNPM 1978).

Within the study area, *A. hodli* is distributed exclusively on the left riverbank, occurring from localities across the river from Fortaleza do Abunã to the level of the Cachoeira do Jirau rapids. Downstream of the Cachoeira do Jirau rapids, and across the right riverbank, *A. hodli* is replaced by *A. femoralis* (Fig. 1b). The two species are easily distinguished by their advertisement calls (Fig. 1c), in addition to characteristic color patterns (Simões et al. 2010).

Although the contact zone between *A. hodli* and *A. femoralis* is restricted to the left riverbank, mtDNA haplotype sharing is known to occur between *A. femoralis* populations on opposite banks in regions near Humaitá

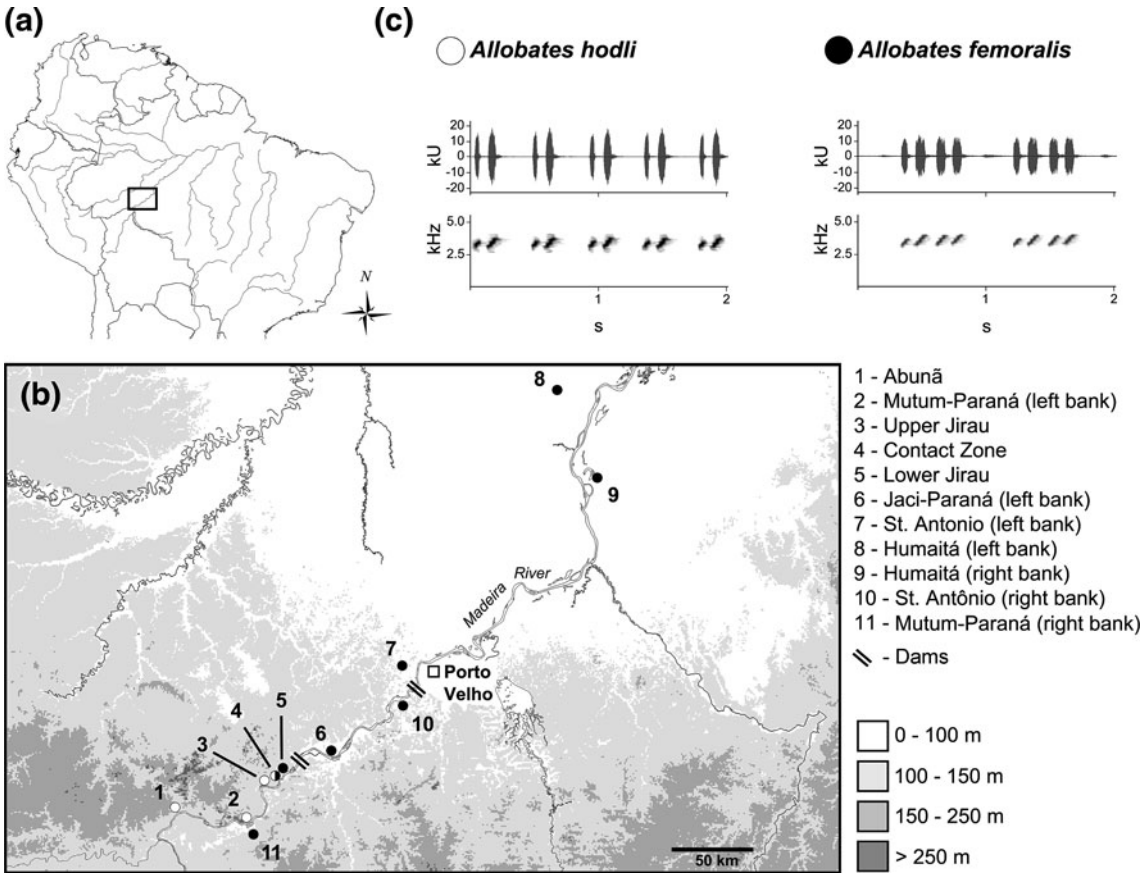

Fig. 1 **a** Relative location of study area in northern South America; **b** Distribution and denomination of 11 sampling sites along the study area in the upper Madeira River, southwestern Brazilian Amazonia. *White filled dots* correspond to distribution of *A. hodli*, and *black filled dots* to the distribution of *A. femoralis*. The two species meet at a contact zone on the left riverbank (sampling site 4) adjacent to the Cachoeira do Jirau rapids; *paired bars point* the locations of the Santo Antônio (downstream) and Jirau (upstream) dams. **c** *A. hodli* and *A. femoralis* can be distinguished by characteristic advertisement calls, constituted by two notes in *A. hodli* and four notes in *A. femoralis*. *Sonograms* represent calls of one individual from Abunã (site 1) and one individual from Lower Jirau (site 5)

(Simões et al. unpublished data). Possible cases of DNA introgression between *A. femoralis* inhabiting the right bank of the upper Madeira River and *A. hodli* have not been verified in previous studies. Therefore, samples from three sites on the right riverbank (9–11, Fig. 1b) were used to evaluate potential genetic admixture between these two groups of populations prior to hybridization analysis, which were restricted to samples collected along the left riverbank.

Molecular data acquisition

Allobates hodli and *A. femoralis* muscle tissue samples were housed at Coleção de Tecidos de Genética Animal at Universidade Federal do Amazonas (CTGA–ICB/UFAM—Appendix I in Supplementary material), Manaus, Brazil, originating from field work carried out at 11 sampling sites along the study area (Table 1; Fig. 1b), which were visited in different occasions between 2004 and 2009 by P.I. Simões and A.P. Lima. Fragments of the 16S rRNA mitochondrial gene for some of these locations were already available on GenBank (Appendix II in Supplementary material). We complemented the available 16S dataset by including additional sequences obtained from samples from the same localities and sequences from the remaining sampling sites. Sequences and microsatellite markers used in population and hybridization analyses were amplified according to the following laboratory protocols.

Total genomic DNA was extracted from preserved muscle tissue samples using a cetyl trimethyl ammonium bromide (CTAB) protocol (modified from Doyle and Doyle 1987). We used primers 16Sar and 16Sbr (Palumbi 1996) to amplify a 507 bp fragment of the 16S rRNA mitochondrial gene via polymerase chain reaction (PCR). PCR reactions used a final volume of 16 μL and contained 6.7 μL ddH_2O, 2.0 μL of 25 mM $MgCl_2$, 1.5 μL of 10 mM dNTPs (2.5 mM each dNTP), 1.5 μL of ×10 amplification buffer (75 mM Tris HCl, 50 mM KCl, 20 mM $(NH_4)2SO_4$), 1.5 μL of a 2 μM solution of each primer, 0.3 μL of *Taq* DNA Polymerase 5 U/μL (Biotools, Spain) and 1 μL of DNA (about 30 ng/μL). PCR conditions had a pre-heating step of 92 °C for 60 s, followed by 35 cycles of denaturation at 92 °C for 60 s, primer annealing at 50 °C for 50 s and primer extension at 72 °C for 90 s. A final extension step occurred at 72 °C for 5 min. Sequencing reactions were performed according to manufacturer's recommended ABI BigDye Terminator Cycle Sequencing protocol, using primer 16Sbr and an annealing temperature of 50 °C. Sequencing was performed in an automatic ABI 3130xl sequencer (Applied Biosystems).

