Shallow phylogeographic structure of Puerto Rico freshwater crabs: an evolutionary explanation for low species diversity compared to Jamaica

CHRISTOPH D. SCHUBART[1], NICOLE T. RIVERA[1], KEITH A. CRANDALL[2] & TOBIAS SANTL[1]

[1] *Biology 1, University of Regensburg, 93040 Regensburg, Germany*
[2] *Department of Biology, Bringham Young University, Provo, UT 84602-5255, U. S. A.*

ABSTRACT

Freshwater crabs constitute a common faunal component of tropical and subtropical river systems. They have a worldwide occurrence in these warmer regions, being represented by different taxonomic lineages on different continents or even within continents. Due to their mostly direct development and assumed dependency on fresh water, freshwater crabs are considered reliable model organisms to genetically reconstruct the hydrographical history of a region. However, very few studies have been carried out to directly document within-river dispersal or overland dispersal of these crabs. Thus the questions remain, in how far the restriction to river systems is comparable throughout the different taxa of freshwater crabs, and if all of the taxa can be used similarly well to reconstruct the history of watersheds, orogeny, island formation, and continental drift. In the current study, we analyze the phylogeographic structure of *Epilobocera sinuatifrons* (Decapoda: Brachyura: Pseudothelphusidae), a freshwater crab species endemic to the Caribbean island of Puerto Rico. Results show limited morphometric and genetic (mitochondrial and nuclear DNA) differentiation among metapopulations along a west-east gradient, paralleling the direction of the main mountain chain. The north-south comparison, in turn, does not show any differentiation, suggesting that the crabs must be able to migrate between headwaters of unconnected river systems. These results are compared to recently published ones on phylogeographic structure within species of *Sesarma* (Decapoda: Brachyura: Sesarmidae) from Jamaican rivers. The Jamaican freshwater crabs are endemic to a much smaller geographic area and show a pronounced genetic-geographical structure with restricted gene flow among many of the studied rivers systems. These results are unexpected, because the colonization of Jamaica occurred much more recently according to geological history and because the Jamaican crabs still have an abbreviated larval development (González-Gordillo et al. 2010) which should favor distribution within a drainage system and possibly among rivers, if able to survive in coastal areas. This comparison gives evidence for different distribution potential in freshwater crabs and cautions about the assumption that these crabs do not migrate between rivers and are thus infallible biogeographic model systems.

1 INTRODUCTION

The Caribbean islands (or West Indies) consist of the four Greater Antillean islands, Jamaica, Cuba, Hispaniola and Puerto Rico, the Leeward Antilles and the Lesser Antilles. The arc formed by the Greater and Lesser Antilles delimits the Caribbean Sea. Different scenarios have been put

forward to explain the geological history of the Caribbean (Buskirk 1985; Pindell 1994; Hedges 2001). Today there is growing evidence that the Proto-Caribbean Plate formed in the eastern Pacific during the Mid Cretaceous around 100 mya. This newly formed plate then moved northeast towards its present position (Pindell 1994). Subduction from the North American Plate under the lighter Caribbean Plate caused formation of the Proto-Antilles which can be considered the ancestral island of the present-day Greater Antilles. The geological history of this region remains very complex and until today there is uncertainty as to which islands were above the sea level at which time (Hedges 1996, 2006). Iturralde-Vinent & MacPhee (1999) claim that no land areas in the Greater Antilles were constantly above sea level before 45 mya, thus not allowing survival of a possible Proto-Antillean fauna and flora after early contact with North and South America. Instead they postulate a mid-Cenozoic vicariant event via the Aves Ridge to South America that allowed land mammals to colonize the Antilles. On the other hand, Hedges (2006, 2010) postulates that all life on the Caribbean islands originated from dispersal, with most terrestrial vertebrates probably arriving via flotsam and prevalent currents from South America, whereas most of the birds, bats, and freshwater fishes appear to have come from North and Central America. The island of Jamaica became submerged around 20 mya, starting in the late Eocene. The limestone and karst formations covering large parts of Jamaica are a result of these submarine epochs. Starting in the late Miocene, Jamaica was lifted again above the sea level (Lewis & Draper 1990; Robinson 1994). The newly emerged island was then available for new biological colonizations, resulting in plenty of endemic animal and plant species. The Greater Antillean island, Puerto Rico, reached its present position around 35 mya. The island lost its connection with Hispaniola in the Miocene (Graham 2003) and was separated from the Virgin Islands due to sea level changes resulting from glacial events in the Quaternary. These changes in water level also altered the amount and distribution of land mass on Puerto Rico, whereas the central mountain range, Cordillera Central, is the result of Eocene volcanism, uplift, and later deformation followed by erosion.

Possibly in consequence of its geological complexity, the Caribbean is today considered one of the biodiversity hotspots of the world (Mittermeier et al. 2004). Within this Caribbean hotspot, the islands of the Greater Antilles harbour a particularly high degree of endemic flora and fauna. These islands cover more than 90% of the 229,549 square kilometres of terrestrial surface in the Caribbean. They also present the highest elevation with 3071 m above sea level, the Pico Duarte on Hispaniola (Orvis 2003). Very different vegetation occurs on the islands, from cactus shrubs, savannahs, evergreen bushland, to freshwater swamps, mangrove forests or lowland rainforests, which are today mostly deforested. In higher elevation, seasonal forest and mountain cloud forest occur (Beard 1955). Among the vertebrate species, frogs show more than 99% endemism (164 out of 165 species). Most of these are endemic to specific islands. The reptiles also bear a high percentage of endemism with around 94% (Hedges 1996). This includes some interesting species radiations, one of which is the genus *Anolis*, with 150 endemic out of 154 species (Roughgarden 1995). In the islands' freshwater systems, 74 species of fish can be found, of which 71 are endemic, some of them even inhabiting single lakes (Hedges 1996). Also the invertebrate fauna of the Caribbean islands has developed a huge amount of endemic species, even if they are not documented as thoroughly as vertebrates. According to Woods & Sergile (2001), the diversity of invertebrates known from the West Indies is only a small fraction of the undocumented present diversity. These authors also remarked that the known species groups tend to be the result of adaptive radiation. As an example, only thirteen species of ostracods were known from Jamaican ponds, most of them widespread in the neotropics. In 1996, Little & Hebert discovered and described eleven new species of ostracods, all from bromeliads, ten of which are endemic to Jamaica. Similarly, the number of endemic millipedes of the genus *Anadenobolus* from Jamaica increased from one to three after the genetic study by Bond & Sierwald (2002). The terrestrial mollusc fauna from Jamaica also has a high percentage of endemic species. Nearly 90%, that is 505 species out of 562, are only found on this island

(Rosenberg & Muratov 2006). In the present study, we will focus our attention on the freshwater crab diversity of two Caribbean islands, the potential of finding undescribed diversity and the mechanisms generating current diversity. In order to do so, we present new data on the Puerto Rican freshwater crab *Epilobocera sinuatifrons* and compare it with already published data on selected Jamaican freshwater crabs (Schubart et al. 2010).

