

Partitioning of molecular variation at local spatial scales in the vulnerable neotropical freshwater turtle, *Hydromedusa maximiliani* (Testudines, Chelidae): implications for the conservation of aquatic organisms in natural hierarchical systems

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Abstract

Hydromedusa maximiliani is a vulnerable freshwater turtle endemic to mountainous regions of the Atlantic rainforest in south-eastern Brazil. Random amplified polymorphic DNA (RAPD) were surveyed with the purpose of assessing the genetic structure and determining the partitioning of molecular variation in *H. maximiliani* across the natural spatial hierarchical scale of its habitat. The goal of the study was to integrate ecological data with estimates of molecular genetics diversity to develop strategies for the conservation of this freshwater turtle. Specimens were sampled from rivers and streams across three drainages. Nine of the 80 primers used generated 27 scoreable bands of which 10 (37%) were polymorphic and produced 16 RAPD phenotypes. Significant heterogeneity was found in the distribution of RAPD molecular phenotypes across the three drainages. Analysis of molecular variance for molecular phenotypes showed that the heterogeneity had a spatial structure since a significant amount (22%) of the total variance was attributable to variation among rivers and streams. Since the genetic variation of this turtle seems to be structured according to the natural hierarchical system of rivers and streams within drainages, it is suggested that local populations should be considered as separate management units. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The degradation and fragmentation of natural ecosystems has produced landscape islands, with the populations in such areas becoming small because of the reduction in the species' original distribution (Primack, 1998). In species with small populations resulting from habitat fragmentation, stochastic factors (demographic and environmental) assume considerable importance in

the population dynamics and in the species survival (Templeton et al., 1990; Lacy, 1993; Primack, 1998). Understanding the ecological and demographic patterns which drive population dynamics is therefore fundamental for conservation efforts (Caughley, 1994), particularly for long lived organisms such as turtles (Dunham et al., 1989; Congdon et al., 1994). Because of their limited dispersal capabilities (exception for marine and some freshwater turtles; Pritchard and Trebbau, 1984; Nichols et al., 2000; Valenzuela, 2001) and their often specific habitat requirements (Pritchard and Trebbau, 1984; Ernst and Barbour, 1989; Cabrera, 1998), turtles are highly vulnerable to becoming

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restricted to habitat-isolated populations with little or no gene flow between them.

Hydromedusa maximiliani is endemic to the Atlantic forest of the coastal region of eastern Brazil and ranges from the State of Espírito Santo to the State of São Paulo (Ernst and Barbour, 1989; Iverson, 1992). The Atlantic forest habitat of *H. maximiliani* has been severely disturbed by humans since the sixteenth century, and this has resulted in extensive fragmentation of the tropical mountain rain forest (Dean, 1996; Chiarollo, 1999; Tabarelli et al., 1999). Throughout its range the species distribution is disjunct, occurring in isolation at elevations above 600 m. Locally, its habitat is topologically complex, with sequences of ridges and valleys each drained by river and stream systems (Souza, 1995).

The natural disjunct distribution of *H. maximiliani* across its range and the increasing fragmentation of its natural habitat, associated with a low reproductive rate and highly seasonal dynamics (Souza, 1995), makes this species particularly susceptible to demographic and environmental stochastic phenomena that may interact to cause local population extinction (McCarthy, 1996; Hanski, 1998; Legendre et al., 1999). Capture–recapture studies have shown that these turtles have a limited dispersal ability, with a mean daily displacement of 2 m. Thus, streams are occupied by the same turtles for a long time (Souza, 1995; Souza and Abe, 1997a). The dispersion or migratory behavior is limited to the rainy season, when temporary or intermittent water systems are connected with the main water courses (Souza and Abe, 1997a). This limited dispersion suggests that each population of *H. maximiliani* inhabits a specific river within a given drainage system, and therefore exists as a metapopulation (sensu Hanski and Simberloff, 1997). The population dynamics of *H. maximiliani* associated with its habitat characteristics led Souza and Abe (1997a) to define the conservation status of this species as Vulnerable.