In addition to 16S rRNA sequences, we used four pairs of primers described by Jehle et al. (2008) in order to amplify four microsatellite loci from samples from both species (Epifem 03, Epifem 05, Epifem 12 and Epifem 13). PCR reactions used a final volume of 10.5 μL, and cointained 2.6 μL ddH_2O, 1.3 μL 25 mM $MgCl_2$, 1.3 μL 10 mM dNTPs, 2.0 μL of ×10 amplification buffer, 1.0 μL of a 2 μM solution of reverse primer, 0.5 μL of a 2 μM solution of forward primer, 0.5 μL of a 2 μM solution of the M13 primer, 0.3 μL of *Taq* DNA Polymerase 5 U/μL and 1.0 μL of DNA (30 ng/μL). PCR conditions used preheating step of 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s and extension at 72 °C for 45 s. The annealing of M13 primers occurred subsequently, applying 15 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s, and extension at 72 °C for 45 s. Final extension occurred at 72 °C for 30 min. PCR products were genotyped in an automatic ABI 3130xl sequencer. Resulting genotypes were inspected in GeneMapper 4.0 (Applied Biosystems), and allele sizes were inferred by comparisons with peaks of known size produced by ROX-labeled size standards (DeWoody et al. 2004).

The six remaining pairs of primers described by Jehle et al. (2008) either rendered monomorphic alleles across the study populations (Epifem 06), or failed to successfully

Table 1 Designations, position according to riverbank, clade/species attribution and coordinates of 11 sampling sites along the upper Madeira River, in southwestern Brazilian Amazon

Site	Locality name	Riverbank	Clade	Latitude	Longitude
1	Abunã	Left	*A. hodli*	9.5160°S	65.3249°W
2	Mutum-Paraná	Left	*A. hodli*	9.5732°S	64.9211°W
3	Jirau	Left	*A. hodli*	9.3347°S	64.7375°W
4	Contact Zone	Left	*A. hodli*/A femoralis	9.3206°S	64.7225°W
5	Lower Jirau	Left	*A. femoralis*	9.3114°S	64.7172°W
6	Jaci-Paraná	Left	*A. femoralis*	9.1694°S	64.4289°W
7	Santo Antônio	Left	*A. femoralis*	8.8309°S	64.0206°W
8	Humaitá	Left	*A. femoralis*	7.0228°S	63.1028°W
9	Humaitá	Right	*A. femoralis*	7.5488°S	62.8772°W
10	Santo Antônio	Right	*A. femoralis*	8.6550°S	64.0195°W
11	Mutum-Paraná	Right	*A. femoralis*	9.6414°S	64.8859°W

amplify the respective microsatellite markers in all (Epifem16, Epifem17) or in a set of particular populations (Epifem 09, Epifem 14, Epifem15). These markers were characterized from a single population from the vicinities of Santarém, State of Pará, Brazil, located at least 1050 km from our study area, and might not be applicable to all populations referred to as *A. femoralis*, which comprise a group genetically divergent cryptic species (Grant et al. 2006; Santos et al. 2009), possibly due to substitutions on primer annealing sites.

Mitochondrial DNA analyses

The 16S rDNA sequences were initially aligned using the ClustalW algorithm (Thompson et al. 1994) implemented in BioEdit (Hall 1999), verified by eye, and corrected manually, when necessary. Gaps and substitutions were checked by comparisons with the original chromatographs. In order to evaluate the genealogical relationships among haplotypes and overall haplotype distributions, haplotype networks were built from the resulting alignment by methods of statistical parsimony (Templeton et al. 1992) using TCS 1.21 (Clement et al. 2000), and applying a 95 % connection limit, considering gaps as a 5th character state. Analysis of DNA polymorphism and estimates of genetic diversity were carried out in DnaSP v.5.10 (Librado and Rozas 2009) for samples of each species and from each sampling site, separately.

We applied a Bayesian analysis of population structure on nucleotide frequencies (Corander and Tang 2007; Corander et al. 2008) over the 16S rDNA database in order to estimate the most probable number of genetic clusters formed by samples along the study area, and to evaluate the existence of sites where mtDNA introgression between clusters occurred. Analysis were run in BAPS 5 (Corander and Tang 2007; Corander et al. 2008), taking the number of clusters as a random parameter and setting the upper bound to one or up to eleven clusters (the latter corresponding to the total number of sampling sites). Five independent runs were performed for each upper bound value, and selection of the most probable cluster configuration was made by comparing the log-likelihood values of the best models. The evolutionary relationships between samples were further verified by reducing the 16S rDNA database to unique haplotypes, from which we obtained a Neighbor-Joining tree (Saitou and Nei 1987) based on Tamura-Nei genetic distances (Tamura and Nei 1993) in MEGA 4.1 (Tamura et al. 2007).

Population structure and hybridization analysis using microsatellites

Description of microsatellite loci variability and evaluation of genetic diversity parameters for each sampling site were carried out in GENALEX 6 (Peakall and Smouse 2006). Measures of Fst between sampling sites based on Weir and Cockerham estimates and heterozygote deficit within populations (Fis) were calculated in FSTAT 2.9.3.2 (Goudet 2001).

We investigated the existence of large scale population structuring and admixture within the study area based on microsatellite markers using Bayesian inference, as implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000). This model-based approach uses information on allele frequencies and assumes Hardy–Weinberg and linkage equilibrium between loci within each inferred genetic cluster to approximate the posterior probability of the actual number of clusters. Once reliable information on the number and distribution of clusters is obtained, it is possible to assign the proportion of each individual's genome that originated from a particular cluster. In a preliminary analysis, we used samples from all sites, and assumed the number of possible genetic clusters formed by these samples (K) to vary from one to ten. This analysis was run for 15 iterations with 1 million MCMC replicates after 100,000 initial replicates which were discarded as burn-in, applying an admixture model and considering allele frequencies to be independent from each other. The most probable number of clusters was selected graphically according to the mean increase in posterior probabilities observed from each value of K to $K + 1$ between all iterations. The results allowed us to confirm the number of clusters suggested by the mtDNA population structure analysis described above and to identify sampling sites where genetic admixture between riverbanks occurred. Results were also used to select populations experiencing no (or very reduced) genetic admixture regimes on the left riverbank, which could be assigned as pure parental populations in the hybridization analyses described below.

