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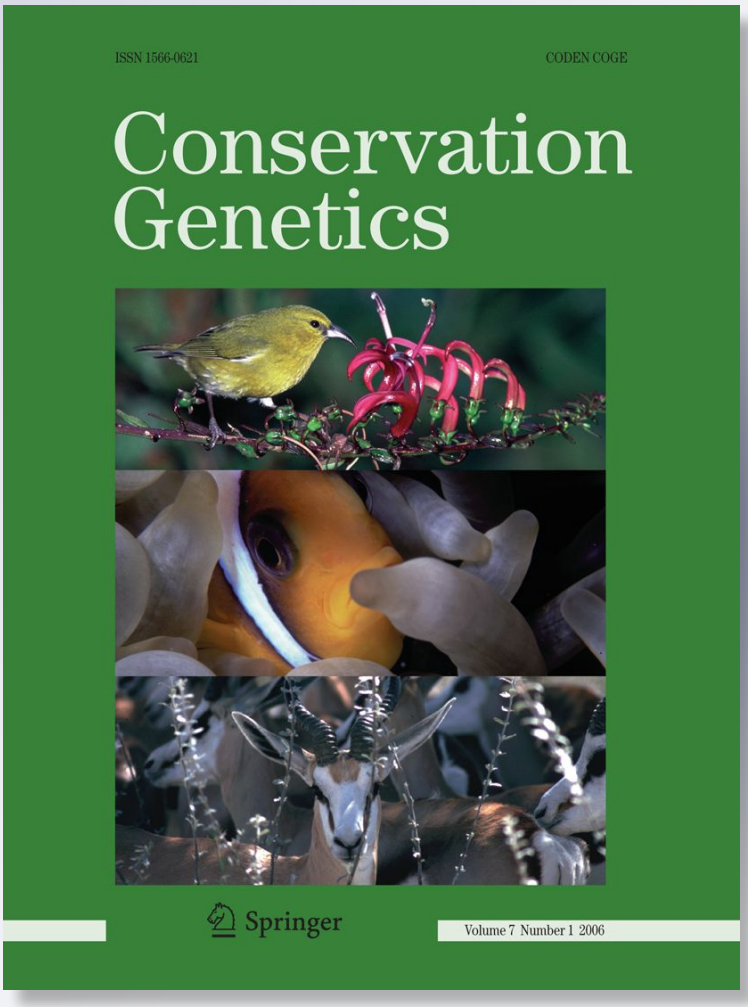

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ORIGINAL RESEARCH

Two frog species or one? A multi-marker approach to assessing the distinctiveness of genetic lineages in the Northern Leopard Frog, *Rana pipiens*

Ryan P. O'Donnell · Karen E. Mock

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Abstract A genetic boundary at the Mississippi River, USA, has been suggested for the Northern Leopard Frog, *Rana pipiens*, which was recently proposed for listing as federally threatened in the western USA. This suggestion was made on the basis of limited geographic sampling of a mitochondrial gene. However, mitochondrial DNA represents a very small part of the genome and is not necessarily indicative of patterns in nuclear DNA. We tested the hypothesis that eastern and western populations are separated by a distinct genetic boundary by sequencing mitochondrial DNA more extensively across the range, including focused sampling in the zone of hypothetical introgression, and by analyzing four nuclear sequences and seven microsatellite loci. We confirmed previous results that eastern and western populations have unique mitochondrial sequences that are deeply divergent (3.8 %) and which overlap only in a narrow region around the Mississippi River. Nuclear sequences also show divergent eastern and western lineages in some cases but with a broader zone of geographic overlap. Microsatellite data correspond closely to mitochondrial data, differing between east and west and changing abruptly near the Mississippi River. These data collectively demonstrate that eastern and western clades of this species introgress considerably in some markers but are distinct and defined by clear and narrow boundaries in others. We demonstrate that the Mississippi River forms an important, albeit somewhat permeable, boundary between genetic lineages in this species. This genetic boundary coincides with previously described discontinuities in morphological features.

Keywords *Lithobates pipiens* · Microsatellites · Northern Leopard Frog · Phylogeography · *Rana pipiens* · Subspecies · Sequencing

R. P. O'Donnell (✉) · K. E. Mock
Department of Wildland Resources and Ecology Center, Utah State University, 5230 Old Main Hill, Logan, UT 84322-5230, USA
e-mail: ryan.odonnell@usu.edu

Introduction

Despite the fact that leopard frogs, the "*Rana pipiens* group" (sensu Hillis and Wilcox 2005), have been a model organism for studies of physiology and phylogenetics for decades, species boundaries in this complex are still incompletely known. For example, many species were only described in the 1970s and 1980s (e.g. Frost and Bagnara 1976; Platz and Frost 1984; Platz and Mecham 1979), and species boundaries in the Chiricahua Leopard Frog (*R. chiricahuensis*) are still being resolved (e.g. Goldberg et al. 2004; Hekkala et al. 2011). The most widespread member of the *R. pipiens* group is no exception: for over a century it has been debated whether the taxon now known as the Northern Leopard Frog (*R. pipiens*) is monomorphic or contains two species with a border between them around the Mississippi River (e.g. Cope 1889; Dunlap and Platz 1981; Wright and Wright 1949). Early descriptions of Northern Leopard Frogs in the western part of the range described them as a separate subspecies (*R. pipiens brachycephala*) having a shorter head and no distinct barring on anterior surface of the limbs, among other features (Cope 1889). Several authors were unable to confirm Cope's morphological features (reviewed in Dunlap and Platz 1981), and therefore Wright and Wright (1949) commented that most herpetologists of their time did not consider Cope's *brachycephala* to be valid. However,

Wright and Wright (1949) considered the matter unsettled and summarized the work of others in showing the range of *brachycephala* to include all of the western part of the range of *pipiens* to the middle of Minnesota and Iowa. No further attempts were made to assess the validity of *R. p. brachycephala* until Dunlap and Platz (1981) analyzed allozymes and calls and also found no consistent difference between eastern and western Northern Leopard Frogs. By this time, *R. p. brachycephala* was widely regarded as invalid.

The issue of whether eastern and western Northern Leopard Frogs were distinct was revived by Hoffman and Blouin (2004a). In a range-wide phylogeny of the Northern Leopard Frog, they detected two mitochondrial lineages that closely supported the distributions mapped 55 years earlier on the basis of morphology (Wright and Wright 1949). Mitochondrial sequence divergence between eastern and western lineages averaged 3 % and reached 4.3 %, which is comparable to species-level divergences in some other members of the genus (Hoffman and Blouin 2004a; Jaeger et al. 2001). Hoffman and Blouin suggested that the deep divergences between eastern and western mitochondrial lineages may indicate that what is currently known as the Northern Leopard Frog in fact consists of two cryptic species, one in eastern North America and one in western North America. However, they did not recommend recognizing eastern and western clades as separate species based on their results alone, citing examples of discordance between mitochondrial and nuclear phylogenies, morphology, and behavior.

The possibility of significant substructuring within this species has been made even more pertinent because of conservation concerns in the western portion of the species' range (e.g. Clarkson and Rorabaugh 1989; Germaine and Hays 2009; McAllister 2005; Moriarty 2009; Werner 2003). If Northern Leopard Frogs in the west are distinct from those in the east, it will be important to begin management actions quickly to preserve this lineage. However, three significant gaps in the understanding of this species preclude an informed decision on whether the western populations are indeed genetically distinct from those in the east. First, it is not known where the geographic border or zone of intergradation between eastern and western mitochondrial clades lies. Second, it is not known whether the nuclear genome shows similar phylogenetic patterns to the mitochondrial genome. Third, it is not known whether eastern and western lineages are reproductively isolated.

The data currently available on the geographic distribution of eastern and western mitochondrial clades are limited to two analyses, one across the range of the species (Hoffman and Blouin 2004a) and one across western Canada (Wilson et al. 2008). Collectively, these analyses showed that most populations sampled were either entirely eastern or entirely western in their mitochondrial DNA. One population was found where eastern and western lineages have come into secondary contact, at the west side of James Bay, Ontario (plus one recent introduction of an eastern frog in Arizona). However, Hoffman and Blouin (2004a) had limited samples that were widely separated in the potential area of overlap between the eastern and western mitochondrial clades (Fig. 1). The mitochondrial haplotypes of frogs in most of Ontario and all of Iowa and Wisconsin are unknown. Furthermore, and perhaps more importantly, the geographic extent of introgression between these lineages is unknown. The two clades may be separated by a discrete boundary, or they may have a broad zone of introgression. A very narrow zone of introgression would imply strong reproductive isolation between the clades. If introgression is extremely limited or does not exist, the two clades should be considered separate species. To determine the geographic extent of the mitochondrial clades and to determine whether the clades meet in a gradual cline of intergradation or discrete parapatry, more sampling is needed at a finer geographic scale between the previously sampled localities.

