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Isolation and differentiation of *Rivulus hartii* across Trinidad and neighboring islands

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Abstract

Diversification of freshwater fishes on islands is considered unlikely because the traits that enable successful colonization—specifically, broad salinity tolerances and the potential for oceanic dispersal—may also constrain post-colonization genetic differentiation. Some secondary freshwater fish, however, exhibit pronounced genetic differentiation and geographic structure on islands, whereas others do not. It is unclear what conditions give rise to contrasting patterns of differentiation because few comparative reconstructions of population history have been carried out for insular freshwater fishes. In this study, we examined the phylogeography of Hart's killifish (*Rivulus hartii*) across Trinidad, with reference to neighboring islands and northern South America, to test hypotheses of colonization and differentiation derived from comparable work on co-occurring guppies (*Poecilia reticulata*). Geographic patterns of mitochondrial DNA haplotype variation and microsatellite genotype variation provide evidence of genetic differentiation of *R. hartii* among islands and across Trinidad. Our findings are largely consistent with patterns of geographically structured ancestry and admixture found in Trinidadian guppies, which suggests that both species share a history of colonization and differentiation and that post-colonization diversification may be more common among members of insular freshwater fish assemblages than has been previously thought.

Keywords: colonization, Guapin killifish, island biogeography, Trinidad

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Introduction

Island archipelagos are cauldrons of evolutionary diversification (Baldwin & Sanderson 1998; Mendelson & Shaw 2005). The Hawaiian Islands, for example, host radiations of plants and animals, including *Lapaula* crickets that exhibit unprecedented rates of speciation (Witter & Carr 1988; Gillespie *et al.* 1994; Kambysellis *et al.* 1996; Baldwin & Sanderson 1998; Mendelson & Shaw 2005; Sakai *et al.* 2006). Evolutionary diversification of freshwater fishes on islands has been considered unlikely (Zink *et al.* 1996; Chubb *et al.* 1998; McDowall 2003, 2004), but recent evidence suggests that it is possible despite potential life history and physiological constraints (Langerhans *et al.* 2007; Doadrio *et al.* 2009).

Traits that enable fish to successfully colonize freshwater ecosystems on islands, including broad salinity tolerances and the potential for oceanic dispersal, may also constrain post-colonization genetic differentiation. Nearly all fishes found on islands are either amphidromous or are considered secondary freshwater species capable of tolerating brackish or oceanic conditions during at least one life history stage (Myers 1938; Burgess & Franz 1989; Changeux 1998; Donaldson & Myers 2002). Both amphidromy and salinity tolerance enable dispersal across long distances, a process that can be inferred from the pan-tropical distributions of species

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like *Awaous guamensis* and *Sicyopterus lagocephalus* (McDowall 2003; Keith *et al.* 2005). Similarly, amphidromy and salinity tolerance may enable oceanic dispersal among geographically proximate islands and across basins within islands (Zink *et al.* 1996; Chubb *et al.* 1998; Covich 2006; Cook *et al.* 2009).

Despite having broad salinity tolerances, some secondary freshwater fishes have diversified on islands, such as *Girardinus*, *Gambusia* and *Limia* poeciliids inhabiting the Greater Antilles (Hamilton 2001; Doadrio *et al.* 2009; Lara *et al.* 2010). It is not clear why these genera are more speciose than others, as the Greater Antilles also supports genera that have undergone more limited diversification including *Poecilia* mollies and *Rivulus* killifish, as well as monotypic endemics like the poeciliid genus *Quintana* (Rauchenberger 1988; Echelle *et al.* 2006). Contrasting patterns of diversification in the Greater Antilles and elsewhere may correspond to drift vicariance (Chakrabarty 2004), but it may also be attributable to the timing of colonization events, or differential success in resource exploitation and adaptability (Briggs 1984; McDowall 2003; Langerhans *et al.* 2004, 2007).

The conditions that give rise to contrasting patterns of differentiation can be inferred from phylogenies and phylogeographic reconstructions of population history (Ricklefs & Bermingham 2007). In a phylogeographic study of the silvereye species-complex (*Zosterops lateralis*) across the south Pacific, for example, Clegg *et al.* (2002) showed that genetic diversity and differentiation of island populations corresponds to the number of founding events. Geographic analyses of phylogenetic relationships have demonstrated that evolutionary diversification of Caribbean *Anolis* lizards corresponds to island area (Losos & Schluter 2000). Other work on Caribbean *Anolis* and *Tetragnatha* spiders across Hawaii indicates that evolutionary diversification can be constrained by community formation, where intra- and interspecific competition results in nonrandom sets of species (Losos 1992; Gillespie 2004). Similarly, analysis of genetic divergence among populations of birds in the Lesser Antilles found evidence suggesting that competition constrains diversification on islands that experience high rates of colonization from continental or nearby large-island sources (Ricklefs & Bermingham 2007).

Few reconstructions of population history have been carried out for insular freshwater fishes, but studies of guppies (*Poecilia reticulata*) on Trinidad suggest that geographic isolation and local adaptation following colonization can give rise to evolutionary diversification. Contrary to predictions based on physiological tolerances, geographic patterns of mitochondrial (mtDNA) sequence divergence (Fajen & Breden 1992; Alexander *et al.* 2006), allozyme and microsatellite variation (Carvalho *et al.* 1991; Shaw *et al.* 1991; Crispo *et al.* 2006; Suk & Neff 2009) and single nucleotide polymorphisms (SNPs; Willing *et al.* 2010) provide evidence of pronounced differentiation within and among drainage basins across Trinidad. Estimates of mtDNA divergence between Trinidad and northern South American populations (Alexander *et al.* 2006) indicate that patterns of differentiation are not attributable to vicariance following island formation. Patterns of allozyme and mtDNA divergence between eastern and western drainages suggest that differentiation is a consequence of Pleistocene glaciation cycles and associated sea level fluctuation, where two major colonization events occurred during periods of maximal glaciation when low sea levels may have led to the confluence of coastal rivers (Carvalho *et al.* 1991; Fajen & Breden 1992; Alexander *et al.* 2006). Multilocus analysis of microsatellite genotypic variation indicates, however, that additional colonization events may have occurred. Suk & Neff (2009) showed that guppies on the northern slope of the Northern Range are highly differentiated from those on the southern slope, and that some admixture occurs between eastern and western drainages. A SNP-based genomic analysis provides further support for multiple colonization events from patterns of shared ancestry and admixture among drainages, but also found evidence suggesting that patterns of geographic variation have been shaped by variable selection pressures (Willing *et al.* 2010).

The information available on Trinidadian guppies provides a foundation for comparative reconstructions of population history to better understand evolutionary diversification of freshwater fish on islands. We examined genetic variation within Guapin killifish (*Rivulus hartii*) across Trinidad, with reference to neighboring islands and northern South America, to test hypotheses of colonization and differentiation derived from studies of *P. reticulata*. Building on prior work (Murphy & Collier 1996, Murphy *et al.* 1999; Jowers *et al.* 2008), we first compared mtDNA sequence and haplotype variation across Trinidad, Tobago, Grenada, Isla Margarita and northern South America to determine whether population divergence is attributable to island formation or oceanic dispersal. We then examined mtDNA and nuclear microsatellite variation across the Northern Range and among major drainages to the south of the Northern Range to infer the population history of *R. hartii* on Trinidad to determine the extent of concordance with patterns of divergence observed in *P. reticulata*. Finding evidence of concordance would provide support for a shared history of colonization and differentiation and would indicate that post-colonization diversification could be more common among members of insular freshwater fish assemblages than has been previously thought.

Materials and methods

Study system and sample collections

Members of the aplocheloid killifish genus *Rivulus* include more than 70 species found in Central America, the Caribbean, and South America (Murphy *et al.* 1996). As many as six species are considered island endemics, some with ranges that encompass single islands such as *Rivulus cryptocallus* on Martinique, and others that occur on two or more nearby islands such as *Rivulus cylindraceus* on Cuba and Isla de la Juventud. Based on mtDNA phylogenetic relationships, Murphy *et al.* (1996) suggested that the origins of the genus are South American, and that drift vicariance events gave rise to species inhabiting the Greater Antilles whereas the Lesser Antilles were colonized from South America. The species found in the Lesser Antilles—*R. cryptocallus* on Martinique and *Rivulus hartii* on Trinidad, Tobago, Isla Margarita, and also possibly Grenada—are not sister taxa. Rather, *R. cryptocallus* is sister to *Rivulus stagnatus* from Guyana, which parallels relationships found in co-occurring species of boid snakes (Henderson & Hedges 1995). It is possible, however, that *R. cryptocallus* is derived from *R. hartii* since Murphy *et al.* (1996) compared *R. cryptocallus* to *R. hartii* from the Paria Peninsula in Venezuela, which groups with other coastal South American species.