Except for the studies of population ecology reviewed above, very little is known on any other aspects of the biology of this species. In this paper, we used RAPD markers (Williams et al., 1993) to investigate the genetic variability in *H. maximiliani* sampled according to the drainage systems typical of the species habitat. Molecular markers targeted by RAPD have been used increasingly to determine the patterns of genetic variation within populations and to partition genetic variation among populations of vertebrate species (Gibbs et al., 1994; Haig et al., 1994; Gibbs, 1998; Mockford et al., 1999; Cooper, 2000; Vucetich et al., 2001). As emphasized by Haig et al. (1994), appropriately sampled RAPD molecular phenotypes can be conveniently tested for homogeneity across populations, and molecular variation can be partitioned into hierarchical levels to yield information on variation and population structure, with important implications for evolutionary and

conservation biology. Here, measures of genetic subdivision were used to determine the partitioning of variation in *H. maximiliani* across the natural spatial hierarchical scale of its habitat, which involved drainages and rivers and streams within drainages. The goal of the study was to integrate ecological records with estimates of molecular genetics diversity to develop strategies for the conservation of *H. maximiliani*.

2. Methods

2.1. Sample collection

Field work was conducted from November 1998 to November 1999 at the Parque Estadual de Carlos Botelho (PECB), state of São Paulo, southeastern Brazil (24°00'–24°15' S, 47°45'–48°10' W). The PECB is a protected reserve that encompasses over 37,000 ha of intact tropical mountain rain forest typical of southeastern Brazil (Whitmore, 1990; Veloso et al., 1991). The region is topologically complex, with ridges and valleys drained by numerous rivers and streams (Pfeifer et al., 1986; Souza and Abe, 1998).

During this study an area of approximately 2700 ha containing three drainages was sampled based on the natural spatial hierarchy formed by the rivers and streams. The three drainages are referred to as I, II, and III (Fig. 1). Within each drainage, specimens of *H. maximiliani* were hand-caught in shallow rivers and streams (Fig. 1). In this Figure, each dot corresponds to the collecting site of each individual. The sampling effort was not equal in the three drainages because of difficulties in reaching rivers and streams in drainages II and III. The number of individuals obtained for this study in each drainage were: drainage I ($n=25$), drainage II ($n=8$), and drainage III ($n=11$).

From each individual, 200–300 μ l of blood was drawn from the scapula vein/brachial artery (Avery and Vitt, 1984) using a 26-gauge needle and a 1-ml syringe. The blood samples were immediately preserved in plastic vials containing 1 ml of absolute ethanol (Miyaki et al., 1998) and stored at room temperature. All turtles not already individually identified by marginal scute notches used in earlier studies (Souza, 1995; Souza and Abe, 1995, 1997a,b, 1998) were marked at the moment of blood sampling as part of a long-term study. The turtles were released at the point of capture after blood sample collection.

2.2. DNA extraction, primer selection, and RAPD-PCR amplifications

Genomic DNA was extracted from the blood samples by two successive organic extractions with phenol:chloroform using the protocol outlined by Bruford et al.