Analyses of hybridization focused on samples from sites 3, 4 and 5, corresponding to the core contact zone between the two species (site 4), and to sites located $\approx$1.5 km upstream (site 3) and downstream (site 5). Assignment tests were carried out in STRUCTURE 2.3.3 and in NEWHYBRIDS (Anderson and Thompson 2002). Like STRUCTURE, the NEWHYBRIDS method is capable of estimating the probability of assignment of each individual to a particular genotype class by accessing allele frequency variation between species (i.e. the two methods do not depend on diagnostic loci, with exclusive alleles fixed in each species). In addition to estimating the probability of assignment of each individual to one of the species that are potentially hybridizing (as in STRUCTURE), the NEWHYBRIDS method estimates the posterior probability of that individual belonging to a particular hybrid generation or category based on expected genotypic frequencies (i.e. F_1, F_2, parental backcrosses). Both methods provide the posterior

probability of membership of each individual to an alternative genotypic category, allowing for posterior inferences about evolutionary mechanisms regulating the hybrid zone dynamics (Jiggins and Mallet 2000). Recent tests show that these methods do not outperform each other when using microsatellite data, producing complementary results (Sanz et al. 2009).

Species assignment probabilities were accessed in STRUCTURE by setting the number of possible genetic clusters (K) to two (*A. hodli*/*A. femoralis*). As very strong data are necessary to overcome misclassification when priors on pure parental populations are provided, we did not include any prior information about parental populations in the STRUCTURE analysis. Analysis parameters were similar to the previous analysis considering all samples, applying 100.000 burn-in replicates followed by 1 million MCMC replicates after 100,000, considering an admixture model and independent allele frequencies. Average values between 20 iterations are presented for all individuals (q_i) and for within-sampling site (Q) membership coefficients.

For the NEWHYBRIDS analysis, we employed four distinct frequency categories: pure *A. femoralis*, pure *A. hodli*, F_1, and F_2 hybrids. As the small number of loci would probably prevent the correct distinction between pure parental lineages and hybrids originating from backcrosses (Boecklen and Howard 1997), parental backcross categories were not considered. Information on species origin was provided for putative parental individuals of pure *A. hodli* or *A. femoralis* origin collected in sampling sites not close to the contact zone, which were selected from the previous population structure analyses (see "Results" section). This was done by applying the "z" option to the input file, as recommended by the software's programmers. NEWHYBRIDS analysis was run for 5 million sweeps after 500,000 burn-in steps, applying Jeffreys-type prior distributions to allele frequency and mixing proportion parameters.

Results

Mitochondrial 16S rDNA sequence analysis

We obtained 16S rDNA sequences from 222 individuals collected throughout the 11 sampling sites in the study area. These corresponded to 47 unique haplotypes, which were generally species-exclusive and constituted independent haplotype clusters in the TCS parsimony network analysis (Figs. 2, 3). Both *A. femoralis* and *A. hodli* haplotypes were found in the core area of the contact zone, but haplotypes associated with the *A. hodli* lineage are more

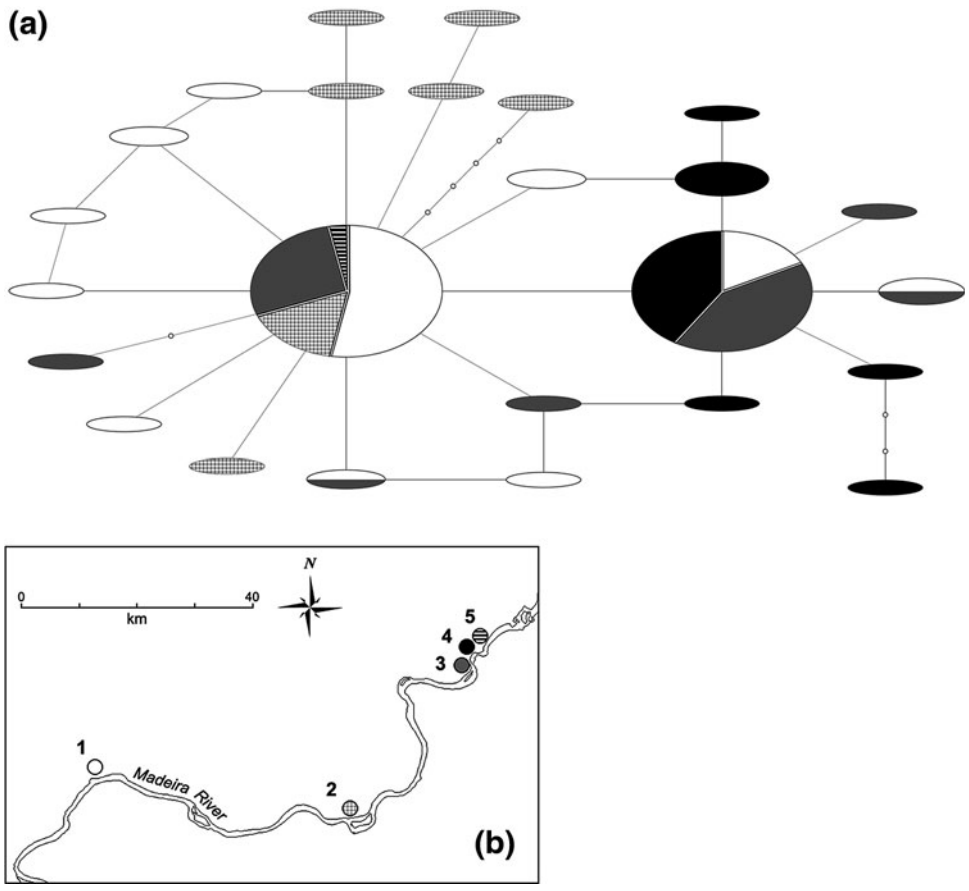

Fig. 2 **a** Haplotype network built from 16S rDNA sequences of *A. hodli* using statistical parsimony. Areas of ellipses proportional to haplotype frequency. *Small dots on lines* represent to missing intermediate haplotypes. *Colors* stand for haplotype origin according to sampling site; **b** relative position of sampling sites along the upper Madeira River