The freshwater crab *Epilobocera sinuatifrons* (A. Milne Edwards 1866) belongs to the family Pseudothelphusidae and is the only freshwater crab of Puerto Rico with a complete freshwater life cycle. It is endemic to the Caribbean islands Puerto Rico and Saint Croix (Chace & Hobbs 1969; Villalobos-Figueroa 1982; Covich & McDowell 1996). Its closest relative is assumed to be the endemic freshwater crab of Hispaniola *Epilobocera haytensis* Rathbun 1893 (see Pretzmann 1974). *Epilobocera sinuatifrons* has a trapezoidal carapace with one anterolateral tooth. Adult individuals can grow to a carapace width of up to 150 mm, maturity is reached with a size of around 30 mm carapace width (Zimmerman & Covich 2003). The species undergoes direct development and females carry relatively large eggs, from which juveniles hatch while the eggs are still carried by the mother. After hatching, the juveniles stay with the mother for some time before they are released into suitable habitats, but do not moult during that time. These habitats can vary greatly. Crabs can be found in rivers of varying structure, from small headwater creeks to large lowland streams, from riverbeds with mainly boulder and rocky composition to sandy and silty ones. According to Zimmermann & Covich (2003), the average flow velocity has an influence on the abundance of juvenile crabs, which tend to prefer higher velocities. Juveniles are often found hiding under rocks, wooden debris or in leaf litter, whereas large adults prefer burrows in sandy or muddy river banks. *Epilobocera sinuatifrons* is omnivorous, with a high percentage of the normal diet comprising palm seeds and fruits, other freshwater invertebrates, and terrestrial snails (Covich & McDowell 1996; March & Pringle 2003). The regular diet of juvenile crabs is unknown (Henry et al. 2000). Unlike its Hispaniolan relative, *Epilobocera haytensis*, *E. sinuatifrons* is no longer a regular component of local human diet, but is more endangered by commercial land use through deforestation and river regulation. The role of humans in translocating animals between rivers is unknown. The phylogeography of the species was described for the first time by Cook et al. (2008) based on representatives of nine rivers. The goal of this study is to describe connectivity in *E. sinuatifrons* among different river systems and to understand the mechanisms of dispersal by comparing morphometric data, mitochondrial, and nuclear DNA of freshwater crabs from 40 localities from throughout Puerto Rico.

2 MATERIALS AND METHODS

Freshwater crabs of the species *Epilobocera sinuatifrons* (Decapoda: Brachyura: Pseudothelphusidae) were collected from 40 localities, corresponding to 23 river systems from throughout Puerto Rico, during four sampling trips between 1997 and 2008 (Figure 1; Appendix, Table A1). For later comparative analyses, these localities were clustered into six (mitochondrial DNA, see Figure 1) or eight (nuclear DNA) geographic regions. These arbitrary regions follow a general west-to-east direction parallel to the central mountain range Cordillera Central, which stretches in the same direction in the southern half of Puerto Rico. In addition, river systems from northern slopes were always distinguished from southern slopes to test the influence of this hydrographic divide. Intentionally, streams with geographically close headwaters, but belonging to either southern or northern drainage systems were sampled. Under the assumption, that each locality should be considered a distinct population, especially if belonging to independent watersheds, as is often the case (see Figure 1), our somewhat artificially created geographic regions will here be regarded as metapopulations. This term was first coined in Levins' (1969) pioneering work, with his own words "a population of populations." It is often applied to species in fragmented habitats and will here be used subsequently for all our geographic clusters.

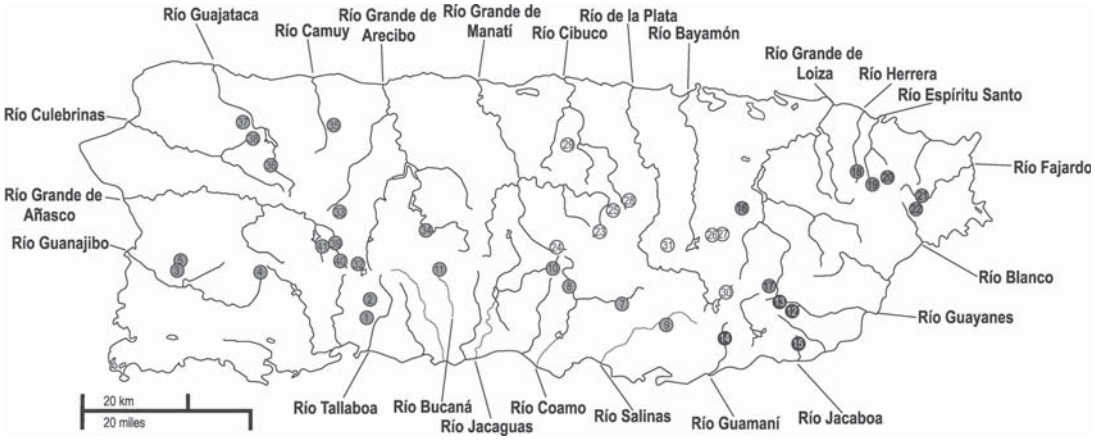

Figure 1. Map of Puerto Rico with 40 (population 6=4) collection points, 23 rivers systems with corresponding names, and the color coding for metapopulations of the freshwater crab *Epilobocera sinuatifrons* as used in the mitochondrial DNA phylogeographic comparisons based on mitochondrial DNA. Green: North-West, blue: South-West, yellow: North-Center, orange: South-Center, red: North-East, dark red: South-East (see Figure 7 in Color insert).

2.1 Morphometry

Morphometric data were collected to detect phenotypic differences and test for their correlation with genetic differences. A mechanical calliper gauge with a digital display was used to take measurements. We measured 111 male and female individuals of the Puerto Rican freshwater crab *Epilobocera sinuatifrons* and recorded the following characters: the width of the carapace measured at the widest point including anterolateral teeth, the length of the carapace measured at the central carapace (CL), body height, the frontal width as the distance between the inner orbits, and the dorsal length and width of the meri from the third and fourth pereiopods. Additionally, the length of the dactyli and the height and length of the propodi of both chelae were measured, recording which of the claws was smaller. During all measurements, extra care was taken not to squeeze the individuals, nor to measure claws or legs that have been regenerated recently or showed signs of damage. To minimize probable errors due to allometric growth (Reuschel & Schubart 2006), only individuals over a certain size were measured and in the end only 100 individuals with CL larger than 2 cm were analyzed (see Appendix, Table A1). All measurements were logarithmically transformed to further minimize the effect of possible allometric growth. Measurements were tested for normal distribution using the one-sample Kolmogorov-Smirnov test. Measurements showing a normal distribution were included in a discriminant function analysis. The variable that had the greatest weight on the outcome of the discriminant function analysis was calculated. The discriminant function analysis was subsequently repeated without this variable to assure that the observed differences were not the result of a single factor. All calculations were performed in SPSS version 16 (SPSS Inc, Chicago IL). Selections of voucher specimens from western, central and eastern Puerto Rico have been deposited at the Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt (SMF); Instituto Nacional de Pesquisas da Amazônia, Manaus (INPA 1530–1533); Naturhistorisches Museum, Wien (NHMW), Natural History Museum London (BMNH); Naturalis Museum, Leiden (RMNH), Zoologische Staatssammlung München (ZSM); Zoological Reference Collection NUS, Singapore (ZRC); Muséum National d'Histoire Naturelle, Paris (MNHN).