Second, while the degree of introgression of mitochondrial genes is an excellent tool to begin to investigate introgression between these lineages, it is unknown to what degree mitochondrial phylogenies represent the phylogeny of this species. Discordances between cytoplasmic DNA and nuclear DNA are well known from many other species (e.g. Fontenot et al. 2011; Jacobsen and Omland 2011; Oyler-McCance et al. 1999; Yang and Kenagy 2009). Range-wide analysis of several unlinked nuclear markers is needed to determine whether the deep divergences in mitochondrial DNA indicate a significant level of genome-wide divergence, which would support the recognition of these clades as separate subspecies or even species.

Third, the extent to which the two lineages are reproductively isolated is not known. Using only a mitochondrial marker, as Hoffman and Blouin (2004a) did, hybrids cannot be detected. Determining whether eastern and western clades can interbreed requires analysis of nuclear DNA from the area of overlap between eastern and western mitochondrial lineages. Extensive interbreeding over a broad area would indicate a lack of reproductive barriers between the two lineages. If nuclear and mitochondrial origins in the zone of overlap are strongly correlated, or if the zone of overlap is narrow, then the hypothesis that eastern and western lineages are reproductively isolated would be supported.

Our objective was to address these three gaps in knowledge by testing three corresponding hypotheses. First, we tested the hypothesis that eastern and western mitochondrial clades overlap over a narrow geographic distance. Second, we tested the hypothesis that the

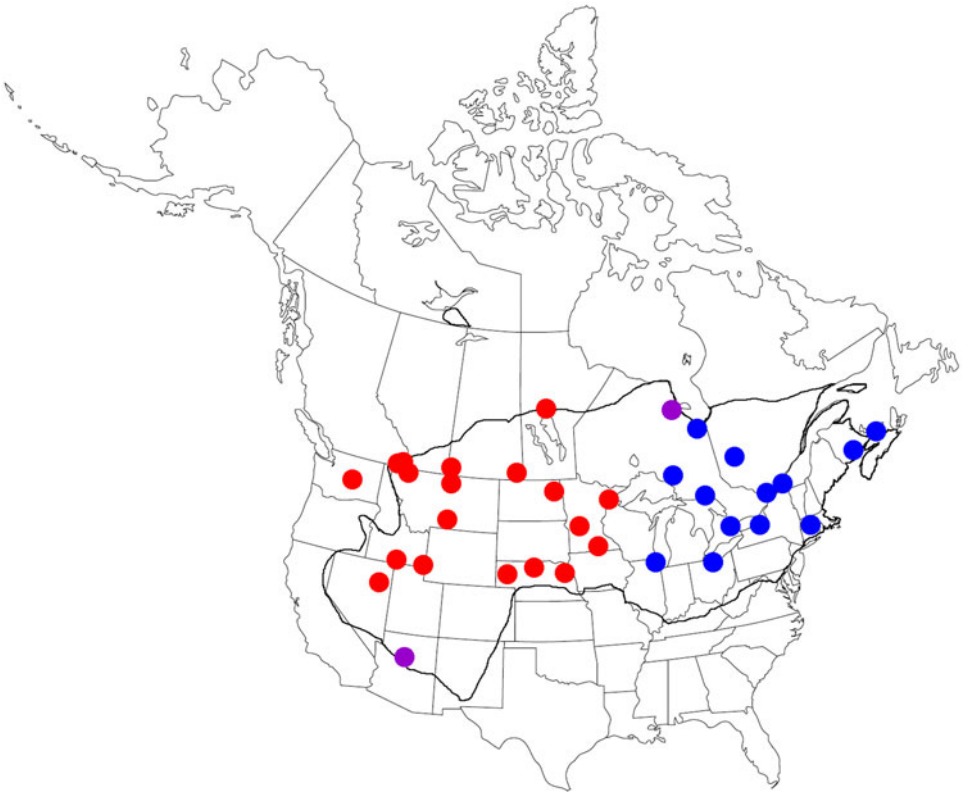

Fig. 1 Mitochondrial haplotype groups from Hoffman and Blouin (2004a). Two major haplotype groups are indicated by two colors, *red* and *blue*. Populations indicated in *purple* contained both haplotype groups. The population in Arizona was reported to contain the eastern group as a result of recent introduction. Sample sizes range from 5 to 15 individuals per population. (Color figure online)

distribution of nuclear markers is congruent with the previously described distribution of mitochondrial clades. Third, we tested the hypothesis that the origin of mitochondrial DNA of individuals in the zone of overlap is significantly correlated with the population of assignment based on microsatellite data.

Materials and methods

We collected tissue samples from 17 to 30 individuals from each of 24 populations around the range of the Northern Leopard Frog, for a total of 567 individuals (Table 1; Fig. 2). Most samples were collected in 2008–2010, but some were acquired from previous collections, with the oldest samples from 2004. Populations were chosen to represent the majority of the species' range, with additional sampling concentrated in the vicinity of the Mississippi River, an area where eastern and western mitochondrial haplotypes were thought to meet (Hoffman and Blouin 2004a). Tissue samples were collected by clipping the tip of the third toe on the right hind foot directly into a microvial containing 95 % ethanol. To minimize the risk of infection, we applied an antibiotic/anesthetic solution to the cut toes before releasing each frog at its point of capture (Green 2001). Surgical instruments were sterilized between each frog to eliminate the risk of sample contamination and to reduce the risk of spreading diseases among frogs. When possible, we collected tissue samples from freshly road-killed specimens rather than live specimens. Samples were then frozen and stored at −80 °C. DNA was extracted from toe clips using a standard chloroform extraction (Müllenbach et al. 1989). The purified DNA was resuspended in a Tris–EDTA buffer (0.1 M Tris, 0.1 M EDTA, pH 9.0) and stored at −80 °C.

We sequenced one mitochondrial gene, to assess the distribution of eastern and western haplotypes in the zone of potential overlap, and four nuclear sequences (including introns and exons) to compare distribution and phylogeny of nuclear genes to the mitochondrial gene. These loci were sequenced for all sampled individuals from populations WAP, TIF, GWM, COP, and OGD, and at least five individuals from each of the remaining populations. Sequence data was obtained for 786 base pairs of subunit 1 of the mitochondrial gene NADH dehydrogenase (ND1) using the primers RpND1F and RpND1R (Hoffman and Blouin 2004a; Wilson et al. 2008). Each 25 mL polymerase chain reaction (PCR) contained 0.4 µmol/L of each primer, 150 µmol/L dNTPs, 1x standard PCR buffer (including 1.5 mmol/L $MgCl_2$), 2.5 units of Taq polymerase, and ~50 ng of genomic DNA. PCR conditions consisted of 5 min of initial denaturation at 95 °C; followed by 35 cycles of 94 °C for 60 s, 54 °C for 60 s, and 72 °C for 90 s; followed by a 5 min final extension at 72 °C. Nuclear

Table 1 Sources of samples

Abbreviation	Location	Longitude	Latitude	*N*
POT	Potholes Lakes, Grant County, Washington	−119.371	47.088	24
TRU	Truxton Wash, Mojave Co., Arizona	−113.640	35.424	28
CHO	Chocolate Pond, Bow City, Alberta	−112.268	50.432	17
OUR	Ouray NWR, Uintah Co., Utah	−109.626	40.144	24
WCO	West Coyote Wash, San Juan Co., Utah	−109.296	38.289	22
SWE	Sweetwater River, Carbon Co. and Natrona Co., Wyoming	−107.289	42.419	24
CUS	Custer NF, Carter Co., Montana	−104.210	45.702	24
HOR	Horsehead Creek, Fall River Co., South Dakota	−103.299	43.121	20
NPL	North Platte River, Keystone, Keith Co., Nebraska	−101.631	41.219	24
WSL	West Shoal Lake, Shoal Lake Rural Municipality, Manitoba	−97.595	50.456	30
TWO	Two Rivers SRA, Douglas Co., Nebraska	−96.343	41.215	24
OGD	Harrier Marsh WMA, Ogden, Boone Co., Iowa	−94.017	42.023	20
MOO	Moose-Willow WMA, Aitkin Co., Minnesota	−93.537	46.952	24
SLA	Sandy Lake First Nation, Ontario	−93.357	53.065	24
TIF	Tiffany SWA, Buffalo Co., Wisconsin	−92.018	44.576	24
WAP	Mill Race Flats WMA, Wapello, Louisa Co., Iowa	−91.189	41.192	22
GWM	Mead WMA, Marathon Co. and Portage Co., Wisconsin	−89.858	44.700	24
COP	Copper Country SF, Houghton Co. and Baraga Co., Michigan	−88.525	46.921	24
KRW	Kentucky River WMA, Henry Co., Kentucky	−84.914	38.426	24
OPI	Opinaca River at Route-du-Baie-James, Quebec	−77.243	52.395	24
HIS	Howland's Island WMA, Cayuga Co., New York	−76.697	43.078	24
LAR	Larose Forest, Co. of Prescott and Russell, Ontario	−75.204	45.396	24
SCO	Scovil, St. John River, New Brunswick	−66.104	45.775	24
JON	Jones Pond, Cape Tormentine, New Brunswick	−63.822	46.130	24