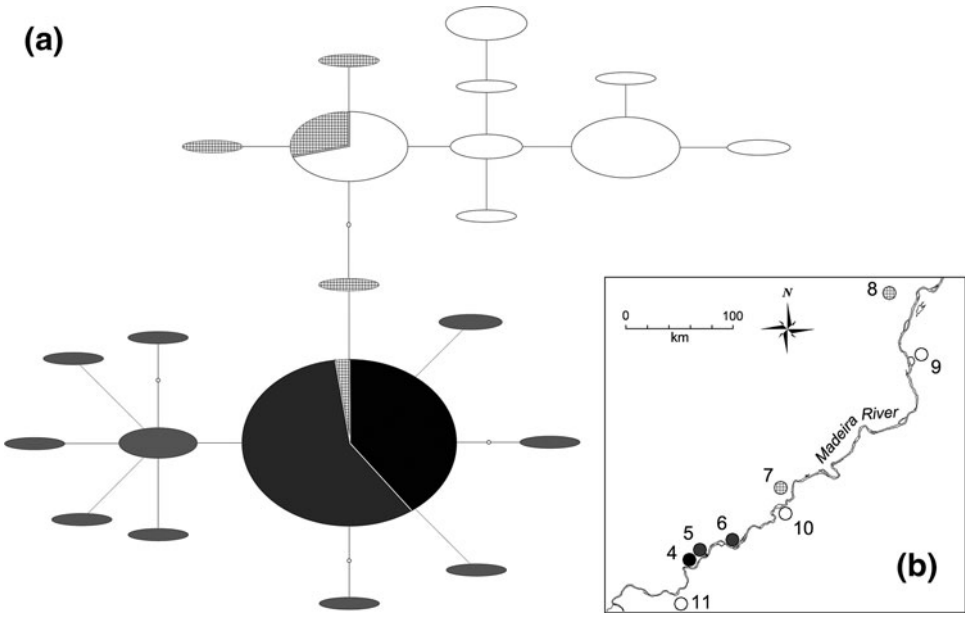

Fig. 3 **a** Haplotype network built from 16S rDNA sequences of *A. femoralis* and **b** origin according to location of sampling sites along the upper Madeira River. Areas of ellipses are proportional to haplotype frequency. *Dots along lines* stand for missing intermediate haplotypes

diverse among Contact Zone samples (six haplotypes, against a single *A. femoralis* haplotype). A single case of mitochondrial DNA introgression between species was detected approximately 1.5 km downstream of the contact zone, at Lower Jirau (site 5, Figs. 1b, 3), where one *A. femoralis* male (original field number/tissue collection number APL-2276) with typical four-note advertisement call carried an *A. hodli* haplotype. No *A. femoralis* haplotypes occurring on the right riverbank were found among *A. hodli* samples or among *A. femoralis* samples upstream of Jaci-Paraná (site 6—Fig. 3).

Genetic diversity estimates were generally lower for pooled samples of *A. hodli*, in comparison to pooled samples of *A. femoralis* (Table 2). Within *A. femoralis*, samples from the right riverbank had lower values for genetic diversity estimates than samples from the left riverbank. Estimates measured for each sampling site separately (Table 2) revealed a sudden increase in nucleotide diversity (π) and genetic diversity estimates (θ_π, θ_S) from Jirau (site 3) towards the Contact Zone (site 4), reflecting the mixed occurrence of *A. hodli* and *A. femoralis* haplotypes at this site. Estimated values drop dramatically from the Contact Zone towards Lower Jirau (site 5), except for the genetic diversity based on the number of segregating sites (θ_S), which increases discretely at this site. Conversely, haplotype diversity drops from the Contact Zone towards Lower Jirau, where only six haplotypes are observed among 33 samples. Additional cases of lowered haplotype diversity are found in Humaitá, at sampling sites on both riverbanks (sites 8 and 9).

Bayesian analysis of population structure on the complete mtDNA dataset indicated the existence of three genetic clusters (log ML = −915.3804; posterior probability = 0.99927—Fig. 4). Two clusters correspond to *A. femoralis* samples, and are roughly structured according to riverbanks (Fig. 4), with some degree of admixture on the left bank, at sampling sites 6–8, reflecting haplotype sharing between localities across the river, as seen above. The third cluster corresponds to *A. hodli* samples from sites 1, 2 and 3, and admixture with the *A. femoralis* cluster exclusive of the left bank occurs in the contact zone at site 4. The same *A. femoralis* individual (APL-2276) reported above as possessing an *A. hodli* haplotype at site 5 was placed in the *A. hodli* cluster.

The Neighbor-Joining tree based on genetic distances between unique 16S rDNA haplotypes revealed two highly supported (bootstrap value = 99 %) monophyletic clades (Fig. 5), corresponding to *A. hodli* and *A. femoralis* samples. No subclades are supported according to the bootstrap analysis (all bootstrap values <40 %). Haplotypes representative of both clades are found at the Contact Zone.

Population structure analysis inferred from microsatellites

The four microsatellite loci were successfully genotyped from samples of a total 195 individuals from the 11 sampling sites. Among these loci, Epifem 05 had the lowest number of alleles and heterozygote genotypes (Table 3). Estimates of microsatellite diversity per locus at each sampling site indicate a slight increase in number of alleles at the Contact Zone and Lower Jirau, while observed heterozygosity was generally lower at both sampling sites immediately adjacent to the Contact Zone (Appendix III in Supplementary material). Values for all diversity estimates decrease abruptly at the level of Santo Antônio, on the left

Table 2 Mitochondrial 16S rDNA genetic diversity estimates along a transition zone between *A. hodli* and *A. femoralis* sampled along the upper Madeira River

Group/sampling site	*n*	nH	Hd	π	*S*	θ_π	θ_S
Study area (all samples)	222	47	0.888 ± 0.011	0.024 ± 0.0004	46	0.0248	0.0157
A. hodli	72	19	0.735 ± 0.041	0.002 ± 0.0003	18	0.0025	0.0075
A. femoralis (samples pooled)	107	23	0.850 ± 0.020	0.006 ± 0.0007	36	0.0061	0.0142
A. femoralis (right bank)	41	8	0.768 ± 0.047	0.003 ± 0.0032	7	0.0032	0.0032
A. femoralis (left bank)	61	15	0.720 ± 0.053	0.005 ± 0.0013	32	0.0047	0.0141
Abunã	34	10	0.717 ± 0.072	0.002 ± 0.0003	8	0.002	0.0039
Mutum-Paraná (left)	11	7	0.818 ± 0.119	0.004 ± 0.0013	9	0.0041	0.0061
Jirau	27	6	0.638 ± 0.068	0.002 ± 0.0003	6	0.0018	0.0031
// Contact Zone //	43	7	0.678 ± 0.052	0.025 ± 0.0009	29	0.0257	0.0138
Lower Jirau	33	6	0.333 ± 0.105	0.003 ± 0.0025	28	0.0036	0.0142
Jaci-Paraná	15	7	0.771 ± 0.100	0.002 ± 0.0005	7	0.0023	0.0043
St. Antônio (left)	5	3	0.700 ± 0.218	0.003 ± 0.0010	3	0.0032	0.0029
Humaitá (left)	13	3	0.295 ± 0.156	0.006 ± 0.0003	2	0.0006	0.0013
Humaitá (right)	6	1	0.000 ± 0.000	0.000 ± 0.0000	0	0	0
St. Antônio (right)	19	5	0.743 ± 0.004	0.003 ± 0.0003	4	0.003	0.0023

Estimates are presented for all samples pooled, for each species (excluding samples from the core area of the Contact Zone, as both species are present at that site, and species attribution is not straightforward), and separately for each one of 11 sampling sites

N sample size, *nH* number of haplotypes, *Hd* haplotype diversity, π nucleotide diversity, *S* number of segregating sites, θ_π genetic diversity according to nucleotide diversity, θ_S genetic diversity according to the number of segregating sites