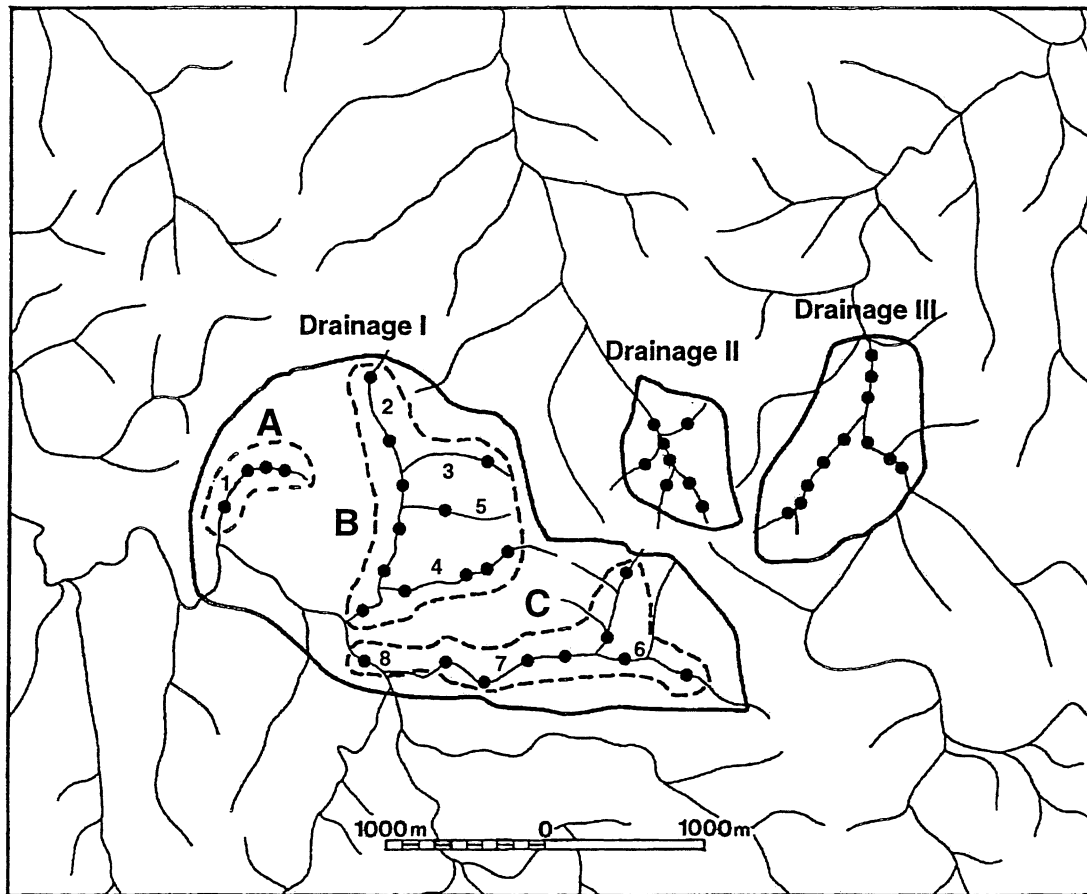


Fig. 1. Map of the study site at Parque Estadual de Carlos Botelho, state of São Paulo, southeastern Brazil, showing the three drainages and rivers sampled for *Hydromedusa maximiliani*. Dots indicate the collecting site of each individual. For drainage I, numbers 1–8 indicate rivers surveyed while dashed lines indicate the three samples sites (A, B, and C) of samples pooled for AMOVA (see text).

(1992) and Miyaki et al. (1998), and then precipitated with 1/10 volume of 1 M sodium acetate (pH 5.3) and two volumes of 100% ethanol. Approximately 100 μ l of blood/ethanol solution from each turtle were placed in Eppendorf tubes containing 300 μ l of 1X TNE (50 mM Tris-HCl, 100 mM NaCl, 6.3 mM EDTA, pH 7.5), 30 μ l 1 M Tris-HCl pH 7.5, 20 μ l of proteinase K (25 mg/ml) and 8 μ l of 25% SDS, and kept at 37 °C for 12 h. The DNA pellet was washed in 70% ethanol and allowed to dry for 5 min before redissolving in 100–150 μ l of sterile. The quality of the extracted DNA was evaluated in agarose gels (0.7%) stained with ethidium bromide and quantified by comparison with DNA standards run in the same gel. DNA was diluted to a working concentration of approximately 0.6 ng/ μ l.

The primers (Operon Technologies, Inc.; Alameda, California, EUA) were selected using a two-step procedure. Initially, 80 primers (Kits A, C, G, N; 20 primers per kit) were surveyed in side-by-side duplicate reactions for two turtles to identify primers that yielded a reproducible and scoreable banding pattern. Only consistently well amplified bands were screened and variation in intensity was ignored. This first approach

revealed nine primers meeting these criteria. These nine primers were used to analyze the full set of DNA samples from the 44 individuals captured in the studied area.

The polymerase chain reaction (PCR) was done in a Perkin-Elmer GeneAmp™ PCR system 9700 with a total volume of 12.5 μ l containing 10 mM Tris HCl (pH 8.4), 50 mM KCl, 3.5 mM MgCl₂, 1 μ l of each dNTP, 2 μ l of primer, 0.3 μ l *Taq* polymerase, 1.2 ng of genomic DNA and sterile water. Negative controls in which DNA was substituted for water were run to check for the possibility of contamination. Reproducibility was assessed by comparing duplicate reactions, which were usually adjacent to one another in the thermocycler, and products were run side-by-side on the same gel. The reactions condition involved: initial denaturation of DNA for 2 min at 94 °C, 39 cycles of 1 min denaturation at 94 °C, 1 min annealing at 40 °C, 2 min extension at 72 °C, and one 5 min cycle at 72 °C for final extension. The amplification products were separated on 1.3% agarose gels stained with ethidium bromide, run in 1X TAE buffer at 80 V for 5 h. Monochrome photographic negatives were taken of the gels and the

individual profiles were scored by two of us (FLS and AFC) for the presence/absence of fragments for each primer.