2.2 Genetics

DNA was extracted from muscle tissue of walking legs from 103 individuals of *Epilobocera sinuatifrons* (see Appendix, Table A1) and DNA isolation was performed using a modified Puregene method from Gentra System. DNA pellets were resuspended in 20 μl of TE buffer and the concentration was ascertained on agarose gels. From the corresponding dilutions of the DNA solution, 1 μl was used for polymerase chain reactions (PCR). Three different genetic markers, the mitochondrial cytochrome c oxidase subunit I (COI), the mitochondrial NADH dehydrogenase subunit 1 (ND1) and the nuclear internally transcribed spacer region ITS1-5.8S-ITS2 (ITS) region were targeted. The primer used for COI were COL6b (5'-ACA AAT CAT AAA GAT ATY GG-3') and COH6 (5'-TAD ACT TCD GGR TGD CCA AAR AAY CA-3') (see Schubart & Huber 2006), and for ND1, the new species-specific primers NDL6Es (5'-CCA ACT ATA AGT AAT TTT ATA G-3') and NDH6Es (5'-ATA AGC TTA TCA TAC CGA AG-3'), which amplified a 608 basepair (bp) ND1 fragment. The ND1 primers were designed based on previous amplifications of *E. sinuatifrons* with NDL4 (5'-AAA AGK CTA ATT RTT TTG TG-3') and NDH2 (5'-GCT AAA TAT ATW AGC TTA TCA TA-3') (see Santl 2009; Schubart 2009). For the ITS region, the primers ITSL1 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITSH1 (5'-TTC AGT CGC CCT TAC TAA GGG AAT CC-3') (corresponding to PT1 and PT3 of Tang et al. 2003) were used. The resulting fragments had a length between 1500 bp and 1700 bp due to several microsatellite-like repeat motifs that are known to occur in other arthropods (Vogler & DeSalle 1994; Harris & Crandall 2000; Schulenburg et al. 2001). For PCR, a standard 25 μl reaction was prepared containing 2.5 μl of 10×

buffer, 2.5 μl of 1.25 mM dNTPs, 0.5 μl of both primers (20 mM), 2 μl of 25 mM $MgCl_2$, 1 μl of $0.5u/\mu$l TAQ and 15 μl of double-distilled water in addition of 1 μl DNA. 40 cycles were run at an annealing temperature of 48 °C for the COI and ND1 primers and 50 °C for the ITS primers. COI and ND1 PCR products were cleaned using QuickClean (GenScript, Piscataway NJ) and sequenced with an ABI-PRISM 310 (Applied Biosystems, Carlsbad CA) or outsourced for sequencing.

Cloning of the ITS genes was carried out at Brigham Young University. Initially, PCR products were treated with an A-Addition kit from Quiagen (Qiagen GmbH, Dsseldorf) to add an A-overhang. Cloning itself was performed using the TOPO-TA cloning kit from Invitrogen (Invitrogen Corporation, Carlsbad, CA). 2 μl PCR product was added to a mix of 2 μlddH_2O, 1 μl salt solution and 1 μl TOPO vector. This mix was incubated for 30 minutes at room temperature. Chemical competent TOP10 One Shot® *Escherichia coli* cells were thawed on ice, and 2 μl of the TOPO cloning reaction was added. The cells were incubated on ice for 30 minutes, subsequently heat-shocked for 30 seconds at 42 °C, and immediately placed back on ice. 250 μl SOC medium was added and reaction tubes were shaken horizontally (200 rpm) at 37 °C. After 1 hour, 25 μl of the cells were spread evenly on pre-warmed LB plates containing 50 μg/ml ampicillin. Plates were incubated overnight at 37 °C. Colonies that had successfully included the vector with the PCR product were picked and transferred to 50 μl of ddH_2O. This solution was denaturized for 10 min at 96 °C, and 1 μl was used for a PCR with 35 cycles and 55 °C as an annealing temperature to check if the correct fragment had been cloned. This PCR product was cleaned with PCR Cleanup Millipore plates (Millipore Corporation, Billerica MA) and thereafter cycle-sequenced in both directions using 1/16th of Big Dye v3.0 reaction and standard protocols. The sequencing was performed on an automated ABI 3730 machine. All sequences obtained were proofread for possible errors made by the sequencer software analyses. We used ChromasLite (http://www.technelysium.com) to read chromatograms and edit possible errors. DNA sequences are deposited at Genbank under accession numbers FR871245–FR871285 (ND1 mtDNA) and FN395370–FN395607 (ITS nDNA).

Due to the lack of indels, the corrected sequences for COI and ND1 could be aligned completely by eye using BioEdit (Hall 1999). The ND1 alignment was exported as a Phylip file to construct a statistical parsimony network using the algorithm outlined in Templeton et al. (1992) and implemented in the TCS software package version 1.21 (Clement et al. 2000). Based on the obtained haplotype network of the ND1 data, a nested clade analysis (NCA) was performed (Templeton et al. 1995; Templeton 2004) to test the null hypothesis of no association between the geographic distributions of the haplotypes. The haplotype network was converted into a nested statistical design using the instructions given in Templeton & Sing (1993) and in Crandall & Templeton (1996). To test for an association between the genetic composition and the geographic distribution of the haplotypes, two distances were calculated. First, the clade distance D_c, which estimates how geographically widespread a clade is and second, the nested clade distance D_n, which measures the relative distribution of a clade compared to the other clades in the same higher clade level (see Posada et al. 2006 for details). All calculations were carried out with the application GEODIS 2.5 (Posada et al. 2000), using 1,000,000 permutations and direct distances. The direct-distances option was favored over river distances as all species in this study are freshwater species without marine forms, which would theoretically be able to maintain gene flow among independent watersheds (Fetzner & Crandall 2003). The direct distances between single sample locations were measured with GoogleEarth. We used the most recent inference key from Templeton (http://darwin.uvigo.es/software/geodis.html) to infer the historical events that caused the observed genetic population structure. To measure the genetic differentiation between populations, Φ_{ST} values were calculated using an Analysis of Molecular Variance (AMOVA) in ARLEQUIN ver. 3.0 (Excoffier et al. 2005).

The alignment of ITS sequences was created with the ClustalW plugin of BioEdit. After this initial alignment, we manually checked the microsatellite regions as they are not always correctly

recognized by the automated alignment. To be able to analyze the ITS dataset, some pre-processing was necessary. An important part of the information provided by ITS sequences is the high number of indels (Simmons & Ochoterena 2000). These indels are necessary to align microsatellite-like positions in the ITS1/ITS2 region in the noncoding region. The simple indel coding method (Simmons et al. 2001) was applied and calculated with the program GapCoder (Young & Healy 2003) to render the indels phylogenetic information for tree search methods. For further analysis, all alignment files were converted to the Nexus file format. The great amount of variation within the ITS datset did not allow the use of the statistical parsimony algorithm of the TCS software package for network calculation. Therefore, the software Splitstree version 4 was used (Huson & Bryant 2006) to construct minimum spanning networks of the gap-coded ITS sequence data.