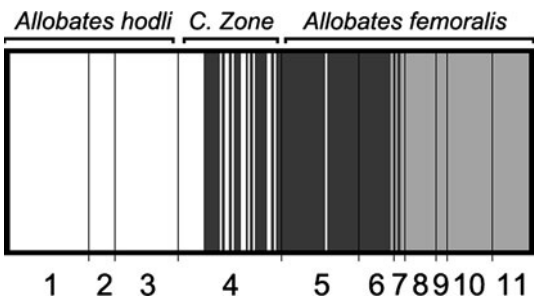

Fig. 4 Bayesian analysis of population structure on a 16S rRNA mitochondrial gene dataset obtained from 222 individuals indicates the existence of three genetic clusters along the upper Madeira River, corresponding to one *A. hodli* cluster and two *A. femoralis* clusters. Genetic admixture between clusters occurs on the hodli/femoralis contact zone on the left riverbank, and between the two femoralis clades downstream on the same riverbank

bank (site 7). Estimates averaged between all loci maintain a similar pattern (Table 4). Heterozygote deficit within sampling sites (estimated as Fis) was relatively pronounced at Jirau (Table 4), approximately 1.5 km upstream the core area of the contact zone, and corresponding to a predominantly *A. hodli* population (see below). At this site, a large number of private alleles are also found, in comparison to adjacent sampling sites. Fst values between sampling sites generally indicated genetic structuring among populations, generally ranging from 0.08 to 0.20 (Table 5). Lower Fst values were observed only among some adjacent sampling sites, and between samples from the right bank at Humaitá (site 9) and sampling sites at Lower Jirau, Jaci-Paraná and Humaitá (sites 5, 6 and 8, respectively), all located on the left bank (Fig. 1).

Based on the average between 15 iterations run in STRUCTURE, the posterior probability among alternative numbers of clusters plateaus at $K = 3$ (Fig. 6), with an abrupt decrease in the magnitude of likelihood change from $K = 3$ to $K = 4$. Selecting three as the actual number of genetic clusters rendered geographic distribution of clusters based on microsatellite markers similar to that obtained with mtDNA data (as suggested by BAPS analysis described above) (Fig. 7). One of the clusters is constituted by *A. hodli* samples, and is distributed from Abunã (site 1) to Jirau (site 3), experiencing admixture with one of the two *A. femoralis* clusters at the Contact Zone. Considering the two *A. femoralis* clusters, one is restricted to the left riverbank, from the Contact Zone to Jaci-Paraná, and the second occurs downstream on the same riverbank, as well as in all sampling sites on the right riverbank. As the two clusters meet at Jaci-Paraná, we removed three samples that had proportions of membership (q_i) to the downstream/right bank cluster superior to 90 % from the Jaci-Paraná pool. The remaining 12 individuals from Jaci-Paraná were used in the subsequent analysis of hybridization as a sample of pure parental *A. femoralis* genotypes. Importantly, no evidence of recent introgression or admixture from the right to the left riverbank was evident upstream of Jaci-Paraná, neither from mtDNA or from microsatellite markers.

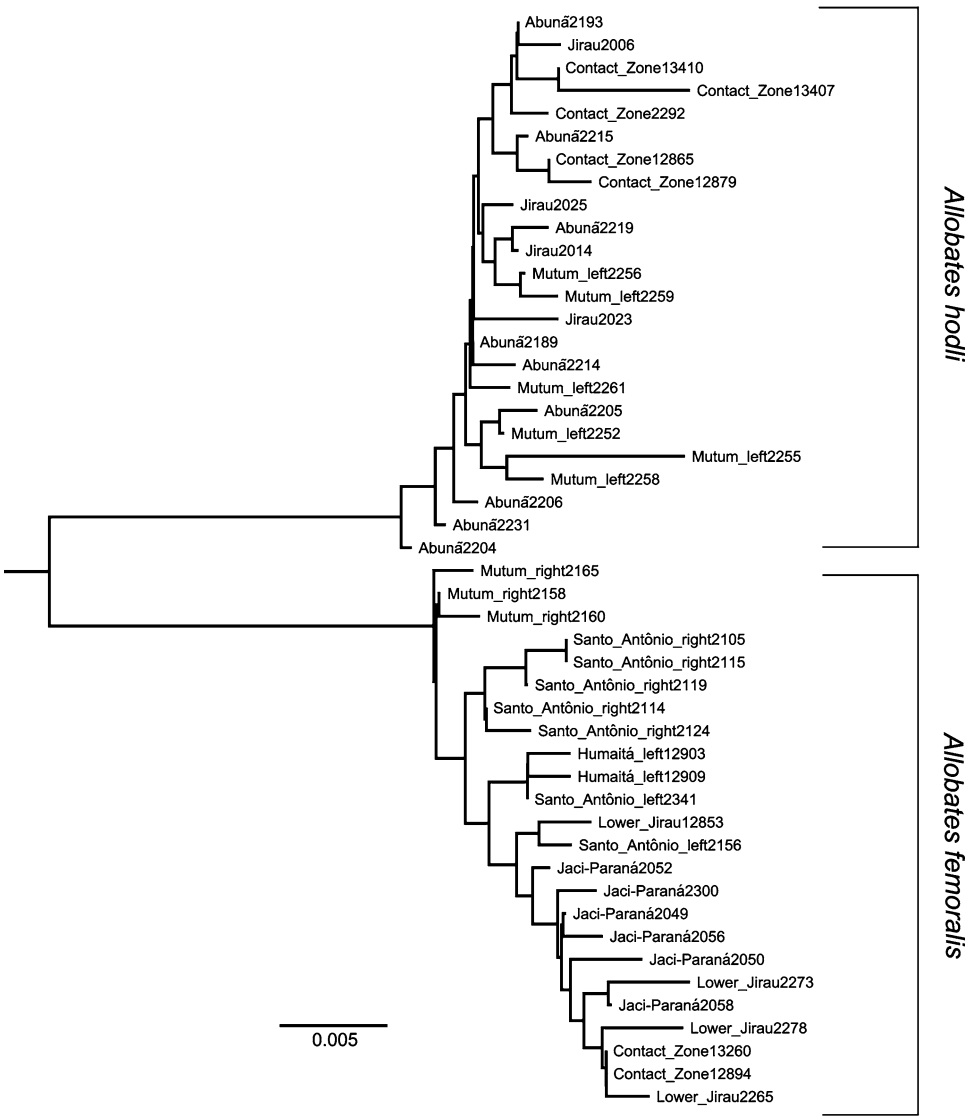

Fig. 5 Neighbor-Joining tree constructed from 48 unique *A. hodli*/*A. femoralis* 16S rDNA haplotypes found along the study area. The two species for monophyletic groups, with samples collected in a contact zone distributed among both clades. *Tip labels* correspond to sampling site followed by tissue collection number. Basal clades are highly supported (bootstrap value = 99 %)