Some location names reference nearby (<5 km) locations. Longitude and latitude are means of all samples, given in decimal degrees, WGS84 projection. Populations are arranged from west to east

NF national forest, *NWR* national wildlife reserve, *SF* state forest, *SRA* state recreation area, *SWA* state wildlife area, *WMA* wildlife management area

sequences included segments of two different exons of the rhodopsin gene (283 bp of Rhod1 and 137 bp of Rhod4), one 507 bp segment of the tyrosinase gene (Tyr), and one 199 bp segment of β-fibrinogen intron 7 (FIB; Bossuyt and Milinkovitch 2000; Di Candia and Routman 2007; Prychitko and Moore 1997). PCR conditions were identical to those for amplifying ND1 sequences with the following exceptions. Taq was reduced to 1.25 units for all nuclear loci. For FIB, final $MgCl_2$ concentration was increased to 2.25 mmol/L. Temperature conditions were also identical to those used for ND1 except that Rhod1 and Rhod4 started with a 10 min initial denaturation, and their extension times were 60 s. Annealing temperatures were 59 °C for Rhod1, 57 °C for Rhod4, and 58 °C for Tyr and FIB. PCR products were visualized on a 0.7 % agarose gel to check for product quantity and size. PCR products were purified with QIAquick PCR purification kit (Qiagen) and sequenced with BigDye chemistry (Applied Biosystems) on an ABI 3730 sequencer. Sequences were edited and aligned with SeqMan II software. Sequences were generated in the forward direction first and in the reverse direction if the sequence could not be confidently read throughout the entire amplicon in the forward direction alone.

Heterozygotes could be identified directly from overlapping peaks in sequencing traces, but haplotypes could not be determined directly in individuals that were heterozygous at more than one base pair. To assign phase (haplotypes association) for these heterozygous sites, we used the program PHASE (Flot 2010; Stephens and Donnelly 2003).

TCS version 1.21 was used to identify unique haplotypes in the dataset and to examine the relationships among those haplotypes using statistical parsimony (Clement et al. 2000). We also examined the relationships among haplotypes using maximum likelihood and Bayesian methods. Maximum likelihood phylogenies were constructed with RAxML 7.2.8 (Stamatakis 2006; Stamatakis et al. 2008) as implemented in CIPRES with default settings (Miller et al. 2010). Bayesian phylogenies were constructed using

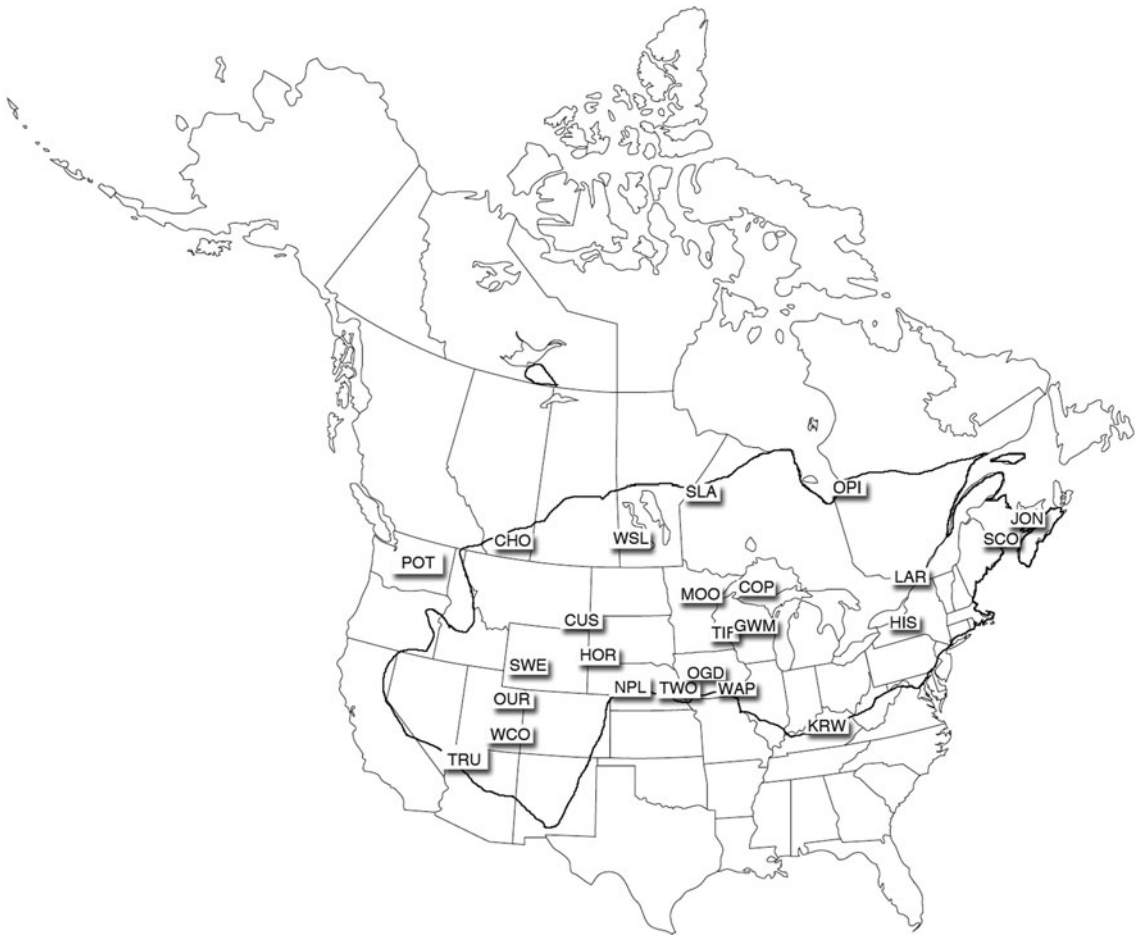

Fig. 2 Populations sampled in this study. See Table 1 for sample sizes, geographic coordinates, and descriptions of localities

MrBayes, sampling at least 1,000,000 generations (or adding 1,000,000 additional generations until the standard deviation of split values was less than 0.01) at every 1000th generation, with nst = 6 and rates with an inverse gamma distribution, and discarding the first 25 % of samples as burn-in.

To further test for geographic patterns in nuclear DNA and to test for evidence of interbreeding in the zone of introgression, we amplified seven microsatellite loci: Rpi100, Rpi101, Rpi102, Rpi104, Rpi107, and Rpi108 (Hoffman et al. 2003), and RP193 (Hoffman and Blouin 2004b). PCR conditions were 2 min of initial denaturation at 95 °C, followed by 30 cycles of the following steps: 95 °C for 30 s, annealing temperature for 30 s, and 72 °C for 1 min; followed by a 10 min final extension at 72 °C. Locus-specific annealing temperatures were as follows: Rpi100: 52 °C, Rpi101: 62 °C, Rpi102: 50 °C, Rpi103: 55 °C, Rpi104: 56 °C, Rpi107: 52 °C, Rpi108: 52 °C, and RP193: 56 °C. The PCR product was visualized on a 0.7 % agarose gel to check for product quantity and size. PCR products were then analyzed on an ABI 3100 or 3730 sequencer. To search for clusters of similar individuals based on microsatellite allele frequencies, we used the program STRUCTURE version 2.3 (Pritchard et al. 2000), following the recommendations for interpreting results from Evanno et al. (2005). After determining the optimum number of groups using STRUCTURE, we then plotted the spatial distribution of these groups using GENELAND (Guillot et al. 2005). To test the significance of structuring both between populations and between larger groups identified by STRUCTURE, we used AMOVA, implemented in GENALEX version 6.4 (Peakall and Smouse 2006). To test whether genetic distance between populations was significantly related to their geographic distance, we used a Mantel test, implemented in GENALEX (Peakall and Smouse 2006). Finally, to test whether genetic differences were greater between groups than expected due to isolation by distance, we used a partial Mantel test, implemented in ZT (Bonnet and Van de Peer 2002). Specifically, we used the partial Mantel test to determine whether genetic distance differed between categories of population pairs (within east, within west, or across the east–west boundary), after controlling for geographic distance. For this test, we excluded populations that were

indicated by STRUCTURE to be in the zone of introgression (see results). We used these varied approaches to help overcome any inherent biases in each individual approach: it is common for various methods to give different results, and a broad perspective is necessary to draw correct inferences from analyses such as these, especially when isolation by distance is evident (Frantz et al. 2009).