3 RESULTS

3.1 Ecology

The collection of *Epilobocera sinuatifrons* during four field seasons between 1997 and 2008 enables us to provide some ecological observations. This species, like other species of *Epilobocera* on Hispaniola and Cuba, is inactive during the daytime, except for cave populations (personal observations). The best way to obtain specimens during the daytime is thus to actively search the river bed and banks of small- to medium-sized rivers. Larger fast-flowing rivers, which probably have a considerable degree of bed-load shift during high water periods and exclusively consist of large boulders, are normally devoid of crabs. On the other hand, it is difficult or impossible to find these crabs in slow-flowing or standing waters (e.g., lakes) with lack of bottom structure. Smaller individuals were easiest to collect from under rocks in the shallow part of rivers by hand, or in deeper parts by placing a hand net downstream of the rock to be turned. In deeper pools of mountainous regions, crabs can be seen and collected by snorkelling and exploring cracks in the rocky walls, which they inhabit together with species of *Macrobrachium* sp. Largest individuals (up to 9.8 cm carapace width) were invariably collected from within caves or relatively deep burrows in the sediment of the water banks with entrances above the water level, but often reaching down to the water table and having more than one burrow opening. This differs from observations by Covich & McDowell (1996) and Zimmerman & Covich (2003), who reported groups of adult crabs under rocks in saturated sections of stream banks. Their findings are from streams of the El Yunque National Forest that are relatively steep, with many pools and often lack muddy banks. This may account for the differences among adult habitats in most of the rivers sampled by us from other regions of Puerto Rico. Our observations on the terrestrial movements of these crabs will be summarized in the Discussion.

Table 1. Classification percentage based on the morphometric classification function for three geographically defined metapopulations of *Epilobocera sinuatifrons* from Puerto Rico. Correct classifications are indicated in bold. The mean correct classification corresponds to 64.4%.

Metapopulation	East	Center	West
East	**66.7**	22.9	10.4
Center	10.5	**84.2**	5.3
West	34.8	21.7	**43.5**

3.2 Morphometry

According to the Kolmogorov-Smirnov test, 10 out of the 15 measured characters in the morphometric dataset of *Epilobocera sinuatifrons* showed a normal distribution; the measurements of the interorbital distance, the carapace height and all measurements from the larger chelae (sexually dimorphic) were not normally distributed. Analyses were continued exclusively with the normal-distributed data: nineteen different populations, from which morphometric data were available, were differently pooled into geographic groups and tested for the highest signal of differentiation. We compared a west-to-east differentiation with a north-to-south differentiation. In addition, we compared the influence of subdividing the dataset into more (five) or less (three) geographic subgroupings. The clearest signal of differentiation resulted from a subdivision into three groups, namely West, Center, and East. These three groups showed significant differences (Wilk's Lambda 0.614; $P < 0.005$) with an overall correct classification of 64.4% (from 43.5 to 84.2%, see Table 1, Figure 2). When the sampling points were pooled into northern, central and southern groups, no significant differences were found (Wilk's Lambda 0.779; $P = 0.557$) and the corresponding classification only revealed no more than 52.2% (from 48.7 to 56.2%) overall correct placement (see Santl 2009). The morphometric data thus show that there are subtle phenotypic differences in *Epilobocera sinuatifrons* following a west-east direction, compared to less and not significant differences in a north-south direction. However, all these morphometric differences are not very pronounced and do not allow consistent distinction of morphotypes.

3.3 Genetics

Amplification of COI resulted in fragments of 658 bp, of which 624 were compared in an alignment. Many of the sequences showed double peaks in several positions in addition to a surprising homogeneity. We thus suspected the presence of pseudogenes and refrained from continuing with this gene (which was furthermore already used in the study by Cook et al. 2008. Instead, we concentrated on the second mitochondrial gene, the ND1. In total, sequences of ND1 were obtained from 103 individuals, which had to be cropped to a length of 572 bp in order to be used for network and AMOVA analyses. The final alignment included 89 variable positions of which 27 were parsimony-informative, resulting in 41 different haplotypes that are distributed over the six defined metapopulations (Table 2). Relatively high haplotype diversities (h), but moderate nucleotide diversities (π) are noteworthy, indicating that at least some populations are out of equilibrium (Grant & Bowen 1998): all six metapopulations have h values of at least 0.83 and the number of haplotypes is always larger than the 50% value of the sample size, suggesting that additional sampling would uncover many more haplotypes. The dataset was furthermore condensed into three metapopulations in a west-east direction or two metapopulations in a north-south direction. While there is a noticeable decrease from west to east in haplotype diversity, such a gradient is not visible from north to south.

We constructed a TCS haplotype network on these haplotypes and documented their occurrence across the six metapopulations (Figure 3). It can be seen that the distribution is not random and thus geographic influence can be determined. However, there is also no clean genetic separation between the six metapopulations. In the upper half of the network plus cluster 1–5, blue and green colors (with the exception of a dark red coded individual comprised in haplotype 24, which needs to be confirmed) indicate that these haplotypes are restricted to the western part of Puerto Rico as delimited by our metapopulations North-West and South-West. This, however, does not mean that western animals are not represented in the rest of the network. They are still very abundant in the most common haplotype 1 (ht1), which they share with animals from North-Center and South-Center. Eastern animals, as defined by metapopulations North-East and South-East, are not represented in the com-