Table 3 Characteristics of the four microsatellite loci described by Jehle et al. (2008) for *A. femoralis* used in this study, sampled from total 195 individuals of *A. femoralis* and *A. hodli* collected in the study area along the upper Madeira River

Locus	Repeat motif	No. of alleles	Allele size range	% of rare alleles (freq. <0.05)	Observed heterozygosity	% missing data[a]
Epifem 03	$(GATA)_{11}$	29	188–294	0.65	0.653	0.01
Epifem 05	$(CATA)_3(AT)_3(AC)_{18}$	11	102–122	0.54	0.241	0.02
Epifem 12	$(TATC)_{15}$	40	134–210	0.92	0.575	0.01
Epifem 13	$(CTAT)_{20}$	49	206–334	0.91	0.774	0.00

[a] Measured as the number of individuals lacking information for the referred locus from a total 195 individuals genotyped

Hybridization analysis

Hybridization analyses were performed on 145 individuals from six sampling sites on the left riverbank. Individuals from Abunã and Mutum-Paraná (sites 1 and 2) were considered pure parental *A. hodli* populations ($n = 34$), while individuals from Jaci-Paraná ($n = 12$) were considered pure *A. femoralis*. The analyses focused on the remaining individuals ($n = 99$), sampled across the contact zone from Jirau to Lower Jirau (sites 3–5).

Bayesian admixture analysis conducted in STRUCTURE revealed a steep trend in the average proportion of species membership associated to each population from Jirau to Lower Jirau, largely concentrated in the core area of the

Table 4 Average microsatellite diversity (±SE) and estimate of heterozygote deficit (Fis) obtained from four microsatellite loci of *A. hodli* and *A. femoralis* from 11 sampling sites along the upper Madeira River

Site	Na	Ne	Np	He	Fis
1	11.250 ± 2.496	7.454 ± 1.922	0.250 ± 0.250	0.790 ± 0.101	0.274
2	9.500 ± 1.893	6.298 ± 1.448	0.000 ± 0.000	0.785 ± 0.083	0.122
3	11.750 ± 2.287	5.759 ± 1.525	1.750 ± 1.109	0.781 ± 0.058	0.431
4	13.000 ± 3.697	8.114 ± 2.422	0.250 ± 0.250	0.809 ± 0.085	0.264
5	13.500 ± 3.697	7.879 ± 2.373	1.250 ± 0.479	0.726 ± 0.173	0.274
6	10.500 ± 2.723	6.909 ± 2.524	1.000 ± 0.408	0.736 ± 0.129	0.292
7	4.500 ± 0.645	3.462 ± 0.527	0.000 ± 0.000	0.689 ± 0.049	0.344
8	10.250 ± 2.810	6.813 ± 1.685	0.750 ± 0.479	0.797 ± 0.080	0.289
9	7.000 ± 1.871	5.532 ± 1.480	1.750 ± 1.109	0.737 ± 0.111	0.343
10	6.750 ± 0.854	5.069 ± 0.588	0.500 ± 0.289	0.795 ± 0.023	0.355
11	8.250 ± 1.436	5.207 ± 1.202	1.250 ± 0.479	0.735 ± 0.107	0.267

Na number of alleles, *Ne* number of effective alleles, *Np* number of private alleles, *He* expected heterozygosity

Table 5 Fst values between 11 sampling sites along the upper Madeira River based on Weir and Cockerham estimators, obtained from four microsatellite loci of *A. hodli* and *A. femoralis*

		1	2	3	4	5	6	7	8	9	10	11
1	Abunã	0.0000										
2	Mutum (L)	0.0280	0.0000									
3	Jirau	0.0835*	0.0800*	0.0000								
4	Contact Zone	0.0965*	0.0846*	0.0898*	0.0000							
5	Lower Jirau	0.1885*	0.1823*	0.1848*	0.0402*	0.0000						
6	Jaci-Paraná	0.1765*	0.1728*	0.1801*	0.0639*	0.0471*	0.0000					
7	St. Antônio (L)	0.1913*	0.1871*	0.2012*	0.1561*	0.1839*	0.1449*	0.0000				
8	Humaitá (L)	0.1397*	0.1307*	0.1551*	0.0742*	0.0788*	0.0676*	0.1138*	0.0000			
9	Humaitá (R)	0.1559*	0.1624*	0.1668*	0.0702	0.0597	0.0375	0.1013	0.0424	0.0000		
10	St. Antônio (R)	0.1281*	0.1309*	0.1161*	0.1080*	0.1574*	0.1268*	0.1451*	0.0908*	0.0893	0.0000	
11	Mutum (R)	0.1624*	0.1353*	0.1802*	0.1673*	0.2084*	0.1832*	0.1994*	0.0970	0.1672*	0.1094*	0.0000

Sampling sites 1–3 correspond to *A. hodli* samples, while sites 5–11 are considered to be exclusively *A. femoralis*. Significant population differentiation was observed between all sampling sites at the level of 0.01 after 50,000 permutations. Asterisks point significant genetic differentiation after standard Bonferroni correction was applied to comparisons

(R) and (L) correspond to right and left riverbanks, respectively, for sites with the same denomination. Numbers correspond to site locations and coordinates as presented in Fig. 1 and Table 1

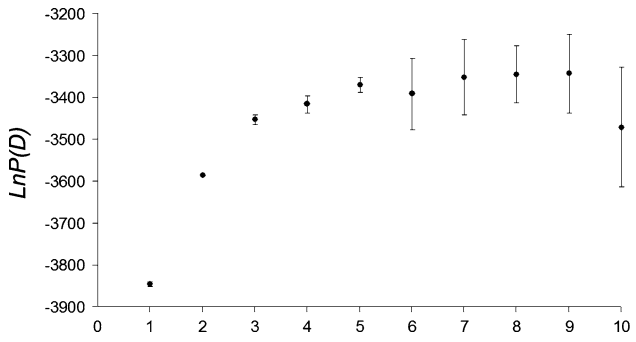

Fig. 6 Posterior probability of data according to the possible number of genetic clusters (K = 1–10, x axis) formed by samples of *A. hodli* and *A. femoralis* from 11 sampling sites along the upper Madeira River. *Signs* for each value of K represent the arithmetic mean and standard deviations between 15 iterations run in STRUCTURE 2.3.3

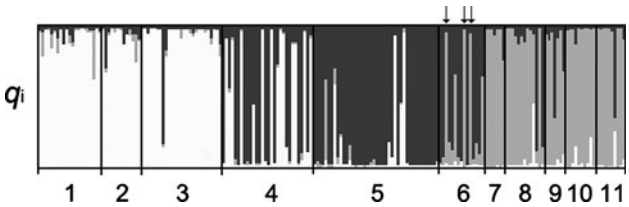

Fig. 7 Barplot of membership coefficients (q_i) obtained in STRUCTURE assigning samples of *A. hodli* and *A. femoralis* to three genetic clusters inferred from data on four microsatellite loci. The two *darker* clusters represent *A. femoralis* clusters (see text). *Numbers* on x axis refer to sampling localities presented in Table 1 and Fig. 1. *Arrows* indicate individuals removed from the Jaci-Paraná sample in posterior analyses of hybridization