Although it is well understood that island populations of *R. hartii* are derived from South America, little is known about the biogeographic and evolutionary history of these populations, including those on Trinidad and Tobago where the species' natural history and ecology have been well-studied (Fraser *et al.* 2001; Gilliam & Fraser 2001). Like other secondary freshwater fishes, *R. hartii* exhibit euryhaline physiological tolerances. The species is also well known for accessing habitats unavailable to other species, including headwater streams and isolated pools. It is not only capable of ascending waterfalls and rapids, but can also bypass in-stream barriers by traveling over land through damp litter (Boulenger 1890; Jordan 1923; Seghers 1978). Despite having the ability to disperse widely through both aquatic and terrestrial habitats, a comparison of mtDNA haplotypes from 36 individuals sampled from 10 locations across Trinidad found evidence of pronounced geographic structure (Jowers *et al.* 2008). Comparison of haplotypes on Trinidad to one recovered on Tobago and to haplotypes found in northern South America also suggests that the islands were colonized by at least two evolutionary lineages (Murphy & Collier 1996, Murphy *et al.* 1999; Jowers *et al.* 2008). This finding is generally consistent with the 'two-arc' hypothesis derived from studies of *Poecilia reticulata*, however, a north-south genetic disjunction appears to occur in *R. hartii* whereas both north-south and east-west disjunctions have been observed in *P. reticulata*. Jowers *et al.* (2008) proposed that the distribution of haplotypes in Trinidad reflects flooding of the Orinoco River leading to high dispersal among watersheds throughout lowland Trinidad and geographic differentiation among lowland and higher altitude (i.e. Northern Range) localities.

To test hypotheses of colonization and differentiation derived from prior work on *R. hartii* and *P. reticulata*, we obtained tissue from 659 *R. hartii* in October 2005 and January 2006 from nearly all of the major drainage basins across Trinidad (Fig. 1). Between 11 and 61 samples were collected at each site. We obtained an additional five specimens from one location in Tobago and two specimens provisionally identified as *R. hartii* from one site in Grenada (Table 1). Either whole specimens or caudal fin clips were taken. All tissues were preserved in 95% ethanol. On Trinidad, samples were collected from at least one location per drainage to evaluate between-basin patterns of geographic differentiation. All individuals were collected using hand held dip nets, and were taken from still water habitats within tributaries, side channels or small pools proximate to mainstem reaches.

DNA extraction, mitochondrial DNA sequencing and multilocus microsatellite genotyping

Genomic DNA was extracted from 30 mg of caudal fin tissue from each individual using the DNeasy (Qiagen, Valencia, CA, USA) commercial extraction kit designed for animal tissue. Approximately 10–50 ng of genomic DNA was used as template for 15 μL PCRs that included 2.5 mM $MgCl_2$, 100 μM each dNTP, 0.5 units *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), 0.5 μM each of a pair of primers and 1× PCR buffer. An approximately 700 bp region of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene was amplified for 205 individuals with primers LCO-1490 and HCO-2198 (Folmer *et al.* 1994). PCR conditions consisted of 35 cycles of 94 °C for 45 s, 52 °C for 30 s, and 72 °C for 90 s, with a final extension stage at 72 °C for 5 min. MJ Dyad thermocyclers (MJ Research, Inc.) were used for all PCRs. Amplicons were purified with 96-well QIAquick Biorobot kits (Qiagen, Inc.) on a Qiagen Biorobot 3000, and served as templates in sequencing reactions utilizing ABI BigDye sequencing kits (Applied Biosystems, Inc., Foster City, CA, USA) following manufacturer protocols. The sequencing reactions were run on an ABI 3730 automated sequencer (Applied Biosystems, Inc.). Raw sequence files were edited, assembled and aligned with Sequencher 4.9 (Gene Codes), and

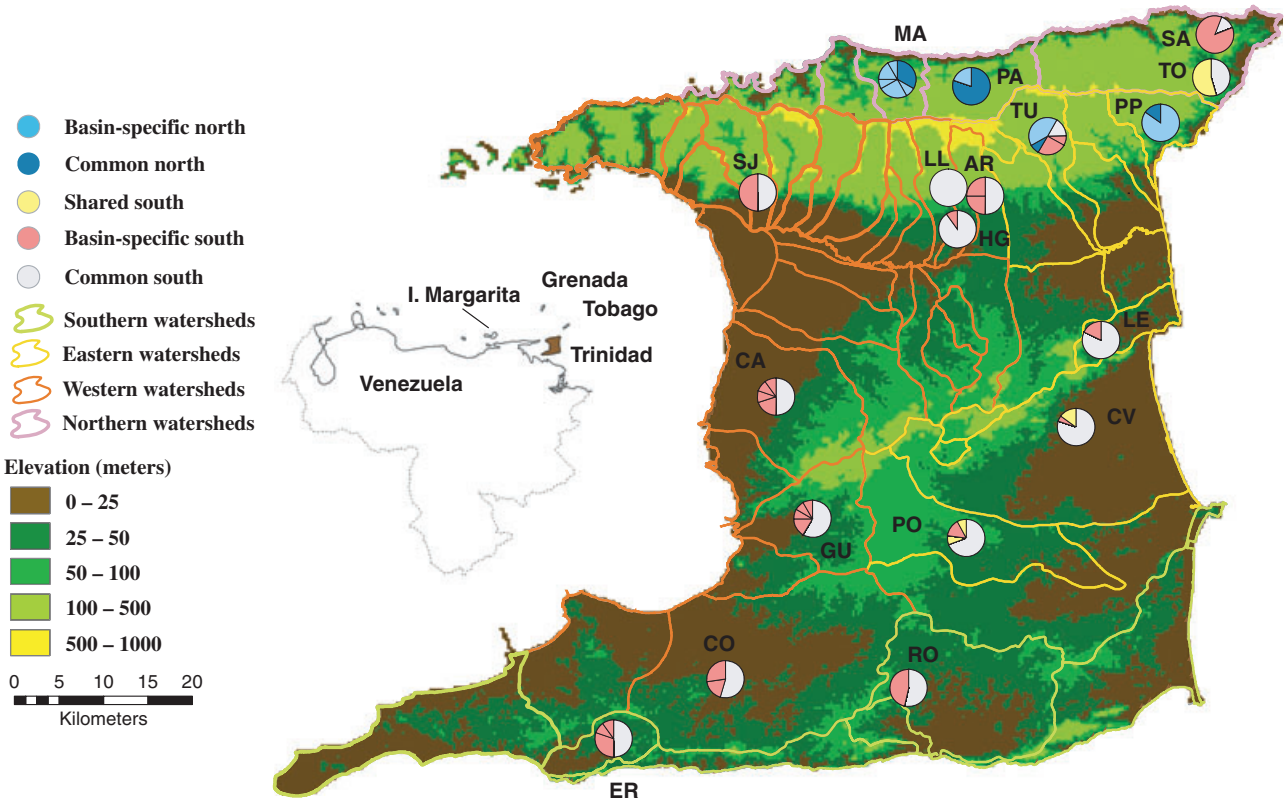

Fig. 1 Distribution and frequency of of cytochrome *c* oxidase subunit I haplotypes in *Rivulus hartii* across Trinidad.

subsequently submitted to GenBank (accession numbers HQ405397–HQ405601).

To compare data from our samples of *R. hartii* with published sequences, we PCR-amplified approximately 800 bp of the cytochrome *b* (cyt *b*) gene for a group of 79 specimens representing all recovered COI haplotypes. Primers L14724 (Kocher *et al.* 1989) and H15557 (Hillis *et al.* 1996) were used in PCRs containing ~10 ng template DNA, 1× buffer, 2.0 mM $MgCl_2$, 100 μM of each dNTP, 1 μM of each primer, and 1 U *Paq* 5000™ polymerase (Stratagene) in a 15 μL reaction volume. PCR conditions consisted of an initial denaturation step at 94 °C for 6 min, followed by 35 cycles of 94 °C for 15 s, 52 °C for 15 s, 72 °C for 15 s, and a final elongation step at 72 °C for 10 min. Amplicons were purified using ExoSAP-It (USB, Cleveland, OH, USA). Cycle sequencing reactions were performed using both forward and reverse primers, DYEnamic ET (GE) chemistry and were analyzed on an ABI 3100XL automated sequencer (Applied Biosystems). Raw sequence files were edited, assembled and aligned with SEQUENCHER, and submitted to GenBank (accession numbers HQ612185–HG612238).