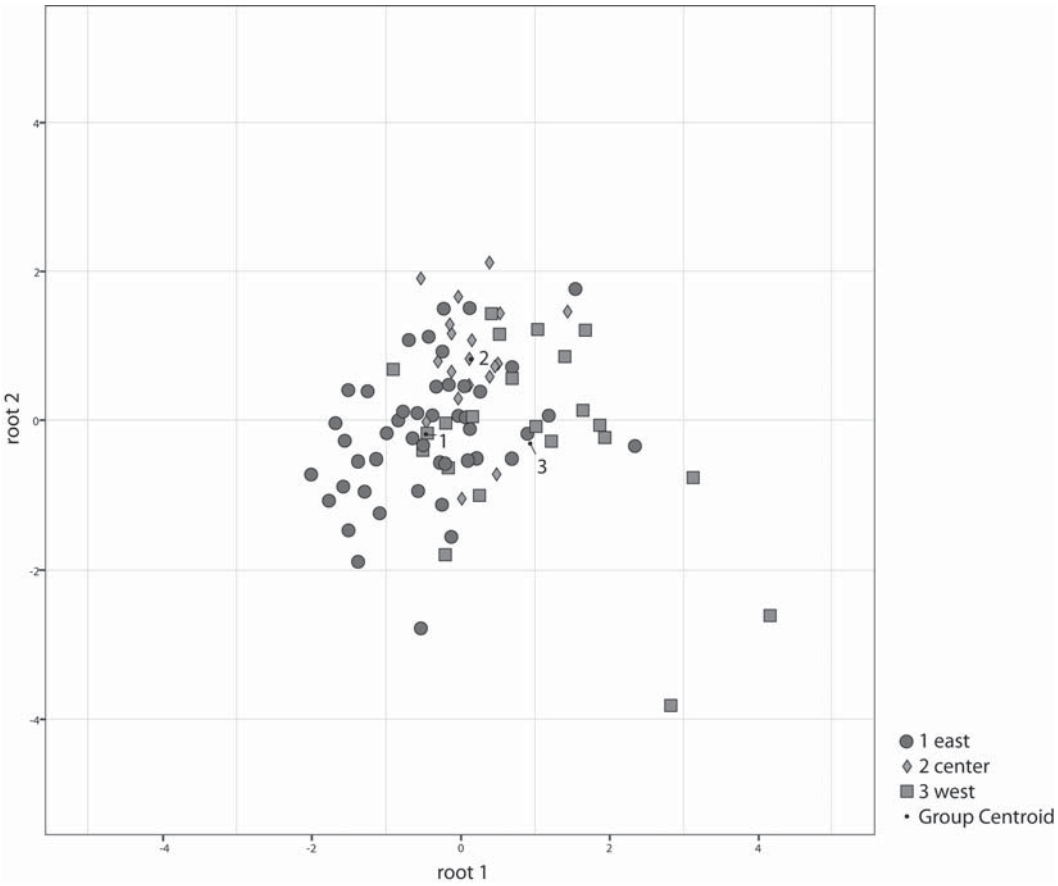

Figure 2. Morphometric analysis of *Epilobocera sinuatifrons* throughout Puerto Rico. Canonical analysis showing plot of the first two discriminant functions. Discrimination based on 10 normally distributed measurements among three metapopulations: West, Center, East. Coloration explained in legend (see Figure 8 in Color insert).

mon haplotype, but they form rare satellite haplotypes 9 and 11 in close vicinity to ht1. Otherwise the lower third of the network, separated by at least five mutational steps from all other haplotypes, is restricted to animals from the central (mainly in haplotype 37) and from the eastern (mainly in haplotype 38) part of Puerto Rico. Table 2 also shows negative and significant Fu's F_S values for all the western populations (North-West -5.096, South-West -7.825, lumped West -12.034), indicating departure from neutrality and population expansion in the west, as also concluded from the increased haplotype diversities in western Puerto Rico. These values, in addition to the structure of the network, suggest that the colonization of Puerto Rican rivers probably took place from west to east.

The estimation of gene flow among the six metapopulations by means of an AMOVA revealed low Φ_{ST} values that nevertheless resulted in restricted gene flow at various significance levels (Table 3). Strongest level of significance ($P < 0.001$) and highest Φ_{ST} values (up to 0.127) were detectable between all pairwise combinations of the three northern populations, between North-Central and South-East and South-West respectively and between North-East and South-

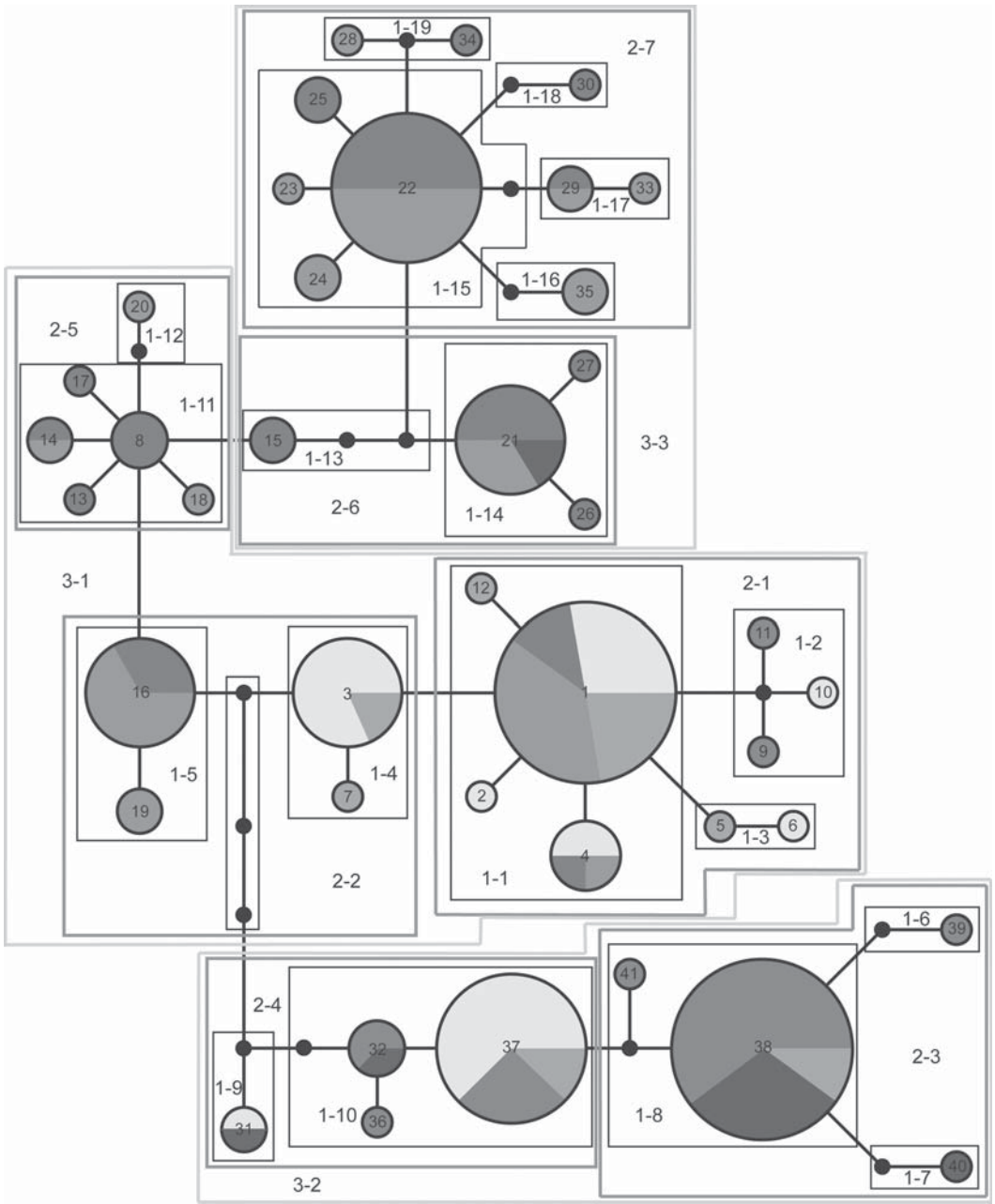

Figure 3. Statistical parsimony network of ND1 haplotypes of *Epilobocera sinuatifrons* ($N = 103$) from Puerto Rico constructed with TCS and the corresponding nesting design for the Nested Clade Analysis. Each black line represents one substitution, dots on lines indicate additional substitutions between haplotypes. The size of a circle represents the frequency of the corresponding haplotype. Coloration according to Figure 1 (see Figure 9 in Color insert).