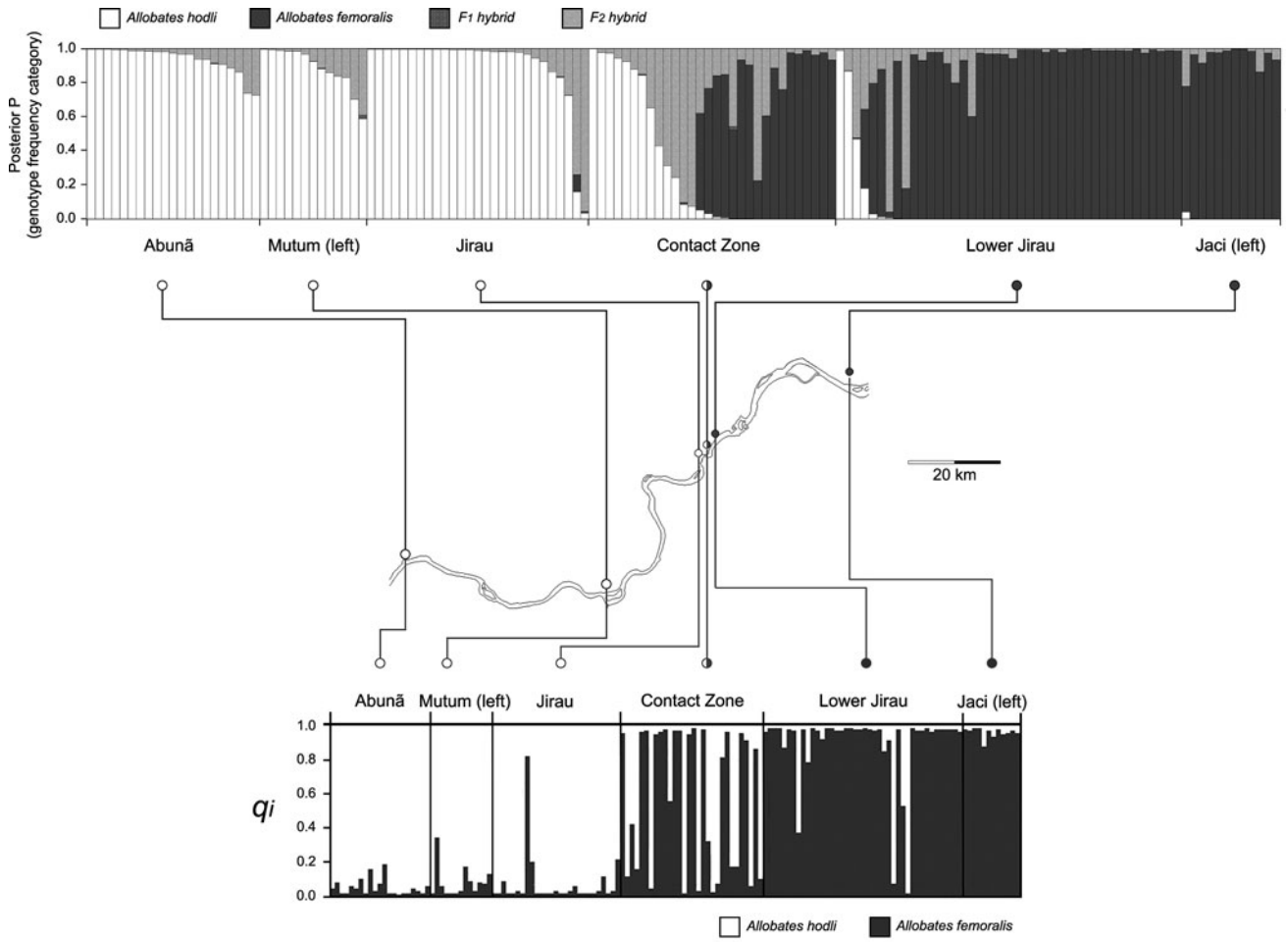

Fig. 8 Barplots of hybrid category assignments generated by NewHybrids (*upper graph*) and membership coefficients generated by Structure (*lower graph*). Each vertical column on both *graphs* represents one of 145 individuals of *A. hodli* and *A. femoralis* originating from six sampling sites on the left bank of the upper Madeira River (*center*). For the NewHybrids analysis, Abunã and Mutum were assigned as containing pure *A. hodli* individuals, while Jaci was regarded as a pure *A. femoralis* sample. Both analysis support the hybrid zone as strongly bimodal, with few putative hybrids greatly restricted to the core of the contact zone. Frequency of F_1 hybrids is almost negligible in comparison to F_2 hybrids (but see text)

contact zone (site 4). Proportions of membership to one of the two species estimated from overall samples at this core area were almost equivalent, indicating a high level of genetic admixture. Estimated average proportions of membership to *A. hodli* (Q_h) increases abruptly upstream, while membership to *A. femoralis* (Q_f) increases on the opposite direction. Average individual membership coefficients highlight the presence of extensive admixture in the core area of the contact zone, with introgression of only a few individuals bearing genotypes attributed to the alternate species at adjacent sites downstream and upstream (Fig. 8).

The NewHybrids analysis confirmed the presence of possible hybrid individuals in the core area of the contact zone (five of 30 individuals with >50 % posterior probability of assignment to hybrid categories at Contact Zone site), but the frequency of individuals bearing hybrid genomes decreases abruptly at immediately adjacent sampling sites (Fig. 8). Among 10 females genotyped from the core area of the contact zone, only one had a posterior probability superior to 40 % of belonging to a hybrid class. Among the 20 males collected at the same site, six (30 % of total males) surpassed this threshold.

All hybrids were strongly attributed to F_2 genotypic class, and are consequently considered to be more closely related to parental genotypes than expected for F_1 generations. The contact zone has a clear bimodal pattern, with a few individuals presenting high probabilities of bearing intermediate genotypes, and parental genotypes being frequent even in the contact zone's core (Fig. 8). The analyses

assigned a 60 % probability of a pure *A. femoralis* origin and 40 % probability of a F_2 hybrid origin to the *A. femoralis* male found to bear an *A. hodli* haplotype collected at Lower Jirau (APL-2276).

Discussion

Results from genetic analyses across the contact zone between *A. hodli* and *A. femoralis* on the left riverbank of the upper Madeira River suggest that it conforms better to a tension zone model than to a case of insipient hybrid swarm or to a clinal model with gradual replacement of genetic characteristics from one species towards the alternate species range (Barton and Hewitt 1985). As typical of such tension zones, genetic admixture and hybridization between the two species is greatly restricted to the core area of the contact zone (namely, to sampling site 4). This is reflected in local genetic diversity estimates, as indexes based on nucleotide and allele diversity increase at the core zone as a result of admixture between genomes of both species. On the other hand, estimates based on haplotype diversity and heterozygosity indicate reduced diversity immediately downstream and severe heterozygote deficit upstream of this area, supporting the existence of selective pressures preventing gene flow past the areas adjacent to the core zone.