To look for genetic evidence of reproductive barriers, we first selected populations where several individuals assigned to more than one group of populations (see results), and then we conducted two separate tests on these populations. First, we compared probability of assignment to one group among individuals with eastern or western mitochondrial haplotypes using a Wilcoxon rank-sum test. Strong genetic isolation would cause the probability of microsatellite-based group assignment to be highly correlated with mitochondrial haplotype. Second, we performed a Principal Coordinates Analysis (PCA) on microsatellite allele frequencies among individuals in these populations. If reproductive isolation were complete, we would expect two discrete groups to be visible in the PCA. If the two lineages can reproduce but produce infertile offspring, we would expect two discrete groups representing each lineage with a third discrete group between them, representing the F1 hybrids. If no reproductive barriers exist, or if reproductive barriers are present but incomplete, we would expect to see all individuals in a single cluster in the PCA analysis.

Results

Mitochondrial sequencing

ND1 sequencing revealed two groups of related haplotypes that differed from each other by 2.7 to 4.5 % sequence divergence (mean = 3.8 %, uncorrected). These two groups did not connect in a 95 % parsimony diagram with a connection limit of 12 steps (Fig. 3). Within-group divergences ranged from 0.1 to 1 % in the eastern group and 0.1 to 0.9 % in the western group. These two haplogroups were strongly supported (98 % bootstrap support), but had little resolution among haplotypes within each group. These haplogroups corresponded to the eastern and western groups described by Hoffman and Blouin (2004a). Only two of the 24 populations we sampled had both eastern and western haplogroups. These two populations were both on the Mississippi River: WAP on the west side of the river in Iowa (4 eastern, 18 western) and TIF on the northeast side of the river in Wisconsin (2 eastern, 22 western). All other populations west of the Mississippi River consisted of 100 % western haplotypes, and all other populations east of the Mississippi River consisted of 100 % eastern haplotypes.

Nuclear sequencing

PHASE was able to assign haplotypes in heterozygotes with varying success. For Rhod1, all haplotypes were assigned with 100 % confidence except for one position in one individual that was ambiguous (50 % confidence for each of two assignments). For Rhod4, all haplotypes were assigned with 100 % confidence. For Tyr, some sites had confidence as low as 58 %. For this gene, we used two approaches for phylogeny reconstruction, one including all haplotypes as determined by majority (>50 %) support, and one including only haplotypes determined with 100 % confidence. For FIB, all haplotypes were assigned with at least 98 % confidence.

For Rhod1, we found only three haplotypes (Fig. 4). One of these was very rare and differed by only one bp from a second haplotype that was widespread in the western part of the range; together these were considered a western group. The third haplotype was found throughout the east, and we considered it an eastern form. Eastern and western haplotypes differed by 1.1–1.4 % sequence divergence (mean 1.2 %). The eastern haplotype was well supported (99 % bootstrap support) as being separate from the western haplotypes. Both eastern and western haplotypes were found at four of the 24 populations we sampled. WAP and GWM had mixed Rhod1 haplotypes (WAP: 3 eastern, 37 western; GWM: 3 eastern, 45 western) and were in the area of overlap between eastern and western lineages according to our mitochondrial sequencing results. Two additional populations, LAR in eastern Ontario, and HIS in western New York, each had a single western haplotype (out of 10 for LAR and 12 for HIS).

We detected four haplotypes for Rhod4 (Fig. 5). Three of these haplotypes were closely related and prevalent in the west. We considered these a western haplogroup. The fourth haplotype was generally limited to the east and we considered this the only representative of the eastern haplogroup. Western haplotypes differed by 1–2 bp (0.7–1.5 %). Western haplotypes differed from eastern haplotypes by 2.2–2.9 % (mean 2.7 %). The distribution of Rhod4 haplotypes was very similar to that of Rhod1 haplotypes. This was not surprising given that Rhod4 is physically located very near Rhod1 and therefore low levels of recombination are expected between these sequences. About 1,700 bp separate these genes in the Western Clawed Frog (*Xenopus tropicalis*; xenbase.org), and the distance is presumably similar in the Northern Leopard Frog. The area of geographic overlap between eastern and western haplogroups was nearly identical to that described for Rhod1. Only one individual had a Rhod4 haplogroup that did not match its Rhod1 haplogroup ($n = 312$), as expected for two sequences that are physically very close in the genome. The only difference from

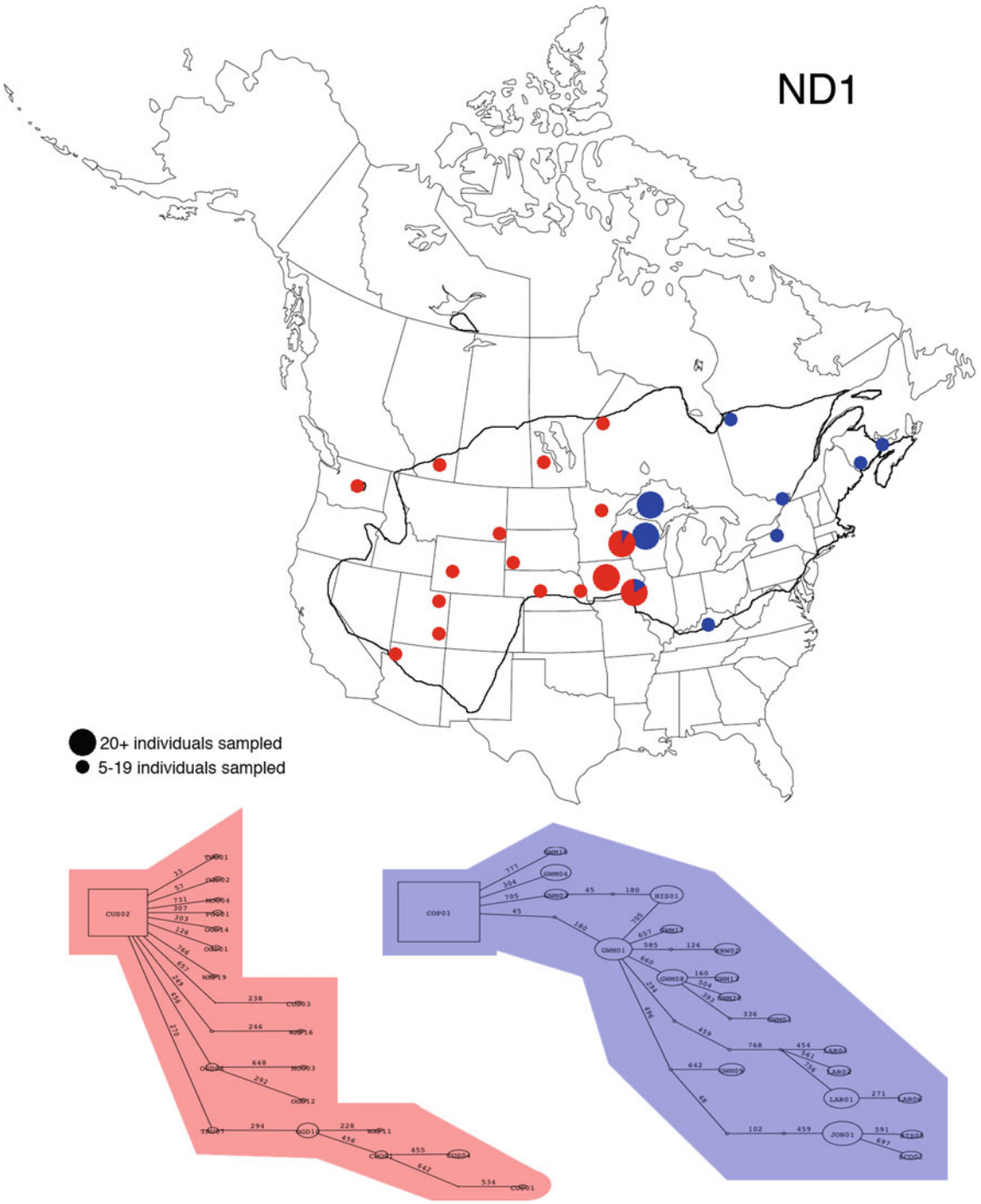

Fig. 3 ND1 haplogroups and their distribution. Two parsimony diagrams did not join (95 %, connection limit 12). One of these haplogroups was limited to the east, and the other to the west. *Black line* marks approximate range of the Northern Leopard Frog. (Color figure online)

the distribution of Rhod1 was one individual at LAR that was homozygous at Rhod1 and heterozygous at Rhod4.