Table 2. Sample size, number of haplotypes, molecular diversities and population demographic statistics of the ND1 gene (572 bp) of different metapopulations of *Epilobocera sinuatifrons* with a sample size $N \geq 7$. Significant values for demographic parameters are shown in bold.

Metapopulation	Sample Size	Haplotypes	h	π	Tajima's D	Fu's F_S
North-West	28	15	0.942	0.0070	−0.7667	**−5.096**
South-West	26	16	0.954	0.0063	−0.6543	**−7.825**
North-Center	20	9	0.874	0.0068	−0.2634	−0.839
South-Center	7	6	0.952	0.0071	−0.4753	−1.540
North-East	15	8	0.838	0.0061	−0.7373	−1.214
South-East	7	5	0.857	0.0072	−0.7865	−0.047
West	54	24	0.948	0.0067	−0.8797	**−12.034**
Center	27	12	0.883	0.0067	−0.5992	−2.402
East	22	11	0.827	0.0064	−1.0905	−2.534
North	63	29	0.956	0.0098	−0.7036	**−11.693**
South	40	24	0.965	0.0092	−0.4433	**−11.393**
Total	103	41	0.960	0.0097	−0.5262	−2.366

West. Three other pairwise relationships show significant limitation of gene flow, but at a much weaker level ($0.01 < P < 0.05$, Φ_{ST} values from 0.036 to 0.085). It is noteworthy that all three comparisons of local gene flow across the Cordillera Central were nonsignificant and thus imply unrestricted gene flow: North-West vs. South-West: $\Phi_{ST} = 0.001$; North-Center vs. South-Center: $\Phi_{ST} = 0$; North-East vs. South-East: $\Phi_{ST} = 0$. These are by far the lowest values in all pairwise comparisons. This phenomenon is also reflected in Table 4 where condensing the dataset in either West-Central-East or North-South metapopulations reveals that highly significant reduction of gene flow is only detectable in an west-east, but not in a north-south direction.

The Nested Clade Analysis (NCA), which is based on the clades as drawn on top of the haplotype network in Figure 3, proposes significant conclusions for clades 1-14, 1-15 (both restricted gene flow with isolation by distance = ibd) and 1-11 (contiguous range expansion = crg) at the first clade level, for 2-2 (crg) and 2-7 (inconclusive outcome) at the second clade level, and for 3-1 and 3-3 (ibd) at the third clade level. The total network is interpreted by NCA to be shaped by long distance colonization or past fragmentation followed by range expansion.

In order to obtain comparable results from the nuclear genome, we amplified the ITS1-5.8S-ITS2 complex with an average length of around 1620 bp in 40 individuals of *Epilobocera sinuatifrons*. These amplicons were then cloned and treated as described in Material and Methods. In total, we obtained 238 clones and the number of clones per individuals varied between one and seventeen. After incorporation of all indel positions in the dataset, the number of aligned sites increased to 1765. In total, we found 236 alleles resulting in 729 variable sites, of which 258 were parsimonious informative. The fact that 236 alleles were obtained from 40 specimens gives clear evidence for the existence of more than two alleles per animal. Furthermore only two alleles were found twice, suggesting that the dataset is by far not exhaustive and many more alleles can be expected within these 40 animals (see Discussion on nonconcerted evolution of ITS genes in arthropods in Schubart et al. 2010). In Figure 4, the minimum spanning network constructed from this data is displayed. Overall, the ITS dataset does not provide a clear picture of geographic distribution of alleles, but the trends already observed in the morphometric and mitochondrial data can be

Table 3. Φ_{ST} (lower left) and P values (upper right) of six metapopulations of *Epilobocera sinuatifrons*; *: $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $P < 0.001$ (in bold); –: $P > 0.05$ (not significant).

P / Φ_{ST}	North-West	South-West	North-Center	South-Center	North-East	South-East
North-West		–	***	–	***	*
South-West	0.001		***	*	***	*
North-Center	0.068	0.077		–	***	***
South-Center	0.013	0.036	0		–	–
North-East	0.107	0.101	0.114	0.037		–
South-East	0.085	0.072	0.127	0.036	0	

Table 4. Φ_{ST} (lower left) and P values (upper right) of three west-eastern or two north-southern metapopulations of *Epilobocera sinuatifrons*; *: $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $P < 0.001$ (in bold); –: $P > 0.05$ (not significant).

P / Φ_{ST}	West	Center	East	North	South
West		***	***		
Center	0.064		***		
East	0.104	0.112			
North					–
South				0.001	

confirmed. Three major groupings can be recognized: a western, an eastern, and a central group, respectively, including alleles from the neighboring regions, and standing in a triangular relation to each other. There is no pattern that would allow separating northern from southern metapopulations.

4 DISCUSSION

In the present study, we present morphometric and genetic data describing intraspecific geographic structure of the Puerto Rican freshwater crab *Epilobocera sinuatifrons*. Three datasets (morphometry, mitochondrial DNA, and nuclear DNA) agree in proposing a west-eastern orientation of differentiation processes, alongside the central mountain range (Cordillera Central). On the other hand, in three or four subjectively defined geographic regions of Puerto Rico (e.g., West, Center, East), no restriction of gene flow across the Cordillera Central (northern versus southern drainages) could be determined, thereby giving evidence for faunal exchange across the water divide, not only in one case, but in several regions independently. This finding supports previous observations that this species of freshwater crabs has well-developed capacities for overland dispersal.

Overland movement of *Epilobocera sinuatifrons* has been previously documented by Covich & McDowell (1996) and March & Pringle (2003), with observations that adult crabs often feed on leaf-based detritus, forest fruits, and terrestrial invertebrates on the rainforest floor. Several locals in Puerto Rico also report that during the rainy season, crabs can be found crossing roads or even wandering through human settlements, if adjacent to forest with streams (pers. comm. to authors).