Individuals (Table 1) were also genotyped at six microsatellite loci developed for *R. hartii*, referred hereafter as Rha3, Rha5, Rha10, Rha 16, Rha18, and Rha30 (Appendix 1). Approximately 10 ng of genomic DNA was used as a template in 15-μL PCRs composed of 1 U of *Paq* polymerase, 1× *Paq* PCR buffer, 2.0 mM $MgCl_2$, 100 μM of each dNTP, and 1 μM of each primer. PCR conditions consisted of an initial denaturation at 94 °C for 6 min, followed by 34 cycles of 94 °C for 60 s, locus specific annealing temperature for 60 s (63 °C for Rha3 and Rha18; 60 °C for Rha5 and Rha10; 50 °C for Rha16; and 58 °C for Rha30), extension at 72 °C for 60 s, with a final extension at 72 °C for 7 min. Sizes of labeled PCR amplicons were determined using an ABI 3100 automated sequencer, with CST ROX 50–500 standard (Bioventures, Inc., Murfreesboro, TN, USA), and scored with GENEMARKER software (Softgenetics, LLC, State College, PA, USA). MSA 4.0 (Dieringer & Schlötterer 2003) and MICROCHECKER (Van Oosterhout *et al.* 2004) were used to check for genotyping errors and the presence of null alleles. Tests for departures from linkage equilibrium among loci, and departure from Hardy–Weinberg equilibrium were performed in 20 000 permutations in GENEPOP 4.0 (Raymond & Rousset 1995).

Genetic data analysis

Identification of unique haplotypes, haplotype frequencies at each sample location, and construction of a

statistical-parsimony haplotype network using the COI data were performed in TCS 1.21 with the probability of the network set at 95% (Clement *et al.* 2000).

Geographic patterns of genetic variation were initially inferred from the distribution and frequency of COI haplotypes within and among sample sites. Patterns of genetic variation were assessed according to the frequency of haplotype classes, where haplotypes found at multiple locations were distinguished from haplotypes that only occurred at a single location. Haplotypes were also categorized according to number of intervening mutation steps in the haplotype network. Pairwise genetic differentiation (Φ_{ST}), with equal weighting attributed to transitions and transversions, among sample sites was calculated using ARLEQUIN 3.1 (Excoffier *et al.* 2005). The statistical significance of pairwise Φ_{ST} values was determined using both Bonferroni correction and graphically-sharpened false discovery rate (Benjamini & Hochberg 2000). To evaluate the proportion of genetic variance explained by alternative biogeographic hypotheses, hierarchical Analyses of Molecular Variance (AMOVA) were performed with 10 000 permutations in ARLEQUIN. Sites were grouped geographically in accordance with three alterative biogeographic hypotheses: (i) 'Two-arc' hypothesis, or East-West split; (ii) island formation and drainage vicariance; and (iii) the Northern Range acting as an isolating barrier.

Previous studies by Murphy & Collier (1996) and Jowers *et al.* (2008) found significant genetic structuring in *R. hartii* among both South American and Trinidadian populations according to sequence variation across the cyt *b* gene region. For comparative purposes, we aligned our cyt *b* sequence with published sequences (Murphy & Collier 1996; Jowers *et al.* 2008) to produce datasets of 349 bp ('short') and 745 bp ('long'), respectively. Two independent Bayesian estimations of phylogeny were performed in MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003) for each dataset. Conditions for analyses were the same for both datasets. We applied the GTR+I+G model following a likelihood ratio test and Akaike Information Criteria (AIC) implemented in MRMODELTEST 2.0 (Nylander 2004). The Bayesian posterior probability density was estimated in four independent runs. Each run consisted of four incrementally heated chains which ran for 5 million generations (sampled every 1000).

We calculated microsatellite allelic diversity, expected heterozygosity (H_E), and observed heterozygosity (H_O) with GENALEX 6.3 (Peakall & Smouse 2006). AMOVA and pairwise F_{ST} values (Weir & Cockerham 1984) were performed in ARLEQUIN with 10 000 permutations. The hierarchy of groupings used in the AMOVA was the same as in the haplotype analysis. Isolation-by-distance (IBD) analyses were performed using a Mantel test to test for significant relationships for straight-line geographic distance and linearized F_{ST} ($F_{ST}/(1-F_{ST})$ in GENALEX. The Bayesian clustering software Structure 2.1 (Pritchard *et al.* 2000) was also used to infer the number of populations (K) independent of spatial sampling. Given the possibility of gene flow among drainages, and because evidence of admixture has been found in co-occurring *P. reticulata* (Suk & Neff 2009; Willing *et al.* 2010), analyses were performed using the admixture model with correlated allele frequencies in three independent runs from K = 1–20, with a burn-in of 500 000 iterations followed by another 500 000 iterations. Selection of K was determined using two methods: (i) by plotting the negative log likelihoods [−ln P(D)] versus K, and (ii) using the ΔK method described in Evanno *et al.* (2005).

Results

Analysis of COI haplotype diversity and distributions

We found evidence of greater mtDNA haplotype variation across Trinidad, Tobago and Grenada than did other studies of the same regions (Murphy & Collier 1996; Collier *et al.* 1998; Murphy *et al* 1999; Jowers *et al.* 2008). The COI data yielded 39 unique haplotypes from 592 bp of sequence among the 205 individuals sampled from 20 locations. Sixty-one variable sites differentiated haplotypes that fall into two major groups, hereafter referred to as haplotype groups *A* and *B* (Table 1). A single A/T transversion distinguishes haplotype groups *A* and *B*. The majority of samples (n = 162) fall within haplotype group *A*. In comparison, only 41 individuals were recovered in haplotype group *B*. Among haplotype group differentiation was estimated at 0.6% sequence divergence. Variation among individual haplotypes within each group was slightly more pronounced within haplotype group *B* compared to haplotype group *A*, with average sequence divergence estimates of 0.3% and 0.2%, respectively.

Haplotypes were unequally distributed across the island of Trinidad (Table 1, Fig. 1). The geographic distribution of haplotypes groups generally conformed to a North-South split. Individuals from sites located south of the Northern Range Mountains largely exhibited haplotypes falling within group A, and individuals sampled to the north of the Northern Range Mountains, consisting of individuals collected at MA, PA, PP and Tobago, exhibited haplotypes in group *B*. The total number of haplotypes per site ranged from 1 to 6, with the highest number of haplotypes per site occurring at locations on the Caribbean slope of the Northern Range. The most common haplotype, which falls within haplotype group A (Table 1, Fig. 1), was found at every sample site in Trinidad except PP, MA, and PA. This

Table 1 Summary of mtDNA haplotype and microsatellite data

Code	Waterbody	Latitude	Longitude	Drainage basin	N_{msat}	A_R	H_O	H_E	N_{seq}	Haplotype group A	Haplotype group B
HG	Guanapo tributary	10.5865	−61.2369	Caroni	17	1.6	0.314	0.301	10	10	0
LL	Guanapo R.	10.7094	−61.2683	Caroni	19	1.4	0.184	0.163	10	10	0
AR	Aripo R.	10.6925	−61.2286	Caroni	10	2.9	0.75	0.727	4	4	0
SJ	St. Joseph R.	10.6892	−61.0350	Caroni	5	2.8	0.633	0.669	4	4	0
CA	Caparo R.	10.5039	−61.3722	Gulf of Paria	11	3.0	0.732	0.747	10	10	0
GU	Guaracara R.	10.3111	−61.3808	Gulf of Paria	12	3.1	0.872	0.776	12	12	0
CO	Coora R.	10.1575	−61.4747	Gulf of Paria	11	3.1	0.77	0.776	11	11	0
ER	Erin R.	10.1103	−61.5667	South slope	11	2.9	0.664	0.699	10	10	0
RO	Rocky R.	10.1297	−61.3006	South slope	15	3.0	0.847	0.765	15	15	0
PO	Poole R.	10.3311	−61.3000	Ortoire	16	3.0	0.781	0.772	13	13	0
CV	Charuma Village R.	10.4031	−61.1542	Nariva	20	3.1	0.902	0.806	20	20	0
LE	L′ Ebranche R.	10.4833	−61.0453	Nariva	11	2.6	0.566	0.661	11	11	0
TU	Turure R.	10.6756	−61.1650	N. Oropouche	12	2.5	0.532	0.622	12	6	6
PP	Primera Pria R.	10.7063	−61.0237	Atlantic Ocean	13	3.0	0.754	0.763	13	0	13
TO	Tompire R.	10.7814	−60.9508	Atlantic Ocean	11	2.5	0.643	0.619	11	11	0
SA	Salybia R.	10.8247	−60.9394	Carbbean slope	15	2.6	0.735	0.663	15	15	0
PA	Paria R.	10.7478	−61.2597	Carbbean slope	11	2.6	0.673	0.644	5	0	5
MA	Marianne R.	10.7625	−61.3056	Carbbean slope	11	3.0	0.865	0.758	12	0	12
TB	King's Bay, Tobago	11.1949	−60.7290		5	2.1	0.392	0.445	5		
GR	Grenada	12.1275	−61.6672		2	1.3	0.333	0.167	2		