2.3. Statistical analyses

Statistical analyses were done based on the spatial hierarchy (rivers and streams within drainages, and among drainages) of sampling defined by the topographic characteristics of the *H. maximiliani* habitat. The null hypothesis that the distribution of RAPD molecular phenotypes was homogeneous across the three drainages was tested with Fisher's exact test because of the small number of individuals in some cell frequencies in the contingency tables (Sokal and Rohlf, 1981). The heterogeneity in RAPD phenotype distribution among rivers and streams within each drainage was also verified with Fisher's exact test and, whenever appropriate, sequential Bonferroni adjustment for multiple comparisons was applied (Sokal and Rohlf, 1981). The partitioning of molecular variation at the hierarchical levels for which significant departures from the null hypothesis were detected was done by the analysis of molecular variation (AMOVA; Excoffier et al., 1992). The theoretical foundation for AMOVA as derived by Excoffier et al. (1992) is framed in the context of *U*-statistics (Hoeffding, 1948; Pinheiro, 1997), and is based on the fact that the sum of squares in conventional analysis of variance can be written as the sum of squared distances between pairs of phenotypes. The distance between individual RAPD molecular phenotypes used here was a simple Euclidean metric and was calculated by summing the squared differences of pairwise vectors of zeros (band absent) and ones (band present) over all polymorphic loci (Excoffier et al., 1992). The phenotypic distances were partitioned into components of variance in the AMOVA framework and the Φ statistics (Excoffier et al., 1992) was calculated from the variance component representing variation at the hierarchical levels defined by the sampling design for this study. Fisher's exact test was computed with PROC FREQ of SAS (SAS/STAT, 1989) and the AMOVA was computed with Arlequin ver 1.1 (Schneider et al., 1997).

3. Results

Nine of the 80 primers used yielded band patterns that were clear and could be scored with confidence (Table 1). These primers produced 27 potentially scoreable bands (range 1–6; mean 3), of which 10 bands (37%) were polymorphic and produced 16 RAPD phenotypes. Twelve of the 16 phenotypes were represented in the 25 individuals sampled from drainage I, and eight phenotypes were found only in the rivers and streams of this drainage. Five phenotypes were represented in the

eight individuals sampled from drainage II, of which one phenotype occurred only in this drainage. Five phenotypes were represented in the 11 individuals sampled from drainage III, of which three phenotypes were found only in this drainage. Three phenotypes were common to all three drainages.

The null hypothesis that RAPD molecular phenotypes were distributed homogeneously across drainages was rejected by Fisher's exact test for comparison of drainages I, II, and III simultaneously ($P < 0.000656$). To determine which of the three drainages differed significantly in the distribution of RAPD phenotypes, pairwise comparisons between the three drainages were done with Fisher's exact test. The appropriate level of significance that controls for Type I error was obtained through sequential Bonferroni adjustment for multiple comparisons by dividing our α level of 0.05 by the number of comparisons (3), which yielded an adjusted significance level of $P < 0.016$. Comparison of drainages I and II, and of drainages II and III resulted in non-significant differences (P -values of 0.095 and 0.263, respectively), whereas drainages I and III differed significantly ($P < 0.000195$) in the distribution of RAPD molecular phenotypes.

The organization of variation in molecular phenotypes at the level of rivers and streams within drainages was also evaluated with Fisher's exact test. A deviation from the null hypothesis of homogeneous distribution of phenotypes among rivers and streams was found for drainage I ($P < 0.015$), whereas for drainages II and III the results were not significant ($P = 0.871$ and $P = 0.558$, respectively).