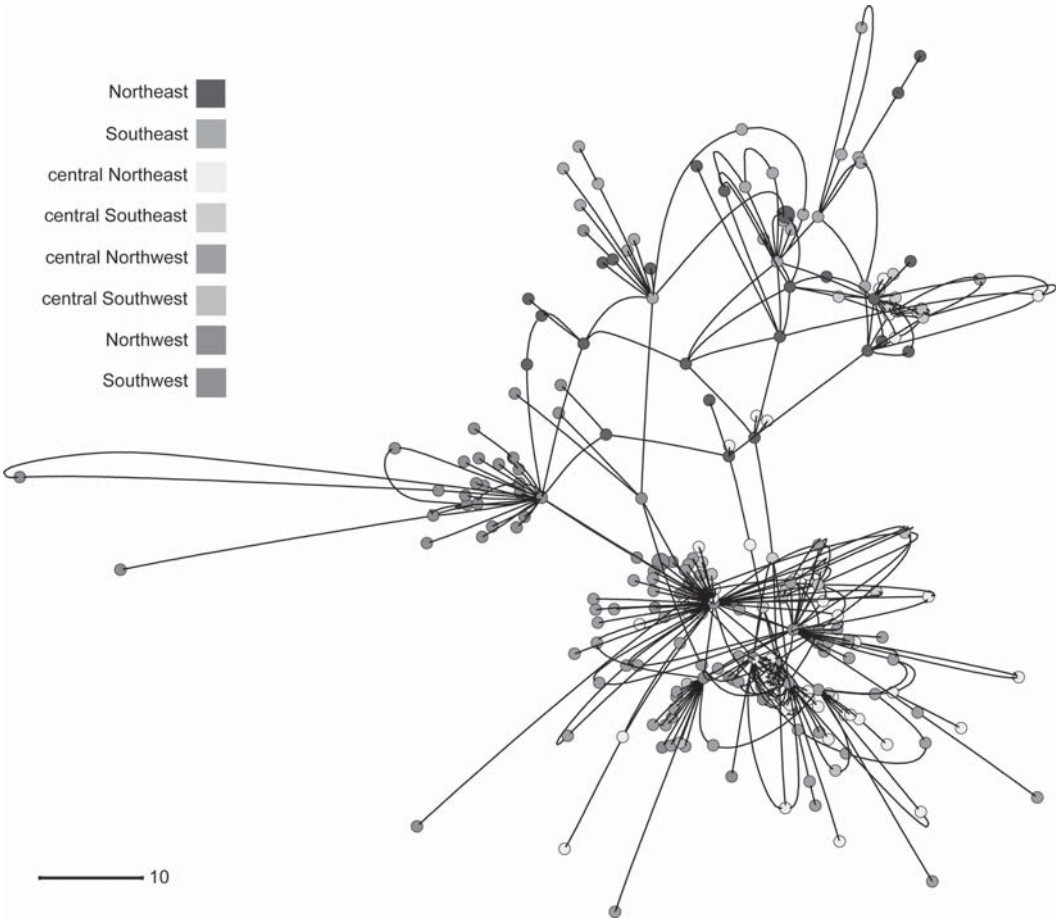

Figure 4. Minimum spanning network based on 238 clones of 1765 gap-coded basepairs of the ITS1-5.8S-ITS2 nuclear DNA region of *Epilobocera sinuatifrons* ($N = 40$) from Puerto Rico. Coloration explained in legend (see Figure 10 in Color insert).

In the Bosque Estatal de Guajataca, we found crabs inhabiting rock rubble in karst sinkholes, thriving in natural crevices and burrows that are probably connected with subterranean water; they are also abundant in several cave systems, which are only partially flooded (personal observations). Unpublished trapping experiments by Alan Covich, and Nicole Rivera and colleagues (pers. comm.) give further evidence that crabs venture out of the streams and thrive on the forest floor at night or during periods of rainfall. This behavior explains how crabs may reach headwaters of one river system after having left their home waters. Forest floor is probably more predominant in the upper rainforests of the Cordillera Central than in the lower mountainous areas or coastal plains, providing an explanation, why exchange in a north-south direction seems to be more frequent than in a west-eastern one.

The population genetic structure of *Epilobocera sinuatifrons* has previously been described by Cook et al. (2008) based on the mitochondrial cytochrome c oxidase subunit I gene (COI). They compare nine river systems from throughout Puerto Rico with a total of 126 individuals and 548 basepairs of aligned sequences. Direct comparison allows concluding that either our sampling scheme of less individuals from more rivers allowed to detect more genetic variability or alter-

Table 5. Comparison of variability in mitochondrial markers in two independent studies on population geographic structure within *Epilobocera sinuatifrons* from throughout Puerto Rico. COI = cytochrome c oxidase subunit I; ND1 = NADH dehydrogenase sununit 1.

Mitochondrial marker	**COI (Cook et al. 2008)**	ND1 **(This study)**
Number of individuals	126	103
Number of catchments	9	23
Alignment length	548	572
Variable positions	25	89
Percent variable positions	4.56	15.37
Haplotypes	26	41
Maximal haplotype distance	7	19

natively that the ND1 gene is more variable than the COI gene used by Cook and co-workers (see Table 5). A forthcoming study by Rivera et al. (unpublished data), comparing fewer populations and more individuals of *E. sinuatifrons* with the ND1 gene, and without lumping river systems into metapopulations, will allow a more direct comparison of haplotype and nucleotide diversities of the two genes and determine the influence of sampling strategy or the selected gene. Since the number of mutational steps between haplotypes is clearly higher in the ND1 gene (Table 5), despite including the same geographic extremes, we assume that ND1 in this case is more variable and thus has a higher resolution power.

Cook et al. (2008) recorded an absence of genetic population subdivision among the western rivers, whereas they found restricted gene flow among most other populations, with sample sizes ranging from 4 to 22. They conclude that the western region experienced relatively recent geographic dispersal. What they do not mention in the text is that their pairwise comparisons fail to show differences between Río Guayanés and Río Espíritu Santo in the east of the island and between Río Coamo and Río Grande de Manatí in the central part of the island. Interestingly these rivers, as well as the three western ones, are all north-south counterparts of each other. Only one of Cook et al.'s (2008) north-south river pairs (Río Guayanés versus Río Grande de Arecibo) has restricted gene flow, with a low Φ_{ST} value of 0.189 (P values are not specified in this study, except for being below 0.05). This seems to reflect the same phenomenon as recorded in our paper: limited differentiation in a north-south direction as opposed to much higher differences in an east-west direction. Cook et al. (2008: 160–161) doubt that walking ability alone is likely to facilitate among-river dispersal in this species and propose that "recent gene flow between rivers at the western end of the island was facilitated by recent (Holocene) drainage rearrangements associated with faulting and erosion processes." A conclusion in the same direction is later made (p. 162): "The geologically dynamic history of Puerto Rico appears to have sporadically and repeatedly facilitated continuity in riverine habitat over evolutionary time, thereby prohibiting strong divergence among populations of *E. sinuatifrons* in different rivers." We challenge this explanation and do not think that extrinsic factors are necessary to explain the population structure of *E. sinuatifrons* in Puerto Rico. We recognize in our data as well as in those of Cook et al. (2008) more instances of genetic homogeneity than the ones reported by them in the west of the island. Since most of these cases are in a north-south direction, there seems to be a pattern that can be explained by overland motility alone. This would be more parsimonious than proposing several independent drainage rearrangements via erosion and fault processes, especially, if they are claimed to have occurred in geologically recent times (Pleistocene: 0.6 mya). Clearly, the Eocene volcanism in Puerto Rico cannot have played a role in these postulated hydrographic changes.

One of the rationales to study population genetic structure within *Epilobocera sinuatifrons* from Puerto Rico and *E. haytensis* from Hispaniola (latter data in Rivera & Schubart, in preparation)

was to compare and understand the apparent lack of diversity (at least at the species level) of the pseudothelphusid freshwater crabs from these islands (1–2 species/island) with species diversity of sesarmid freshwater crabs from Jamaica (10 species, see Schubart & Koller 2005). Two underlying questions were: 1) is the lack of diversity the consequence of morphological stasis in the genus *Epilobocera*, and is genetic diversity possibly much higher, revealing the existence of old cryptic lineages, and 2) is the different potential for diversification of Jamaican versus Puerto Rican crabs already expressed at the population level?