N_{msat}, number of individuals microsatellite genotyped; A_R, mean allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity assuming Hardy–Weinberg equilibrium; N_{seq}, number of individuals sequenced.

haplotype was also absent from samples collected from Tobago and Grenada. Only four haplotypes were shared among sites (Haplotypes 1, 12, 21, 28; Table 1), and 33 haplotypes were drainage-specific. Only one haplotype in group A (Haplotype 1; Table 1) was found at Caribbean slope sites, and four haplotypes in group B (Haplotypes 31, 35, 36, 37; Table 1) were found to the south of the Northern Range. Only one location, Turure River (TU) harbored both northern and southern haplotypes (Table 1, Fig. 1). Individuals sampled from Grenada and Tobago exhibited haplotypes that did not place within either of the two major groups.

The number of basin-specific haplotypes per drainage-basin ranged from 0 to 8, with the Gulf of Paria, the Caribbean slope, and PP (from the Atlantic) showing the highest number of basin-specific haplotypes and highest amounts of both within and among basin differentiation. Of the haplotypes found only in Caribbean slope locations, five were found exclusively within the Marianne River (MA), which itself comprises two divergent haplotype subgroups (*B1*, *B2*). Subgroup *B1* consisted of four MA-specific haplotypes that differed in one A-G transition from all other haplotypes within haplotype group B (Fig. 2). The more divergent subgroup *B2* consisted of four haplotypes sampled from both the MA and PA sites (Fig. 2). Among the southern haplotypes within the Gulf of Paria drainage, sites CA and GU each support four unique haplotypes.

A mean estimate of genetic differentiation (Φ_{ST}) among sites based on COI haplotype frequencies was 0.368. Pairwise Φ_{ST} estimates revealed significant differentiation among all but one pairwise comparison of Caribbean slope and Atlantic sites according to graphically sharpened FDR *q*-values (Table 2). Fewer pairwise comparisons were significant following a more conservative Bonferroni correction, including most comparisons among Southern sites (Table 2). For Caribbean slope sites, both MA and SA showed significant pairwise differentiation with all sites, except AR, and SJ. The Marianne River (MA) did not show significant differentiation from the Paria River (PA). PA showed no significant differentiation with any sites within the Gulf of Paria drainage despite high Φ_{ST} values, which may reflect reduced power due to low sample size. From the Atlantic drainages, PP showed a pattern similar to MA and SA, with significant differentiation with all sites except AR and SJ from the Caroni drainage. TO showed no significant differentiation with any site except for MA. No significant differentiation was detected for pairwise comparisons among Tobago and all other sites,

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
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except MA, SA, TU and the Guanapo sites (HG, LL). The individuals from Grenada were significantly differentiated from those sampled at SA, MA, LE and TU.

Analysis of molecular variance based on COI haplotype frequencies found evidence of significant differentiation among Northern locations (MA, PA), Southern locations (all other pops), Tobago and Grenada.

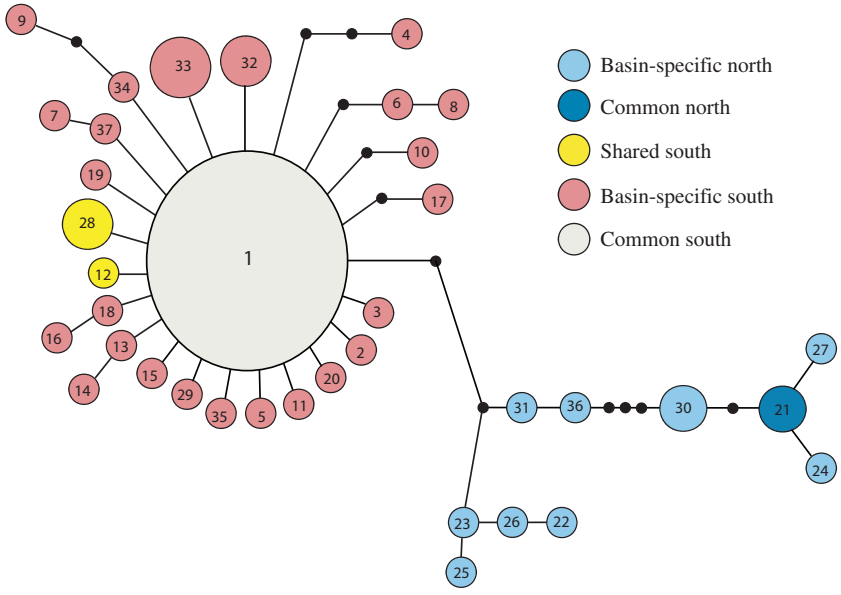

Fig. 2 Statistical-parsimony haplotype frequency network of cytochrome *c* oxidase subunit I sequence variation for *Rivulus hartii* sampled across Trinidad. Numbers refer to respective haplotype numbers from Table 1.

Table 2 Pairwise estimates of population differentiation by watershed. a) mtDNA Φ_{ST} values. Below diagonal are Φ_{ST} values, bold values indicate comparisons that are not significant ($P > 0.05$) following Bonferroni correction. Above diagonal are graphically-sharpened FDR q-values (P-values). (b) Microsatellite-based estimates are below diagonal (F_{ST}, Weir & Cockerham 1984), bold values indicate comparisons that are not significant ($P > 0.05$) following Bonferroni correction. Above diagonal are graphically-sharpened FDR q-values (P-values)