Tyr was much more variable than any of the other sequences studied. Haplotypes found in the east generally grouped together but with very weak bootstrap support (8–39 %, depending on the haplotype), therefore we did not have the power required to detect a geographic difference had there been one. Because we could not assign haplotypes to groups with confidence, we were unable to spatially map their distribution.

We used FIB because previous authors have shown it to be informative for phylogenetic questions in the same genus (Di Candia and Routman 2007). However, contrary to these authors' results, we did not find that the variation

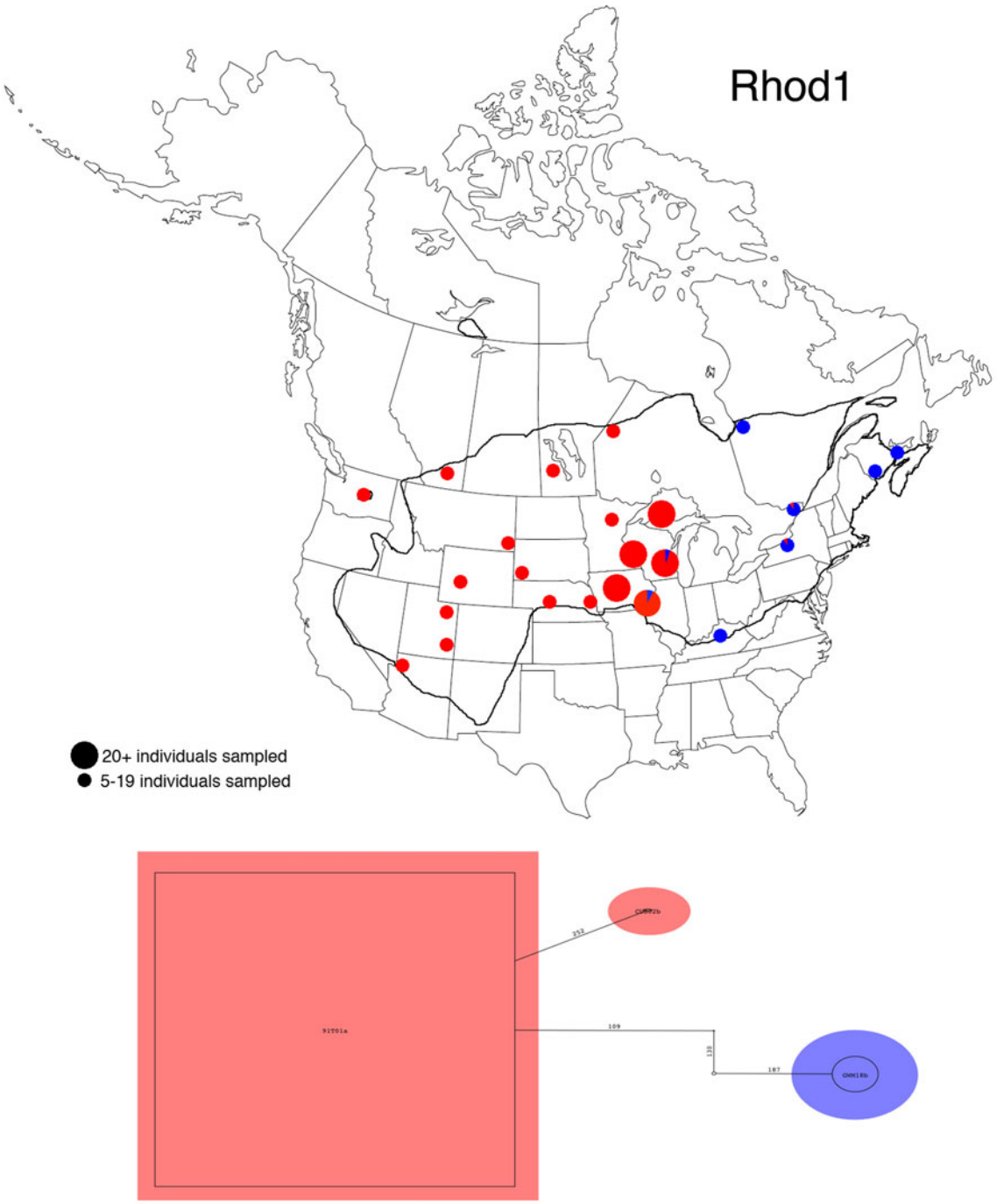

Fig. 4 Rhod1 haplogroups and their distribution

in length of this gene was indicative of species. The insertion/deletion (indel) Di Candia and Routman (2007) described as being diagnostic of Plains Leopard Frogs (*R. blairi*) also was widespread among western Northern Leopard Frogs, which greatly complicated the analysis of heterozygous individuals. There were at least two different indels in the sequences we amplified from Northern Leopard Frogs. Because PHASE is not well suited to determining phase in the presence of indels, we excluded the part of the gene with the indels, leaving a much shorter segment for analysis than previous authors have used (199 bp vs up to 615 bp). Despite this short read, we found 10 different haplotypes among our FIB sequences (Fig. 6). These grouped into four haplogroups, but there was no clear distinction between east and west in this locus. One haplogroup (blue in Fig. 6) was the only one present in the