Drainage I, which gave a statistically significant result in Fisher's exact test, was investigated with AMOVA in order to provide insight into the partitioning of RAPD phenotypic variation among rivers and streams in drainage I. Since some rivers in drainage I were sampled for only a few individuals, samples were pooled according to the spatial hierarchy of the main rivers and their tributaries, resulting in three sample sites, as follows: sample A including river 1, sample B including rivers 2, 3, 4, and 5, and sample C including rivers 6, 7, and 8 (Fig. 1). For this sampling design, the phenotypic AMOVA analysis was used to derive variance compo-

Table 1
RAPD primers, their sequences, and the size (molecular weight) of the polymorphic marker bands generated for *Hydromedusa maximiliani*

Primer	Sequence (5'–3')	Polymorphic bands
OPA07	GAAACGGGTG	625
OPA09	GGGTAACGCC	425
OPA10	GTGATCGCAG	425, 475
OPA20	GTTGCGATCC	450
OPN01	CTCACGTTGG	725, 750
OPN09	TGCCGGCTTG	550, 850, 950

nents and to calculate the value of Φ_{ST} that quantifies the level of variation among sample areas A, B, and C. The level of Φ_{ST} was statistically significant ($P < 0.00684$) and accounted for 22% of the total variance in the molecular phenotypes among rivers and streams within drainage I (Table 2).

To gain further insight into the pattern of molecular variation in drainage I the relationship between pairwise RAPD phenotype distances, computed as Euclidean distances squared (Rohlf, 1994) and spatial distance, measured in river distance (measurements taken with a swivel handle map measurer), was assessed with Mantel's (1967) test using NTSYS-pc (Rohlf, 1994). The correlation between spatial and molecular phenotype distances ($r = -0.125$) for all individuals sampled in drainage I was not significant ($t = -1.228$; $P = 0.110$). Mantel's test was also applied to all individuals samples in the three drainages. The pairwise RAPD phenotype distances were calculated as above, whereas the geographic distances were calculated as great circle (or straight-line) distances because rivers in different drainages are not connected. The correlation between spatial and molecular phenotype distances ($r = 0.068$) for all individuals sampled in drainages I, II, and III was also not significant ($t = 1.201$; $P = 0.885$).

4. Discussion

The level of polymorphism of *H. maximiliani* (37%), based on the RAPD molecular phenotypes observed in this study, was eightfold higher than that reported for Blanding's turtle, *Emydoidea blandingii* (4.5%), the only turtle species for which RAPD data are available (Mockford et al., 1999). Mockford et al. (1999) suggested that the low polymorphism detected in *E. blandingii* was not surprising since early studies with turtles had shown low rates of mutation in both mitochondrial (Avise et al., 1992; Bowen et al., 1993) and nuclear DNA (FitzSimmons et al., 1995). Mockford et al. (1999) also regarded the low levels of polymorphism in

E. blandingii to be characteristic of Testudines. Estimates of polymorphism based on RAPD markers are sensitive to a certain level of subjectivity during the scoring of polymorphic bands (Grosberg et al., 1996), and this limits the effectiveness of comparisons across studies. Nevertheless, the data obtained here for *H. maximiliani* indicated that the levels of polymorphism in Testudines, as measured by RAPD molecular phenotypes, may not necessarily be as low as currently thought (see also Seddon et al., 1998). This finding is all the more remarkable considering the fine spatial scale of sampling of *H. maximiliani* which involved distances of about 5 linear (airline) km, whereas for *E. blandingii* the sampling scale ranged over 1000 km.

There were significant differences in the distribution of RAPD phenotypes in *H. maximiliani* between drainages and among rivers and streams within drainages. At the spatial scale of drainages there was also significant heterogeneity in the distribution of phenotypes between drainages I and III, which were located further apart (Fig. 1). The distribution of RAPD molecular phenotypes deviated from the null hypothesis of homogeneity at the scale of rivers and streams within a drainage. This result was found only for drainage I and may reflect the fact that this drainage had the largest sample size, which would increase the power of the statistical test. The AMOVA results for the three sampling areas within drainage I produced a significant estimate of Φ_{ST} , providing genetic evidence for population substructuring in *H. maximiliani* on very local spatial scales; that is, among rivers and streams within a drainage.

The evidence obtained for substructuring derived from molecular markers mirrors the evidence available from ecological studies. Direct estimates of dispersal for *H. maximiliani* based on mark-recapture data gathered during a 1993–1994 study (Souza and Abe, 1997a) showed that the movement of individuals was fairly restricted, with a mean daily displacement of 2 m, suggesting that turtles from each river and stream within a drainage could be structured as metapopulations (Hanski and Simberloff, 1997). In fact, individuals from which blood samples were taken for the present study in 1999 were found only a few meters from the site where they had been marked in 1993–1994.