	Caroni				Gulf of Paria			South Slope		Ortoire	Nariva		Oropouche	Atlantic		North Slope			Tobago	Grenada
	HG	LL	AR	SJ	CA	GU	CO	ER	RO	PO	CV	LE	TU	PP	TO	SA	PA	MA	TB	GR
(a) *Sequence-based* Φ_{ST} *values*																				
HG		**0.053**	0.011	0.005	0.006	0.008	0.001	0.005	0.002	0.014	0.017	0.019	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.001
LL	**0.000**		0.000	0.005	0.003	0.003	0.000	0.003	0.002	0.006	0.006	0.012	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.001
AR	**0.191**	**0.420**		0.035	0.042	0.016	0.015	0.031	0.014	0.015	0.005	0.008	0.005	0.000	0.009	0.000	0.002	0.002	0.001	0.008
SJ	0.328	**0.570**	**0.000**		0.016	0.012	0.009	0.011	0.006	0.008	0.003	0.007	0.002	0.000	0.006	0.000	0.001	0.001	0.001	0.012
CA	**0.131**	0.244	**−0.052**	**0.041**		0.018	0.006	0.017	0.004	0.014	0.003	0.005	0.001	0.000	0.003	0.000	0.000	0.000	0.000	0.002
GU	**0.076**	0.174	**−0.040**	**0.059**	**−0.003**		0.005	0.010	0.003	0.023	0.008	0.011	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.002
CO	0.247	0.344	**0.048**	**0.131**	**0.080**	**0.094**		0.004	0.001	0.002	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.001
ER	**0.172**	0.289	**−0.017**	**0.076**	**0.022**	**0.028**	**0.107**		0.003	0.009	0.003	0.005	0.001	0.000	0.002	0.000	0.000	0.000	0.000	0.001
RO	0.268	0.370	**0.119**	**0.208**	**0.130**	**0.132**	0.207	**0.159**		0.002	0.001	0.002	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
PO	**0.026**	**0.114**	**0.005**	**0.113**	**0.025**	**0.001**	**0.133**	**0.059**	**0.160**		0.020	0.018	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.002
CV	**−0.001**	**0.057**	**0.120**	0.238	0.105	**0.059**	0.220	**0.142**	0.235	**−0.005**		0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
LE	**−0.002**	**0.088**	**0.108**	0.233	**0.089**	**0.046**	0.203	**0.126**	0.226	0.013	**0.014**		0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000
TU	0.401	0.500	**0.121**	0.190	0.158	0.194	0.204	0.181	0.281	0.260	0.372	0.349		0.000	0.000	0.000	0.000	0.000	0.000	0.000
PP	0.754	0.841	0.557	0.610	0.501	0.531	0.508	0.522	0.586	0.596	0.677	0.697	0.464		0.000	0.000	0.000	0.000	0.000	0.001
TO	0.362	0.485	**0.150**	**0.236**	0.160	0.174	0.227	0.189	0.289	**0.110**	0.314	0.305	0.276	0.594		0.000	0.000	0.000	0.000	0.001
SA	0.741	0.831	0.562	0.616	0.497	0.520	0.507	0.519	0.580	0.582	0.658	0.683	0.489	0.736	0.595		0.000	0.000	0.000	0.000
PA	0.731	0.870	0.403	0.480	0.384	0.430	0.410	0.410	0.510	0.517	0.635	0.648	0.358	0.682	0.507	0.709		0.004	0.001	0.003
MA	0.458	0.549	0.157	0.218	0.196	0.242	0.232	0.218	0.317	0.316	0.433	0.406	0.182	0.441	0.300	0.472	0.100		0.000	0.000
TB	0.865	1.000	0.630	0.706	0.526	0.561	0.534	0.552	0.627	0.641	0.732	0.778	0.491	0.799	0.641	0.817	0.800	0.457		0.004
GR	0.826	1.000	0.420	0.529	0.401	0.458	0.435	0.433	0.551	0.560	0.688	0.720	0.372	0.753	0.550	0.780	0.695	0.331	1.000	
(b) *Microsatellite-based* F_{ST} *values*																				
HG		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
LL	0.429		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
AR	0.378	0.491		0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.018
SJ	0.490	0.642	0.137		0.116	0.002	0.001	0.000	0.000	0.001	0.006	0.002	0.000	0.001	0.000	0.000	0.001	0.000	0.008	0.048
CA	0.377	0.472	0.082	**0.035**		0.005	0.002	0.000	0.000	0.004	0.001	0.006	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.010
GU	0.380	0.478	0.099	**0.085**	**0.039**		0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
CO	0.352	0.478	**0.070**	**0.118**	**0.070**	**0.059**		0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.006
ER	0.407	0.540	0.131	0.190	0.122	0.106	**0.057**		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.012
RO	0.366	0.442	0.109	0.121	0.059	0.054	0.069	0.098		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
PO	0.368	0.452	0.087	**0.097**	**0.041**	**0.047**	0.063	0.131	0.061		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.007
CV	0.363	0.462	0.099	**0.060**	**0.047**	0.054	0.062	0.116	0.067	0.060		0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.005
LE	0.448	0.531	0.121	**0.088**	**0.060**	0.088	0.147	0.209	0.129	0.130	0.096		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011
TU	0.456	0.556	0.191	0.241	0.187	0.220	0.201	0.277	0.212	0.189	0.188	0.249		0.000	0.000	0.000	0.000	0.000	0.000	0.012

Table 2 (continued)

	Caroni				Gulf of Paria			South Slope		Ortoire	Nariva		Oropouche	Atlantic		North Slope			Tobago	Grenada
	HG	LL	AR	SJ	CA	GU	CO	ER	RO	PO	CV	LE	TU	PP	TO	SA	PA	MA	TB	GR
PP	0.397	0.483	0.094	**0.096**	**0.050**	0.057	0.078	0.106	0.070	0.078	**0.038**	0.106	0.215		0.000	0.000	0.000	0.000	0.000	0.009
TO	0.467	0.553	0.184	0.227	0.173	0.157	0.154	0.167	0.157	0.156	0.164	0.253	0.308	0.180		0.000	0.000	0.000	0.000	0.015
SA	0.389	0.483	0.146	0.176	0.094	0.145	0.107	0.138	0.160	0.139	0.144	0.216	0.250	0.172	0.149		0.000	0.000	0.000	0.009
PA	0.461	0.575	0.204	**0.188**	0.158	0.160	0.197	0.179	0.191	0.211	0.148	0.177	0.307	0.132	0.268	0.224		0.000	0.000	0.010
MA	0.400	0.507	0.103	0.138	0.116	0.089	0.118	0.165	0.120	0.116	0.092	0.123	0.230	0.071	0.202	0.213	0.105		0.001	0.006
TB	0.576	0.743	**0.305**	**0.304**	0.283	0.263	0.289	0.315	0.277	0.276	0.263	0.344	0.376	0.286	0.394	0.368	0.314	**0.283**		0.050
GR	**0.706**	**0.832**	**0.382**	**0.354**	**0.344**	**0.340**	**0.301**	**0.406**	**0.337**	**0.311**	**0.299**	**0.405**	**0.416**	**0.311**	**0.445**	**0.406**	**0.446**	**0.368**	**0.584**	

Partitioning of genetic variation among sites grouped by the Island formation (drainage-vicariance) hypothesis, and the 'Two-arc hypothesis' produced less pronounced and non-significant estimates of structure, respectively (Table 3). Nearly three times more genetic variation was explained by grouping sites according to the Northern Range barrier hypothesis (27.09%) versus the drainage-vicariance (9.44%) or the 'Two-arc' (5.44%) hypotheses. This result suggests a strong North-South pattern of differentiation in *Rivulus hartii* from Trinidad.

Analysis of cytochrome b *sequence variation*

A total of 126 variable sites and 35 haplotypes were identified from the 745 bp region of cyt *b* obtained for 79 individuals representing all recovered COI haplotypes. Of the 35 recovered 'long' cyt *b* haplotypes, only one matched a haplotype (of eight) recovered by Jowers *et al*. (2008). This haplotype was reported to be common and widespread in Jowers *et al*. (2008), being collected from the following sites: LaLune, Lopinot, Mt Harris, Mt Benedict and Mt Tamana. However, this cyt *b* haplotype sequence corresponded to five COI haplotypes (CA-H8, CV-H12, GU-H17, RO-H32, TU-H35). Only five haplotypes were recovered from MA using cyt *b* compared with six haplotypes using COI; both COI haplotype 23 and 25 from MA (Table 1) were found to form a single haplotype with cyt *b*. Five cyt *b* haplotypes were also recovered from CO in the South Gulf of Paria drainage; each corresponding to a respective COI haplotype.

Table 3 Results of comparative AMOVAs for three geographically hypothesized relationships among *Rivulus hartii* populations

Source of variation	mtDNA COI	Microsatellites
'Two-Arc' East-West split		
Among groups	5.44*	2.82*
Among populations within groups	34.17	22.23
Within populations	60.29	74.88
Island formation and drainage vicariance		
Among groups	9.44	3.35*
Among populations within groups	30.43	20.97
Within populations	60.13	75.68
North Range Barrier		
Among groups	27.09	5.64
Among populations within groups	24.13	21.77
Within populations	48.78	72.59

COI, cytochrome *c* oxidase subunit I.
*Denotes a non-significant result.

Both 'long' and 'short' analyses recovered four groups among the individuals sampled from Trinidad (Figs 3 and 4). Bayesian phylogenetic analyses recovered topologies that are indicative of significant differentiation among individuals from Blue Basin, other Caribbean slope drainages, Atlantic drainages, and all other Southern drainages (Figs 3 and 4). The four individuals from Blue Basin, representing haplotype 1 in Jowers *et al.* (2008), were the most divergent samples within the dataset (~8% from Tobago and all other samples). The Northern-Southern geographic split among COI haplotypes (Figs 1 and 2) was evident within the 'long' cyt *b* analysis; however, no diagnostic base pair substitutions characterize the observed split, and northern Tucker Valley samples from Jowers *et al.* (2008) nests within the lineage that includes southern haplotypes (Figs 3 and 4).

The 'short' analysis revealed strong differentiation among mainland and island samples. Mainland samples were highly differentiated from island samples. Of the mainland samples, an individual from the Paria Peninsula in Venezuela aligned closest with the Blue Basin samples from Jowers *et al.* (2008), although a haplotype recovered by Murphy *et al.* (1999, GenBank accession no. AF002496.1) from the New River nested within the most divergent MA lineage sampled on Trinidad. *Rivulus hartii* individuals from Caracas exhibited 10.1% sequence divergence from the Blue Basin-Paria Peninsula clade. Other species of *Rivulus* from the mainland, such as *Rivulus amphoreus* and *Rivulus stagnatus*, yielded divergence estimates in excess of 20% from all *R. hartii* samples. *Rivulus cryptocallus*, the rivuliid found on the island of Martinique, exhibited 21% sequence divergence from *R. hartii* individuals sampled from the mainland and all islands, as compared to 6.7% sequence divergence from its putative sister species, *R. stagnatus* from Guyana (Murphy & Collier 1996).