A single case of mtDNA introgression was observed from *A. hodli* towards the distribution of *A. femoralis* at Lower Jirau (site 5), where one *A. femoralis* male carried an *A. hodli* haplotype. This represents a frequency of less than 4 % of introgressed *A. hodli* haplotypes into *A. femoralis* distribution, only 1.5 km away from the core hybrid zone. This individual was subsequently assigned to a *A. femoralis*/F_2 hybrid origin by analysis of microsatellite markers, and probably results from a considerable number of backcrosses involving hybrid individuals and *A. femoralis*. Haplotypes characteristic of *A. hodli* prevail in frequency and richness at the core area, while no *A. femoralis* haplotypes are found upstream (and consequently within *A. hodli* geographic distribution).

Narrow contact zones with a bimodal pattern of genotypic distribution are usually related to prezygotic barriers to gene flow, mediated by assortative mating or fertilization (Jiggins and Mallet 2000). Strong prezygotic female choice for conspecific males is a phenomenon common to anuran hybrid zones, which are often characterized by marked character displacement of call traits, and its reinforcement driven by sexual selection (Höbel and Gerhardt 2003; Pfennig 2003; Hoskin et al. 2005). Although the artificial manipulation of advertisement calls are known to have effects on male to male aggressive behavior in the *A. femoralis* group (Hödl et al. 2004; Göd et al. 2007), playback experiments broadcasting natural calls within territories of *A. femoralis* and *A. hodli* males along the Maderia River contact zone detected no differences in aggressive (phonotactic) behavior towards conspecific or heterospecific calls (Erdtmann et al. 2011). Tests addressing female mate choice are still needed in order to corroborate the existence and the strength of a behavioral reproductive barrier. However, the available evidence obtained so far from male response to playback experiments, and the presence of hybrids along the contact zone, suggest that any behavioral prezygotic barriers between the parental species are, at least, leaky.

Thus, current data offer better support to the hypothesis that the maintenance of the current contact zone is related to postzygotic isolation mechanisms. Among these, the existence of genetic incompatibilities over multiple loci, or reduced fitness of F_1 hybrids (which are apparently rare across the contact zone area) are examples of possible intrinsic and extrinsic factors regulating contact zone position and width. For instance, *A. femoralis* is considered to be a color mimic of sympatric poison-frogs (Darst et al. 2006), and color differences between *A. femoralis* and *A. hodli* are conspicuous (Simões et al. 2010). Ongoing research supports the existence of spectrum overlap in body color between these species and the sympatric poison-frogs *Ameerega picta* and *Adelphobates quinquevittatus* from Jirau to Lower Jirau (Amézquita et al. unpublished data). Thus, hybrid individuals presenting intermediate color patterns might be more susceptible to predation by visually oriented predators.

While a reduced number of polymorphic genetic markers are generally sufficient to point out the existence of hybrid individuals along a contact zone, discrimination between alternate hybrid classes will often demand many more markers. Particularly, the distinction between parental populations and backcrosses might require several dozens (Boecklen and Howard 1997).

Although fitness can vary between hybrid generations, and reduced F_1 survival does not necessarily imply in absence of F_2 hybrids, hybrid classification according to NewHybrids should be viewed with caution due to the small number of microsatellites employed. This analysis is conservative in the sense that hybrids are classified according to genotype frequencies among all loci expected under Mendelian laws of inheritance (Anderson and Thompson 2002). Thus, although useful for revealing the frequency and extent of hybridization between both study species along the left bank of the upper Madeira River, these results allow us to make no strong assumptions about genetic bottlenecks affecting particular hybrid generations, as some F_1 hybrids might have been misclassified as F_2.

It is important to stress that all individuals collected in the core area of the contact zone were adults: males

emitting advertisement calls and females carrying mature oocytes. Most of the individuals to which considerable probability of belonging to a hybrid class was attributed were males (probabilities larger than 50 % of belonging to a hybrid class were attributed to five out of 20 males, and one out of 10 females). These observations suggest possible sexually-related trends on hybridization dynamics, such as increased viability or survival of hybrid males. Future analysis including sex-linked genetic markers will be useful for clarifying these trends. A broader array of neutral markers should also be applied to precisely confirm the assignment of individuals to distinct hybrid classes, ruling out more elaborate hypothesis such as hybrid breakdown by unviable admixture of genetic backgrounds among F_2 or backcross progeny (Burton et al. 2006).

Conservation implications

Natural or human induced environmental changes can rapidly shift the prevailing balance between gene flow from parental populations and localized selection against hybrids along narrow hybrid zones by their effects on available resources and/or population density (Grant and Grant 1992, 2002; Haig et al. 2004; Keller et al. 2008; Genovart 2009). Currently, the *A. hodli*/*A. femoralis* hybrid zone on the left bank of the upper Madeira River seems to be stabilized by selective pressures against hybrids or genetic incompatibility mechanisms, being restricted in width to less than three kilometers, largely coincident with the transition zone between distinct geomorphological compartments.

Contemporary developmental projects have been increasing along the upper course of the Madeira River, among which the Santo Antônio and Jirau dams stand out (Clemons 2007). The area supposedly affected by the latter overlaps with the location of the *A. hodli*/*A. femoralis* hybrid zone studied herein. Apart from the direct effects of dam building on populations inhabiting the vicinities of the current Contac Zone, power-line and road systems associated with these power plants will much probably induce fast human colonization along this entire section of the Madeira River basin (Laurance et al. 2004; Perz et al. 2008). Habitat loss or micro-climatic alterations following changes in land use could break the ongoing balance described for the *A. hodli*/*A. femoralis* hybrid zone, for example, by altering the distribution or availability of tadpole rearing sites (temporary rain puddles in clay soil).

Early reports of these findings were addressed to Brazilian environmental agencies and companies responsible for building and operating the Madeira river dams, which settled permanent sampling grids along the impacted area. One of them consists of two 5 km parallel trails, 1 km distant from each other, and perpendicular to the Madeira River, covering the core area of this hybrid zone. Sampling grids follow specifications of the Program for Biodiversity Research—PPBio (http://ppbio.inpa.gov.br) and will be used for regular surveys for at least 2 years after flooding. During this time, *A. femoralis* and *A. hodli* populations can be re-sampled in order to evaluate immediate changes in hybrid and parental genotype frequencies along this system, as well as effects on genetic diversity parameters.

Thus, our results represent a valuable record with direct application in monitoring short-term effects of the recently established power plant systems and human-induced environmental changes on a well-delimited evolutionary system.

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