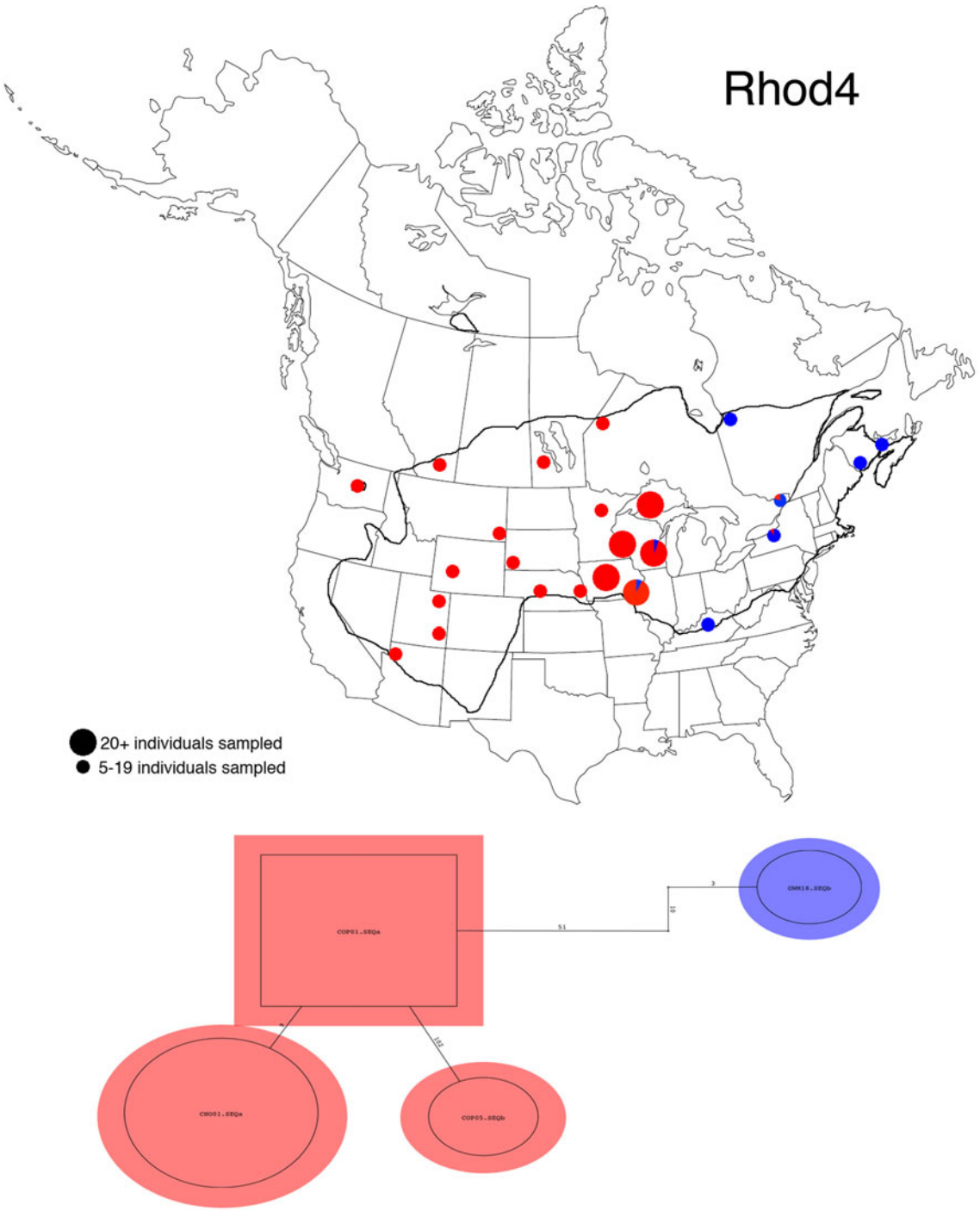

Fig. 5 Rhod4 haplogroups and their distribution

easternmost populations. Defining this group as eastern and the remainder as western, there was as much variation among western haplotypes as there was between eastern and western (mean 1.6 % divergence). This eastern haplogroup was found as far west as central Nebraska, northern Minnesota, and western Ontario (Fig. 6). This was further west than the western limit of eastern haplotypes in ND1, Rhod1, and Rhod4. Conversely, western FIB haplotypes were found as far east as eastern Ontario and central New York. This finding is similar to the eastern limit of eastern haplotypes of Rhod1 and Rhod4.

Microsatellite loci

Seven microsatellite loci were used to assess the phylogeography of the nuclear genome by simultaneously using

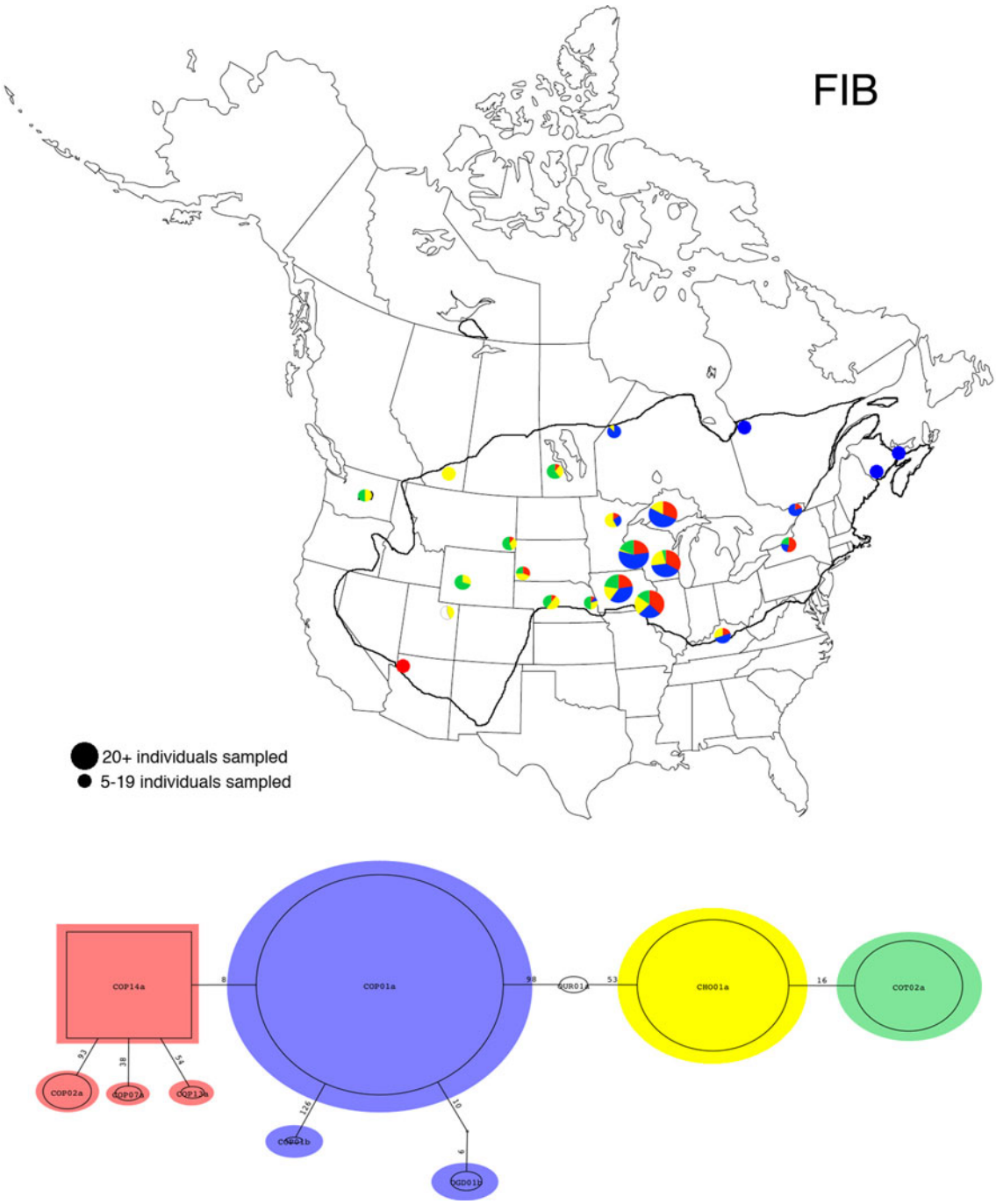

Fig. 6 FIB haplogroups and their distribution. Five samples at WCO did not amplify

a greater number of loci. Using a Bayesian clustering algorithm, STRUCTURE, individuals optimally divided into two groups (Fig. 7). These groups corresponded well with the eastern and western clades identified by mitochondrial sequencing. Only two populations (WAP and TIF, both on the Mississippi River) showed mixed assignments of eastern and western individuals. These are the same two populations that contained eastern and western mitochondrial haplotypes. Ninety-one percent of the remaining individuals assigned to eastern or western groups with >90 % confidence. Assignment of individuals to two groups by Geneland aligned very closely with results from mitochondrial, Rhod1, and Rhod4 sequences (Fig. 8).

We used AMOVA as a secondary method to test the significance of genetic differences between the two groups that were indicated by STRUCTURE and to test for differences between the pre-defined populations from which we sampled. There were significant differences in allele

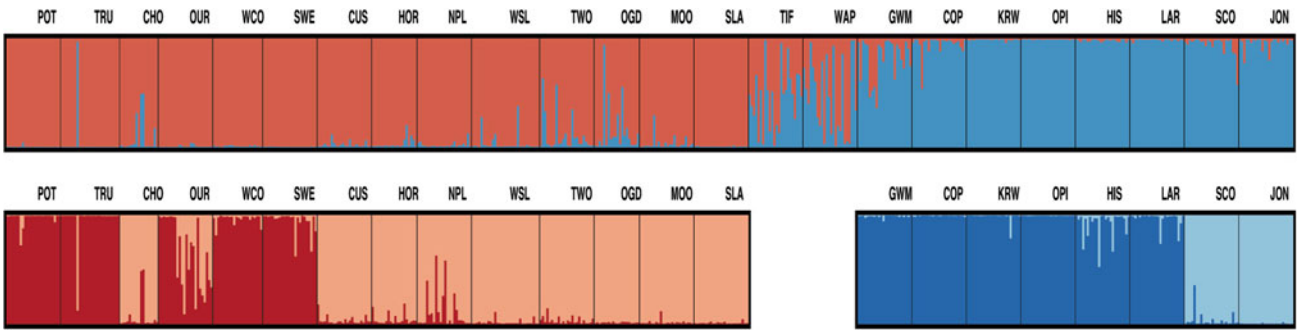

Fig. 7 Probability of assignment of individuals, represented by colors of vertical bars, to two groups by STRUCTURE. Populations are arranged by geographic proximity, generally from west (*left*) to east (*right*). *Top bar* is a single run of all individuals; *lower bars* represent additional runs on subsets of the whole dataset. Individuals tend to assign to eastern or western groups, with mixed assignments in the two populations on the Mississippi River, WAP and TIF. Within the western group, populations sort into a northeast and southwest group. Within the eastern group, two populations from New Brunswick, SCO and JON, are distinct from the others. See Fig. 2 for geographic distribution of populations