Divergence among islands was extensive, with samples from Grenada being more divergent than those from Isla Margarita, Trinidad and Tobago. Samples from Grenada were more strongly differentiated (6.7% sequence divergence) from individuals sampled from Tobago and Isla Margarita than were individuals from Trinidad and Isla Margarita (3.6% sequence divergence). Samples from northern Trinidad were 3.1% divergent from individuals that we collected from King's Bay, Tobago, whereas the haplotype recovered by Jowers *et al.* (2008) from the Argyle River on Tobago nested among samples collected from the Caribbean (MA, PA) and Atlantic slope (PP) of Trinidad. Individuals from King's Bay exhibit 4.1% sequence divergence relative to those from Argyle River.

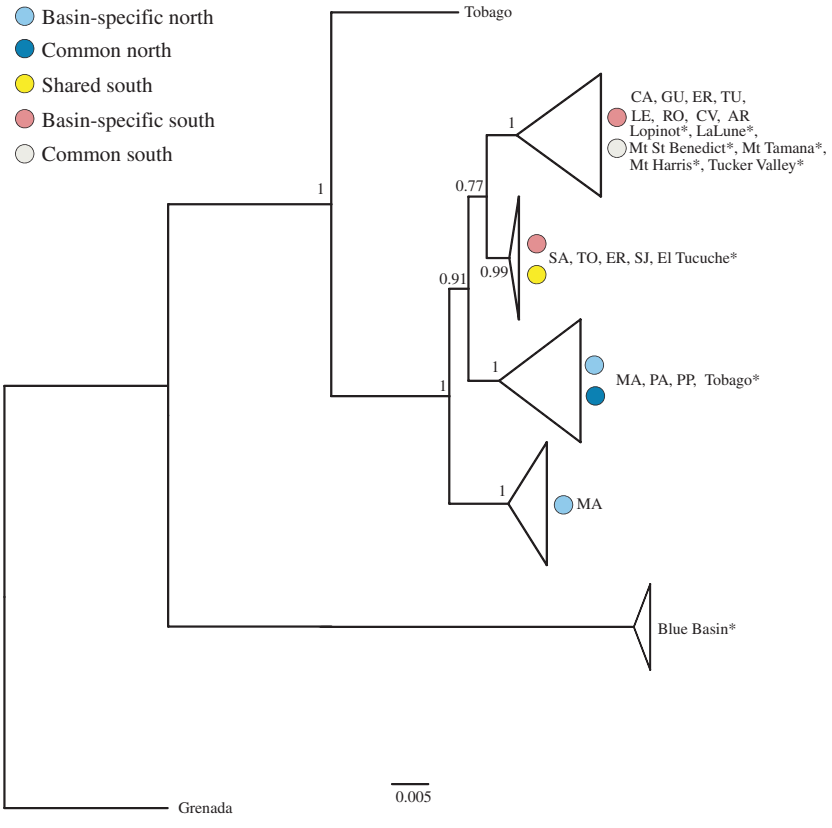

Fig. 3 Bayesian phylogram from combined 'long' dataset of 749 bp cyt *b*. *Denotes samples from Jowers *et al.* (2008).

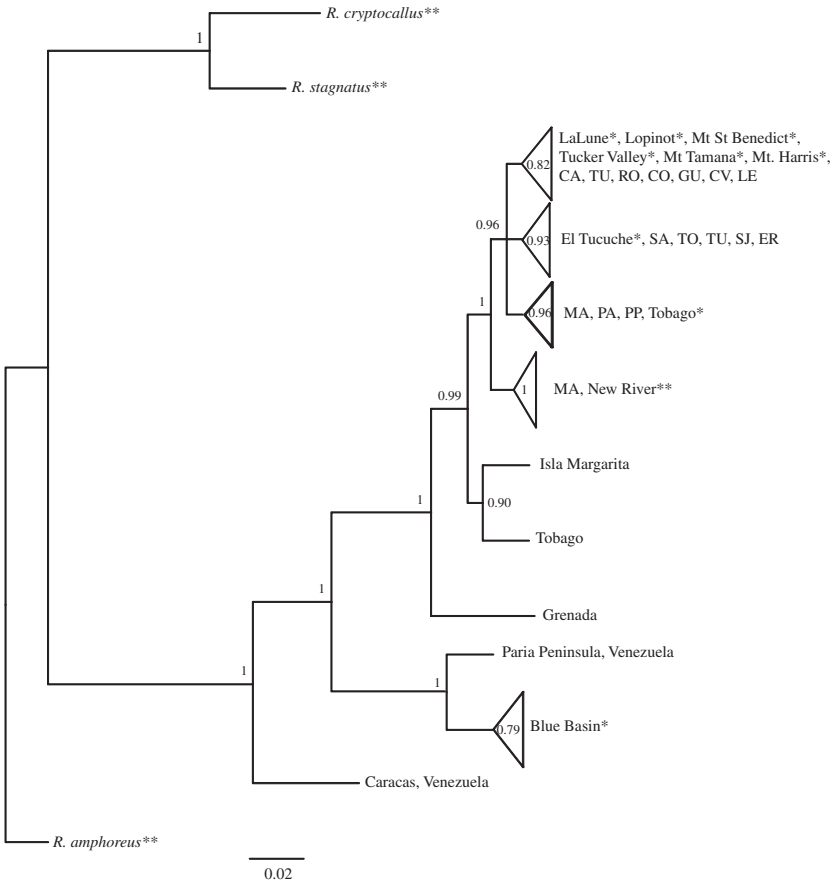

Fig. 4 Bayesian phylogram of the 'short' combined cyt *b* dataset. *Denotes samples from Jowers *et al.* (2008). **Denotes samples from Murphy *et al.* (1999). Samples from Isla Margarita, Paria Peninsula, and Caracas are unpublished sequences used in Collier *et al.* (1998), and Jowers *et al.* (2008).

Microsatellite genetic diversity

Departures from HWE were not significant for all sites following Bonferroni correction except for ER and PO at locus Rha3. No significant linkage disequilibrium among loci was detected. The mean number of alleles per locus (N_A) was 5.6, with a mean allelic richness of 2.6 across all loci. Among Trinidad sites, allelic richness was lowest in the Guanapo River samples (HH, LG), with values of 1.6 and 1.4, respectively (Table 1). Observed heterozygosity (H_O) ranged from low values of 0.184 and 0.314 in Guanapo River populations of the Caroni drainage to as high as 0.872 in the Guaracara River (GU) and 0.902 in the Charuma Village River (CV) (Table 2). Observed heterozygosity (H_O) exceeded expected heterozygosity for 12 of the 20 sampling sites (Table 2).

Microsatellite differentiation and geographic subdivision

We found high genetic differentiation among populations of *R. hartii* with a global F_{ST} = 0.241 (P = 0.0001) based upon variation across all six microsatellite loci. F_{ST} estimates were high for all pairwise comparisons, and all were significant according to graphically-sharpened FDR q-values (Table 2). However, not all comparisons were significant following Bonferroni correction (Table 2). All pairwise comparisons with Grenada were non-significant, likely due to only two individuals genotyped from Grenada. The least differentiated sites were SJ, CO, CA, CV, and GU, as evidenced by a higher number of non-significant pairwise F_{ST} estimates (Table 2). Given the small number of sites within drainages, few within drainage comparisons can be made; however, the Caroni River drainage showed the highest amount of within drainage variation with a mean F_{ST} of 0.442 for all within drainage pairwise comparisons (Table 2). No pattern of IBD was found among populations (r^2 = 0.005, P = 0.520).

AMOVA-based comparisons of hypothesized geographical relationships among sample sites showed significant among group variation only for the Northern Range barrier analysis (5.64%, P = 0.000). In comparison, non-significant results were found for group-level variation according to the 'Two-Arc' hypothesis (2.82%, P = 0.223) and the island-formation hypothesis (3.35%, P = 0.268). Variation among populations and individuals was highly significant regardless of how sample sites were grouped (Table 3).