For naturally subdivided populations such as aquatic organisms, microevolutionary processes are expected to drive the organization of genetic variability, and this may sometimes result in genetic differentiation on a local scale (Johnson & Black, 1991; Perault et al., 1997; Arnaud et al., 1999; Shaffer et al., 2000). For organisms with a sedentary nature and a low dispersal ability, such as snails, the genetic structuring of populations on small geographic scales is frequent (Johnson and Black, 1991; Arnaud et al., 1999). Although many factors, including population history and departures from the equilibrium

Table 2

Analysis of molecular variance (AMOVA) for 25 individuals of *Hydromedusa maximiliani* for drainage I in the Parque Estadual de Carlos Botelho, state of São Paulo, southeastern Brazil^a

Source of variation	d.f.	SSD	Variance	% of total variance	<i>P</i>
Among sites	2	4.308	0.19184	21.99	0.00684
Within sites	22	14.972	0.68056	78.01	0.0001

^a Populations within this drainage were pooled into three samples sites according to the spatial hierarchy of the main rivers and streams (see text). d.f., degrees of freedom; SSD, sums of squared deviations; *P*, probability of obtaining a larger variance by chance under the null hypothesis of zero variance (estimated from 1000 sampling permutations).

between drift and mutation rate, may confound the expected relationship between population structure and dispersal ability (see review in Bohonak, 1999), the general picture emerging from our study is that turtle behavior (sedentary behavior) is closely associated with habitat characteristics (a natural fragmented habitat, with mountain ridges, drainages, and rivers and streams within drainages). This relationship may limit gene flow and results in genetic structuring of the populations on a small spatial scale.

The data obtained from the AMOVA of molecular RADP markers presented here, combined with the ecological data and estimates of dispersal indicates that *H. maximiliani* populations are highly structured in close association with the topographic characteristics of this species habitat. Also it suggests that human-induced habitat fragmentation may not severely impact this species. Nevertheless fragmentation and loss of habitats certainly implies the extinction of local populations, and an understanding of patterns of population substructure combined with knowledge of ecological and natural history data is of fundamental importance for developing management strategies for insuring the long-term persistence of *H. maximiliani*.

The molecular and ecological findings reported here indicate that significant demographic and genetic processes are operating in *H. maximiliani* at the scale of rivers and streams within drainages. These results suggest that such local populations may be considered separate management units (MUs), in the sense that they harbor characteristic demographic processes and genetic variability (Moritz, 1994, 1999; Shaffer et al., 2000). The component populations of MUs are the natural units for population monitoring and demographic study and are, therefore, the target of short-term management (Moritz, 1994; Shaffer et al., 2000). Local population extinction can be counteracted by reintroductions from component populations of MUs (Moritz, 1994; Shaffer et al., 2000). In the case of the samples and geographic area we examined in this study, there was no evidence for isolation by distance in *H. maximiliani* and, therefore, the stocks for re-establishment efforts could be derived from any neighboring population.

Most research on turtles has examined molecular variation and population structure on very large spatial scales (reviewed in Walker and Avise, 1998; Avise, 2000). To our knowledge, this is the first study to document molecular variation on local spatial scales in a neotropical freshwater turtle. The pattern of population substructuring observed seems to be closely associated with the complex topography of the landscape matrix, typical of the habitat of the freshwater turtle *H. maximiliani*. Population phenomena and processes, such as the substructuring of populations on local spatial scales, are now regarded as important for process-oriented conservation approaches including species diver-

sity, ecosystems and landscapes (Moritz, 1999; Poiani et al., 2000; Shaffer et al., 2000), and the results described here could contribute to the goal of conservation of *H. maximiliani*. The scale of sampling available for this study was limited both in geographic area and sample sizes, and most of the inference regarding conservation efforts was based on one drainage for which sample size was larger. Nevertheless, the data obtained provided evidence for potential MUs within *H. maximiliani*. Additional sampling should include not only more drainages, and rivers and streams within drainages, but also the entire geographic range of *H. maximiliani*; and mitochondrial and nuclear sequence markers should also be targeted. Such information will allow the investigation of genetic structure and genealogy at larger spatial scales of sampling to verify the validity of assertions made in this study regarding current population structure, and also to search for evidence of genealogical (i.e. historical) structure. The combination of evidence from both scales, that is, population and genealogy, should provide ecological and evolutionary perspectives for conservation efforts in *H. maximilliani*.

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