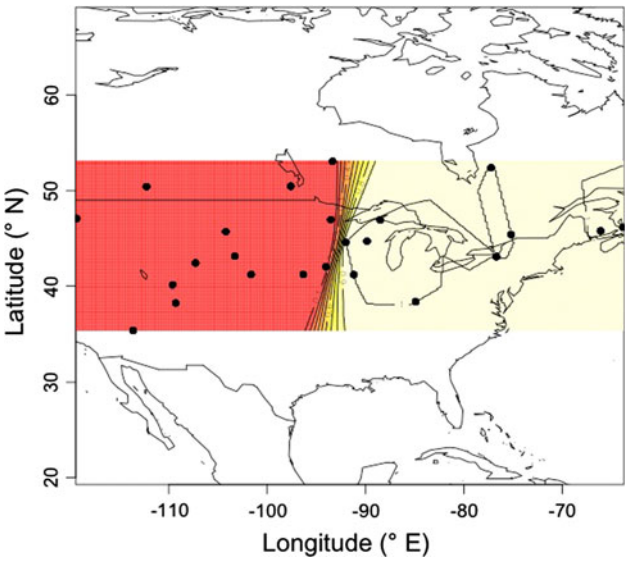

Fig. 8 Posterior probability of assignment of microsatellite allele frequencies to an eastern group, calculated by GENELAND. *Black dots* represent sampled populations. Probability of assignment to a western group is (1-probability of assignment to eastern group). (Color figure online)

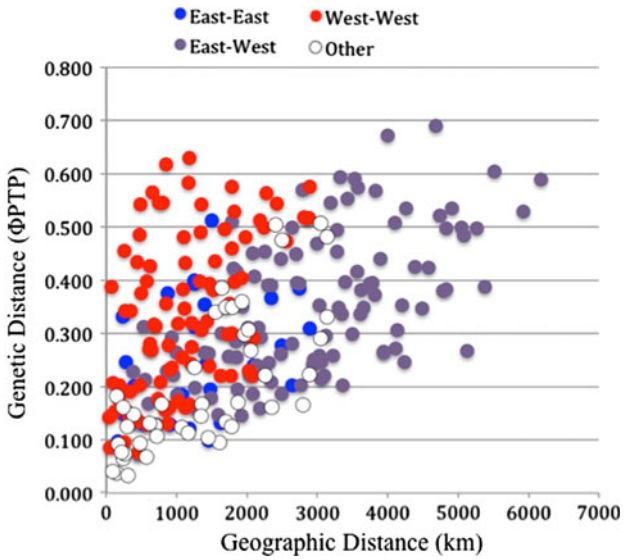

Fig. 9 Isolation by distance. Each point shows the genetic distance (Φ_{PTP}) between two populations as a function of straight-line geographic distance (km), calculated by GENALEX. Populations were defined as east or west according to Fig. 7, any pair including the intermediate populations was considered "other". Across categories, genetic distance is significantly related to geographic distance

frequencies among populations ($p = 0.001$). All pairwise p-values (not corrected for multiple tests) were less than 0.001, the limit of precision for this test. AMOVA also confirmed the presence of a significant difference between the eastern and western groups that had been identified by STRUCTURE ($p = 0.001$).

Isolation by distance was significant across the range (Fig. 9). Genetic distance (Φ_{PTP}) between population pairs was significantly related to geographic distance (Mantel test, $R^2 = 0.263$, $p < 0.001$). Genetic distance between populations was greater within the west than between west and east (partial Mantel test, $r = -0.26$, $p = 0.001$) or within the east (partial Mantel test, $r = 0.62$, $p = 0.0001$). PCA supported the hypothesis that populations close in geographic distance are also generally close in genetic distance. PCA showed a horseshoe shape, which is a common pattern when displaying data across a linear gradient (Fig. 10). In this case, the linear gradient was from eastern to western populations, which formed distinct clusters but which were not widely separated from one another, and which were joined in principal coordinate space by populations with mixed assignments (WAP and TIF, on the Mississippi River).

Hybridization within individuals

To test for genetic evidence of robust reproductive isolation between eastern and western lineages in these two

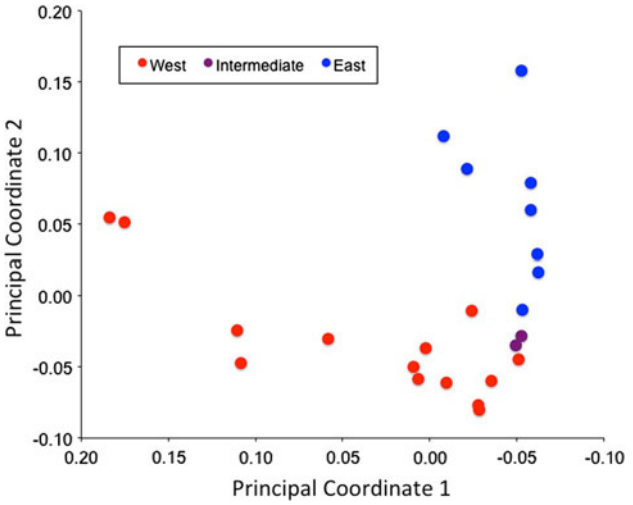

Fig. 10 PCA of 24 populations. Populations are color coded according to assignments from Fig. 7. (Color figure online)

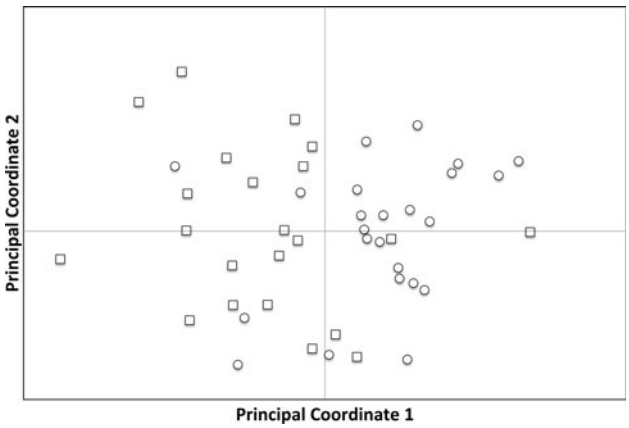

Fig. 11 PCA of microsatellite data from 46 individuals in the intermediate populations, TIF and WAP, on the Mississippi River. *Circles* are individuals from TIF and *squares* are individuals from WAP. The lack of two distinct groups is evidence that in the area of overlap between eastern and western clades, at least some introgression has occurred

populations, we compared the probability of assignment to the eastern group, as computed by STRUCTURE, between two groups of individuals from the area of overlap (TIF and WAP), with eastern versus western mitochondrial haplotypes. Probability of assignment to eastern or western groups on the basis of microsatellite alleles was not related to mitochondrial haplotype (Wilcoxon Rank-Sum Test, $Z = 0.19$, one-tailed $p = 0.43$), indicating that individuals in these populations had a mix of eastern and western ancestry. Secondly, we conducted a PCA of microsatellite alleles from individuals in these two populations. PCA showed a single cluster of individuals, which is expected in the absence of a robust reproductive barrier (Fig. 11).

Discussion

Previous conclusions about subspecific differentiation in Northern Leopard Frogs have ranged from supporting a phylogenetic split within the species on the basis of mitochondrial DNA (Hoffman and Blouin 2004a), to showing no difference between putative subspecies on the basis of allozymes and call characters (Dunlap and Platz 1981). The results presented here provide evidence that Northern Leopard Frogs include two lineages with significant genetic differences, evident in both mitochondrial and nuclear genomes, although the strength of this signal varies among markers used. Nuclear sequencing showed some deep divergences with limited introgression between them, but some loci (FIB and Tyr) lacked evidence of genetic differentiation associated with geography. In contrast, mitochondrial sequencing showed deep divergence between eastern and western clades with very little geographic overlap between them. Significant differentiation was also shown by the combination of seven microsatellite loci, with a narrow range of overlap that coincided closely with the patterns shown by mitochondrial sequencing.