The results of the Bayesian clustering analysis of multilocus genotypes in STRUCTURE showed a peak in posterior probabilities (−ln *P*(D)) at $K = 8$, whereas ΔK values indicated that sampled individuals corresponded to two populations. For both $K = 8$ and $K = 2$ the Guanapo samples (LL, HG) formed a single population. As $K = 2$ precludes finer-scale resolution among the non-Guanapo samples, we evaluated the nature of groupings recovered at $K = 8$. Accordingly, individual *Q* estimates were grouped by drainage and visualized in DISTRUCT 1.1 (Rosenberg 2004). The eight clusters were well defined by the high proportion of membership for sample sites to a given cluster. The clusters did not always correspond to individual drainage basins and major regional breaks; however, some geographic structuring was evident across Trinidad. Samples grouped according to the following configuration: the Guanapo River (LL, HG), Western (CA, AR, SJ, GU) the North (MA, PA), the North-East (SA, TO), two clusters in the South (ER, CO and RO plus PO), and two clusters in the East (LE plus TU). This pattern suggests that genetic variation is partitioned according to the direction of drainages across the island (i.e. drainage to the Caribbean, Atlantic Ocean, Gulf of Paria, etc.). Two island clusters (TB and GR) were also recovered (Fig. 5). Population-level admixture was most evident in Western (CA, AR, SJ, GU) and South sites (CO, ER; Fig. 5). For these sites, the maximum proportion of individuals assigned to one of the eight clusters was 49% (pop $Q < 0.50$). Site CV harbored the highest number of admixed individuals, with nearly 40% of CV individuals exhibiting shared ancestry with individuals from the North cluster. *Rivulus hartii* individuals with mixed ancestry were also more common at sites with elevated population-level admixture (GU, AR, SJ) suggestive of some increased dispersal and gene flow into these sites compared to the others.

Discussion

This study provides one of the most geographically comprehensive perspectives on Trinidadian freshwater phylogeography to date. The population history of *Rivulus hartii* on Trinidad reflects complex patterns of shared ancestry and admixture comparable to recent findings for co-occurring populations of *Poecilia reticulata*. Our results indicate that geographic patterns of genetic differentiation likely reflect multiple founding events, as well as limited exchange across major geological barriers and river drainages. Comparisons to *R. hartii* on neighboring islands also indicate that evolutionary diversification is not necessarily dependent on the number of colonization events, and that dispersal to the Lesser Antilles and continental shelf islands off South America does not always reflect proximity to a mainland source or proximity among islands.

Geographic patterns of genetic variation in Rivulus hartii *on Trinidad*

Biogeographic studies of Trinidadian freshwater fishes have largely focused on understanding genetic relationships among *P. reticulata* populations across the Northern Range. While evolutionary diversification has been demonstrated across small spatial scales (Crispo *et al.* 2006; Willing *et al.* 2010), most studies have focused on patterns of genetic variation among populations from the Caroni, Oropouche and Caribbean slope drainages. Allozyme and mtDNA data provide evidence for two major lineages corresponding to the Oropouche and the Caroni drainages (Carvalho *et al.* 1991; Fajen & Breden 1992), which is more consistent with differentiation following two independent colonization events (the 'two-arc' hypothesis) rather than island formation (Kenny 1995). Despite finding marked divergence among east-

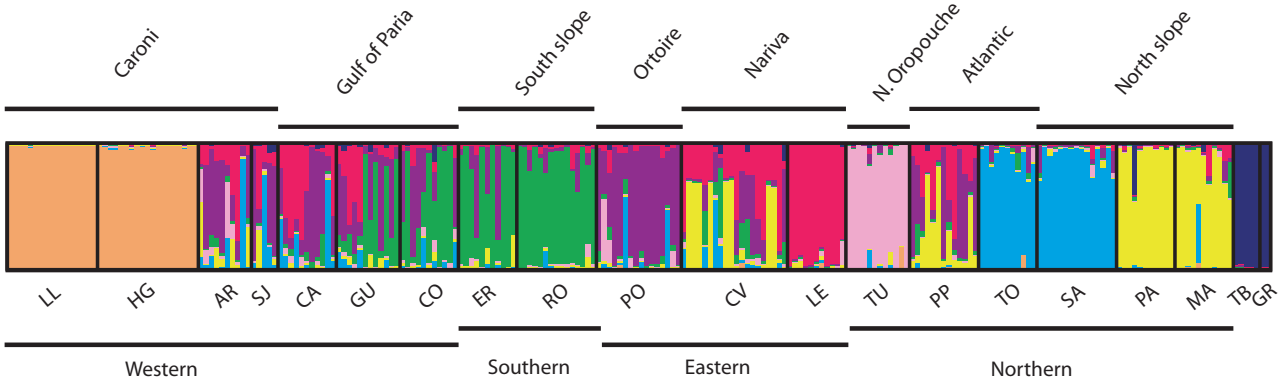

Fig. 5 Bayesian estimate of population structure ($K = 8$) based on microsatellite variation among *Rivulus hartii* populations across Trinidad, Tobago and Grenada by watershed using Structure 2.1.

ern and western *R. hartii* populations, our results do not correspond to an east-west split, and thus do not support the 'two-arc' hypothesis (Table 3). Rather, the distribution of mtDNA haplotypes and geographic patterns of microsatellite genetic variation are more consistent with patterns of 'shared ancestry and admixture' found by Suk & Neff (2009) and Willing *et al.* (2010), who suggest that patterns of genetic variation are attributable to multiple founding events and limited post-colonization dispersal among drainage basins.

Geographic patterns of genetic differentiation among *R. hartii* sampled across Trinidad reflect a history of differentiation and admixture similar to that of *P. reticulata*. We recovered patterns of extensive mtDNA diversification across Trinidad, where haplotypes exhibit as much as 2% pairwise sequence divergence. Both COI and cyt *b* haplotypes have distributions suggestive of site- and basin-level differentiation resulting from geographic isolation (e.g. Willing *et al.* 2010). We did not detect the most common haplotype at three sites (MA, PA, and PP), and almost 85% of haplotypes were found in one basin or one site within a basin, with some sites such as the Marianne River (MA) harboring as many as five exclusive haplotypes. Nonetheless, recovery of several well-supported haplotypes groups in phylogenetic analyses—including two major haplotypes groups that are largely distributed to the north or south of the Northern Range (Fig. 1)—provides evidence of shared ancestry among drainage basins. Similarly, Bayesian analysis of microsatellite variation recovered several distinct genetic clusters corresponding to drainage direction (i.e. Caribbean, Atlantic, Gulf of Paria) rather than site- or basin-specific divergence. However, varying levels of admixture were found among the sampled populations, with admixture being more prevalent among sites to the south of the Northern Range relative to Caribbean slope sites.

The degree of differentiation across Trinidad appears to vary according to the permeability of major geological barriers and drainage direction. Most gene flow occurs among proximal drainages, such as between the eastern sites CV and LE, or between the Caribbean slope sites MA and PA. Little evidence of dispersal was found among more distant locations (e.g. CV and PP), particularly among locations with different drainage directions. Nonetheless, some dispersal occurs across the Northern Range mountains. Southern haplotypes are present at the northeastern peninsular sites SA and TO, and northern haplotypes are present at site PP that drains to the south of the Northern Range. Bayesian analysis of microsatellite variation also recovered evidence of connectivity between the northern MA and PA sites and the northeastern site PP, which suggests the possibility of ongoing dispersal across the northeastern Caribbean and Atlantic slopes of the mountain range. The Northern Range is also permeable near site TU–the only site harboring both northern and southern haplotypes. Unlike the conditions along the northeastern slopes of the Northern Range, admixture at site TU also appears to have resulted in the formation of novel (rather than admixed) genotypes. Further comparisons to other sites within this drainage, however, will be necessary to confirm whether dispersal across the Northern Range promotes evolutionary diversification.

Permeability of the Northern Range at site TU could be due to headwater capture, but it may also reflect active dispersal of *R. hartii* among drainages via overland dispersal. Travel across damp-leaf litter (Seghers 1978) has been described in *R. hartii* with populations documented in pools away from nearby rivers (Reznick 1982; Costa 1987). However, it is likely that overland dispersal would give rise to a pattern of IBD among populations, as sites in proximity are more likely to exchange migrants. IBD has been shown among sites within a single river in *P. reticulata* (Crispo *et al.* 2006), but not among rivers and basins (Suk & Neff 2009). As we found no support for IBD among the sampled populations of *R. hartii*, headwater capture is a more plausible explanation for the observed patterns of admixture at site TU, which is located near a low elevation pass that crosses the Northern Range. Similarly, oceanic dispersal is a plausible explanation for patterns of shared ancestry and admixture among northern and eastern sites.

Prior studies (Jowers *et al.* 2008) proposed that the distribution of genetic variation among Trinidadian *R. hartii* populations reflects elevation, where patterns of divergence are a consequence of episodic flooding and recolonization of lowland regions. Our findings are inconsistent with this hypothesis, although we did find some evidence suggestive of divergence within drainages according to changes in elevation. *Rivulus hartii* are capable of colonizing high elevation reaches by bypassing or ascending waterfalls and rapids (Gilliam & Fraser 2001), therefore populations above in-stream barriers may be genetically divergent from populations at lower reaches as a consequence of genetic drift. Bayesian analysis of population structure recovered a single cluster with no admixture for the HG and LL sites in the Caroni drainage. These sites are high within the Guanapo River basin, and despite low levels of genetic diversity relative to other sites on Trinidad, these populations do not appear to have undergone a recent bottleneck. Comprehensive longitudinal comparisons will be necessary to determine the extent to which sites HG and LL are differentiated from lower Caroni sites, but studies of *P. reticulata* in the Caroni drainage and elsewhere (Crispo *et al.* 2006; Barson *et al.* 2009)

indicate that in-stream barriers are largely responsible for within-basin patterns of genetic divergence.

Although the observed geographic patterns of genetic variation in *R. hartii* are largely consistent with those reported for *P. reticulata* (Suk & Neff 2009; Willing *et al.* 2010), some subtle differences are apparent. For example, we found no evidence that *R. hartii* within the Oropouche drainage are closely related to those in the Caroni drainage, as Suk & Neff (2009) found for *P. reticulata*. Suk & Neff (2009) also report lower levels of divergence among *P. reticulata* populations within the Oropouche drainage compared to populations in the Caroni drainage. We found that *R. hartii* populations to the east of the Central Range (Oropouche, Nariva) exhibit higher levels of differentiation across microsatellite loci compared to populations to the west of the Central Range (Caroni drainage). It is possible that reduced differentiation among western sites may be the result of interbasin dispersal facilitated by seasonal reductions in salinity (>50%) due to elevated discharge from the Orinoco river into the Gulf of Paria (Alkins & De Souza 1984; Read 1987; Kenny 1995; Jowers *et al.* 2008). However, eastern sites also showed evidence of gene flow with populations on the Caribbean slope (a pattern that was not found among western populations), which raises the possibility that higher estimates of differentiation among eastern populations reflect the presence of novel genotypes at site TU.

Freshwater fish island biogeography

Comprehensive phylogenetic comparisons of *Rivulus* species have established the South American origin of species inhabiting continental shelf and islands in the Lesser Antilles (Murphy & Collier 1996). Our findings detail patterns of diversification across populations of *R. hartii*, providing evidence of independent founding events within and among islands. Phylogenetic and phylogeographic analysis of new and published mtDNA sequence data indicate that haplotypes from Trinidad, Tobago, Grenada, Isla Margarita, and South America are highly differentiated from one another. Relationships among mainland and Trinidadian haplotypes also indicate that southern Trinidadian haplotypes likely arose on Trinidad from one recent common ancestor, and that southern and Caribbean slope haplotypes do not share a recent common ancestor. It remains unclear, however, whether Caribbean slope haplotypes originate from one or more different founding populations. The distribution of haplotypes that we recovered across Caribbean slope drainages could be attributable to a single, diverse founding population, but comparisons of our findings with data from previous studies provide evidence for multiple founding events. Resolving the number of independent colonization events across Trinidad will require additional information on mainland populations, particularly from coastal areas of Venezuela.

Colonization of continental shelf and oceanic islands by *Rivulus* does not necessarily follow a stepping stone model where the progression of island colonization reflects proximity to a mainland source or proximity among islands. Rather, island occupancy appears to have resulted from a combination of independent colonization events and stepping stone colonization. Some islands, such as Grenada and perhaps Martinique, appear to have only been colonized a single time, whereas others appear to have been colonized multiple times. Like on Trinidad, individuals of *R. hartii* sampled from Tobago do not appear to be derived from a single founding population. Jowers *et al.* (2008) described a relationship between Tobago samples from the Argyle River and those sampled from the Paria River (PA) on the Caribbean slope of Trinidad. The haplotype we recovered from individuals sampled from King's Bay on Tobago aligned with an *R. hartii* haplotype recovered from Isla Margarita, indicating that Tobago has been colonized multiple times from different source populations.

Our findings suggest that evolutionary diversification of *Rivulus* on continental shelf and oceanic islands is not necessarily dependent on the number of colonization events. Although genetic variation appears to be lower on islands that likely have only been colonized once (e.g. Grenada), within-island genetic variation also reflects post-colonization diversification. For example, *R. hartii* to the south of the Northern Range on Trinidad are probably all derived from a single founding event, but these populations nonetheless exhibit within and among basin patterns of genetic differentiation. Like other studies of genetic variation among *R. hartii* populations on Trinidad that recovered patterns of differentiation suggesting the importance of post-colonization isolation (Jowers *et al.* 2008), our results indicate that post-colonization differentiation across Trinidad reflects isolation across major geological barriers (e.g. the Northern Range) as well as microgeographic vicariance among river basins and perhaps among sites across elevational gradients within basins. Inter-basin exchange might also be influenced by historical conditions, like glacial cycling, that can alter major drainage patterns (Clapperton 1993). Climate driven changes in sea level, for example, has repeatedly isolated and connected continental shelf islands off northern South America—including Trinidad, Tobago and Isla Margarita—that are part of a single mountain range (Bellizzia & Dengo 1990; Clapperton 1993; Alexander *et al.* 2006). Yet the extent of genetic structuring among islands and among *R. hartii*

populations on Trinidad suggests that, although some contemporary dispersal may occur due to seasonal variation in freshwater flows into coastal waters, extensive dispersal via river confluences during periods of low sea level is unlikely.

It is also possible that genetic differences among *R. hartii* populations on Trinidad are due to adaptive rather than neutral processes. The species' secondary freshwater life history and ability to disperse over land would be expected to constrain divergence among populations, particularly among southern sites that lack recognizable barriers to dispersal. It is therefore possible that divergence reflects differential selective pressures facilitating local adaptation. For example, recent work in Trinidad points to fitness differences among *R. hartii* populations that co-occur with guppies compared to those that occur in the absence of guppies. Walsh & Reznick (2009) found that *R. hartii* co-occurring with *P. reticulata* exhibit accelerated growth rates and higher egg production, and demonstrated a strong association between resource availability and growth. This finding suggests that gene flow could be limited among sites if life histories (i.e. spawning periodicity) shift in response to microgeographic variation in conditions affecting resource availability (i.e. density-dependent intraspecific or interspecific competition). The possibility of selection driving differentiation among *R. hartii* populations remains speculative, but since adaptive divergence does contribute to patterns of geographic variation among *P. reticulata* across Trinidad (Willing *et al.* 2010), it would be worthwhile to investigate how selection might be acting on *R. hartii*. Doing so would help determine whether multiple evolutionary processes have given rise to the comparable patterns of differentiation in *R. hartii* and *P. reticulata*, and whether adaptive diversification is more common among members of insular freshwater fish assemblages than has been previously thought (Langerhans *et al.* 2007).

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Appendix 1

Rivulus hartii microsatellite library construction summary:

Four DNA libraries enriched for tetranucleotide sequences (2× GACA, 2× CATC) were prepared using the magnetic bead enrichment procedure of Fischer & Bachman (1988).

Table A1 Summary of microsatellite primers for *Rivulus hartii*, H_O = observed heterozygosity, H_E = expected heterozygosity

Locus	GenBank accession no.	Repeat motif	Sequence 5′–3′		Range	No. of alleles	H_O	H_E
Rha3	HQ634861	$(CATC)_{10}$	F	GATACAGTAGTTTGGATGGCTCA	90–238	34	0.631	0.657
			R	CAGTGATTGCCAGAGATGG				
Rha5	HQ634862	$(GATG)_{14}$	F	GTTGCAGGAGGAAAAAGTGG	102–198	26	0.743	0.719
			R	AAAAGCAGCTGGTCAGAAGG				
Rha10	HQ634863	$(CCAA)_{11}$	F	TCAAACCAGTCAGCCATTCA	78–202	23	0.662	0.662
			R	TTCTGTAGCCTTAGCAGGTTTTT				
Rha16	HQ634864	$(CATC)_{10}$	F	GAGCCCCTTAATGCATGTGT	114–214	22	0.755	0.675
			R	CACCAGTGACCTCTGTGACC				
Rha18	HQ634865	$(CATC)_{13}$	F	CCTGTGACCCTGAAAGGAAG	108–168	17	0.695	0.644
			R	GCCCACTCCCCACAATCTA				
Rha30	HQ634866	$(TGGA)_8$	F	GTAAAACGACGGCCAGTGAGA CACCAGCTCGCCCTGAC	88–120	8	0.395	0.406
			R	TGCCCCACAAATATTTCCAATCA				

Reference

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