For two cryptic species that are completely reproductively isolated, a pattern that looks like introgression can be the result of partial geographic overlap between the species. Our individual-level analysis of populations WAP and TIF confirm that a robust barrier to reproduction does not exist between these two lineages, because PCA did not identify two isolated groups within these populations and because mitochondrial haplotype was not significantly correlated with group assignment based on microsatellites. This does not exclude the possibility of reduced hybrid fitness or partial reproductive isolation—experimental matings would be required to address these possibilities—but we can conclude from these data that any reproductive barriers are at least somewhat permeable.

Because our results differ between loci and marker types (sequencing vs microsatellite), it is important to take a holistic approach to assessing what these data reveal about subdivision within this species (Sites and Marshall 2003, 2004). The extent of introgression that we found ranged from very narrow for mitochondrial sequences and microsatellite allele frequencies, to more extensive for nuclear sequences. There are at least four possible explanations for this apparent discordance. First, our microsatellite analyses simultaneously incorporate data from seven different loci, whereas sequencing analyses are done on a locus-by-locus basis, and are more susceptible to locus-specific stochastic effects of gene flow and genetic drift. Gene trees are not equivalent to species trees, and any one gene is likely to have a phylogeny that does not precisely

match the phylogeny of the organism that contains it, due to these stochastic factors.

Second, our nuclear and mitochondrial sequences are also more likely to be subject to the effects of selection than microsatellite loci, because of their location in or proximity to coding regions. Selection acting on genes can affect the geographic distribution of alleles in ways that are not typical of the rest of the genome. For example, in intergrading subspecies of house mice, the extent to which alleles are exchanged between subspecies depends on the selective advantage of the allele being exchanged (Selander et al. 1969). This potential may explain our results for fibrinogen, which showed the broadest introgression of all nuclear loci we sequenced. Fibrinogen is important in the clotting of blood after an injury. A significant heterozygote advantage can promote the spread of genes across a hybrid zone (e.g. Fitzpatrick and Shaffer 2007). Significant heterosis has been demonstrated for the fibrinogen gene in cattle, where heterozygotes have higher plasma levels of fibrinogen than inbred cattle (Qiu et al. 2007). If a similar heterozygote advantage exists in frogs, this could explain the greater spread of fibrinogen genes across the zone of contact than expected on the basis of microsatellite and mitochondrial data.

A third possible explanation for the wide distribution of some of our nuclear sequences across the zone of contact is that it results from incomplete lineage sorting rather than introgression. Because each individual has two copies of nuclear DNA, and mitochondrial DNA is generally single-copy and maternally inherited, the effective population size for mitochondrial DNA is only one-fourth that of nuclear DNA. This smaller effective population size means that mitochondrial alleles from pre-Pleistocene contact are more likely to be lost due to drift than nuclear alleles. Compounding this effect is the fact that substitution rate for mitochondrial DNA is approximately four to six times faster than for nuclear DNA (Johnson and Clayton 2000; Weibel and Moore 2002), so new mitochondrial haplotypes are more likely to emerge in a period of isolation. The strongest evidence for introgression is the geographic distribution of haplogroups. If two lineages were present in a geographic area because they had not completely sorted, both lineages would be expected to be randomly distributed within the range of the clade. Strong geographic trends in allele frequency are evidence of introgression, and can be discerned in our results for ND1, Rhod1, Rhod4, and the seven microsatellites. In contrast, FIB and Tyr do not show distinct clades associated with geography, and are more likely to be explained by selection or incomplete lineage sorting between recently diverged lineages.

Finally, evidence from a well-studied hybrid zone in mice (*Mus musculus* and *M. domesticus*) indicates that mitochondrial genes are less likely than nuclear genes to introgress because of the lack of recombination in the mitochondrial genome (reviewed in Sage et al. 1993). Individual nuclear genes are more likely to introgress across a hybrid zone because they have only a small effect on coadapted gene complexes, but because mitochondrial DNA is inherited as an entire cytoplasmic genome, the negative effects of a mitochondrial genome mismatching with a nuclear genome are more dramatic and detrimental. Thus, cytoplasmic DNA typically shows a steeper differentiation across a hybrid zone than does nuclear DNA.

Together these factors make it apparent that, despite introgression or incomplete sorting in some nuclear sequences, there are significant genetic differences between eastern and western Northern Leopard Frogs. In light of these genetic differences, it may be worth reevaluating whether there are also phenotypic (morphological, immunologic, metabolic, or behavioral) differences between the east and west in this species. Western populations (eastern Great Plains to Sierra Nevada) were initially considered a subspecies, *R. p. brachycephala*, separate from nominate *R. p. pipiens* on the basis of a variety of morphological features (Cope 1889). The western subspecies was elevated to species status by Kauffeld (1937) on the basis of Cope's morphological features and a few additional features. Kauffeld (1937) extended the range of *brachycephala* as far as the eastern coast of North America, south to parts of Pennsylvania. Using this definition, Moore (1944) failed to find any consistent morphological differences between the species of leopard frogs. He thus considered *brachycephala* to be a synonym of *pipiens*. Wright and Wright (1949) agreed with Cope in considering *brachycephala* to be a valid subspecies and argued that its distribution was limited to the west; they considered populations in the northeastern United States and southeastern Canada to be *R. p. pipiens*. They mapped the ranges of *R. p. pipiens* and *R. p. brachycephala* with parapatric distributions that coincide very closely with the genetic discontinuities noted here. Dunlap and Platz (1981) attempted to address reported differences between *R. p. pipiens* and *R. p. brachycephala* by studying allozyme variation and two acoustic properties of calls across a transect from Idaho to Wisconsin. They found no consistent differences in allozymes ($n = 85$) or calls ($n = 10$) and concluded that *R. pipiens* should be considered monotypic. However, calls of this species are among the most complex of any temperate anuran (Larson 2004), and analysis of only two acoustic parameters from ten individuals may be insufficient to detect regional differences in calling behavior. Furthermore, allozyme analyses may be insufficient because there are several cases in other species where analysis of allozymes failed to detect genetic structuring that was later revealed by analysis of DNA sequences (Avise 2000). For example, allozymes did not significantly differ between Japanese and Korean Pond

Frogs (*R. nigromaculata*), but mitochondrial DNA showed that Japanese *R. nigromaculata* are more closely related to *R. plancyi chosenica* than to Korean *R. nigromaculata* (Kim et al. 2004). For this reason, we are not surprised to see that our analysis, based on a combination of mitochondrial sequencing, nuclear sequencing, and nuclear microsatellites, revealed more significant substructuring than previous work with this species based on allozymes. Perhaps because of the lack of differentiation in allozyme frequencies, the morphological features of Cope and Kauffeld have not been critically evaluated in the context of a strictly western *R. p. brachycephala*. Changes in morphology have been noted across the zone of introgression in northern Canada (Schueler 1973), and an analysis of morphological features generally separated eastern and western frogs, with frogs from the Great Lakes region intermediate (Fig. 10 in Schueler 1982). Given the genetic differences reported here and by Hoffman and Blouin (2004a), and their correlation with previous mapped distributions (Wright and Wright 1949) and, roughly, with spotting patterns (Schueler 1982), additional analysis of phenotypes is warranted. It seems likely that such work will reveal morphological or acoustic traits helpful in distinguishing these two genetic lineages, perhaps justifying the resurrection of *R. p. brachycephala*.

These findings contribute to the growing understanding of phylogeography of North American anurans, and of North American species in general. The Mississippi River occurs as a recurring biogeographical barrier among clades in anurans. For example, both American Bullfrogs (Ranidae: *R. catesbeiana*) and Spring Peepers (Hylidae: *Pseudacris crucifer*) show evidence of intraspecific clades having secondary contact after the Pleistocene around the Mississippi River (Austin et al. 2004). Species-level differentiation among the chorus frogs (*Pseudacris* spp.) also shows east–west divergences, separated by the Mississippi River (Moriarty and Cannatella 2004). Indeed, the Mississippi is an important barrier in many taxa (reviewed in Soltis et al. 2006), for example, turtles (Walker et al. 1998), shrews (Brant and Ortí 2003), and trees (Al-Rabab'ah and Williams 2002). It appears that the same barrier is important, albeit partially permeable, in the Northern Leopard Frog, separating two unique genetic lineages with independent evolutionary histories.

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