The results of the present study on *Epilobocera sinuatifrons* from Puerto Rico, and the ones by Rivera & Schubart (in preparation) on *E. haytensis* from Hispaniola, show that there is no deep intraspecific structure within the two species and thus the first question finds an easy and straightforward response: the apparent lack of diversity in these species is not only based on morphological stasis, but is real and reflected in the genomes. The second question shall be answered by comparing the here presented results of *E. sinuatifrons* with those of a previous study by Schubart et al. (2010) on intraspecific divergence within three freshwater crab species from western and central Jamaica, *Sesarma dolphinum*, *S. meridies*, and *S. windsor*.

Sesarma dolphinum was described as a distinct species from rivers in westernmost Jamaica by Reimer et al. (1998) in order to separate it from the freshwater crab species *S. fossarum* from the neighboring western Cockpit Country. Likewise, *S. meridies* was described by Schubart & Koller (2005) after obtaining evidence from mtDNA and, to a lesser extent, from morphology of its distinctness from *S. windsor* from the eastern Cockpit Country. Preliminary genetic analyses showed that mtDNA in *S. meridies* and *S. windsor* (12S and 16S rRNA genes: Schubart & Koller 2005), *S. dolphinum* (ND1: Santl 2009) and all other Jamaican endemic species (Schubart group, unpublished) often allows to distinguish intraspecific geographically separated populations by diagnostic differences in their DNA. That means that some sequence positions allow to unmistakeably assigning sequences to specific populations, thus resulting in reciprocally monophyletic groups, if represented as phylogenetic trees or networks. This is not really the case in *Epilobocera sinuatifrons* (Figure 3). Even if the distribution is not even and shows clear geographic trends, there is not a single branch that would separate one specific metapopulation from the others and at the same unite all individuals from this metapopulation. The present pattern is thus paraphyletic and reflects recent separation with incomplete lineage sorting (Neigel & Avise 1986) or incomplete separation due to ongoing mixing among neighboring populations.

Nuclear DNA sequence data also confirm higher differentiation potential in Jamaican crabs from freshwater streams (*Sesarma dolphinum*, *S. meridies*, and *S. windsor*) when compared to *Epilobocera sinuatifrons*. The best evidence for this is obtained when comparing the separation of populations in the networks based on the ITS1-5.8S-ITS2 gene regions from *S. dolphinum* (see Schubart et al. 2010: Figure 4) and the one based on the homologous genetic region in *E. sinuatifrons* (see present study: Figure 4). The corresponding F_{ST} values in *S. dolphinum* among single rivers lie between 0.04 and 0.63 (see Schubart et al. 2010: Table 3), whereas between metapopulations (lumping nearby river systems) of *E. sinuatifrons*, these values lie between 0.03 and 0.36 (see Santl 2009). This is even more striking, when considering that *S. dolphinum* only inhabits the western tip of Jamaica and thus an at least five-fold smaller region than *Epilobocera sinuatifrons*, which is distributed throughout Puerto Rico.

Referring back to the second question, stated earlier in this Discussion, whether the different potential for diversification of Jamaican versus Puerto Rican crabs is already expressed at the population level, we can confirm that this is really the case. Pseudothelphusid crabs have probably been present on Puerto Rico, Hispaniola, and Cuba much longer than endemic sesarmid crabs on Jamaica, the latter becoming independent from their marine relatives approximately 4.5 mya (see Schubart et al. 1998). This means that the Pseudothelphusidae did not "use" the available evolutionary time for differentiation and adaptive radiation in the same way as the Jamaican Sesarmidae. The Jamaican endemic crab species still have an abbreviated larval development that should favor

distribution within a drainage system and even among rivers, if able to withstand higher salinities in coastal areas. Nevertheless, results from Santl (2009) and Schubart et al. (2010) show that in different species of *Sesarma*, genetic differentiation takes place within single river systems and thus suggest a high local retention of adult as well as larval stages.

The most likely explanation for the observed differences in genetic differentiation potential according to the current findings is that species of *Epilobocera* migrate more regularly between river systems, thereby crossing watersheds, and thus experience less allopatric differentiation and less local specializations due to regular genetic mixing. This differs from distribution models for other freshwater crab families from Africa (Potamonautidae) and Asia (Gecarcinucidae and Potamidae), for which published evidence exists that strong genetic differentiation regularly occurs even among neighboring river populations (Daniels et al. 2001, 2006; Shih et al. 2006; Klaus, Koller & Schubart, unpublished data). However, genetic similarity has been found among widely separated populations of the European freshwater crab *Potamon fluviatile* (see Jesse et al. 2009). These comparisons give evidence for different dispersal potential in different freshwater crab lineages and cautions about the assumption that these crabs do not migrate between rivers and are thus infallible biogeographic model systems.

ACKNOWLEDGEMENTS

The authors are grateful to Silke Reuschel for her participation in one of the collecting trips. Owen McMillan hosted Tobias Santl during several weeks laboratory work at the University of Puerto Rico, campus of Rios Piedras and Nikolaos V. Schizas hosted Nicole T. Rivera during several months laboratory work (Feb.–Aug. 2008) at the University of Puerto Rico, Department of Marine Sciences, Isla Magüeyes Laboratories (funding provided by NSF-Epscor Puerto Rico to NVS; 31 DNA sequences run at the sequencing facility of UPR, Río Piedras, supported in part by NCRR AABRE grant no. P20 RR16470, NIH-SCORE grant no. S06GM08102, University of Puerto Rico Biology Department and NSFCREST grant no. 0206200). Collection permits were issued by the Puerto Rico Department of Natural and Environmental Resources. In Puerto Rico we also enjoyed the hospitality of Florentina Cuevas Rivera, Yogani Govender and Dani Dávila. Special thanks are also due to the team of the Crandall lab in Provo (Utah), who supported T. Santl, while cloning a maximum of ITS alleles in relatively short time intervals and associated support from the U.S. National Science Foundation (EF-0531762). Lapo Ragionieri commented on the methods. This study was financially supported by a six year research project to Christoph D. Schubart (Schu 1460/3) through the Deutsche Forschungsgemeinschaft within the priority program 1127: "Adaptive Radiation—Origin of Biological Diversity." We acknowledge constructive criticism by Sebastian Klaus, one anonymous reviewer, and Stefan Koenemann. Marco T. Neiber was of invaluable help with the formatting.

REFERENCES

Beard, J.S. 1955. The classification of tropical American vegetation-types. *Ecology* 36: 89–100.

Bond, J.E. & Sierwald, P. 2002. Cryptic speciation in the *Anadenobolus excisus* millipede species complex on the island of Jamaica. *Evolution* 56: 1123–1135.

Buskirk, R. 1985. Zoogeographic patterns and tectonic history of Jamaica and the northern Caribbean. *J. Biogeogr.* 12: 445–461.

Chace Jr., F.A. & Hobbs Jr., H.H. 1969 The freshwater and terrestrial decapod crustaceans of the West Indies with special reference to Dominica. *Bull. U.S. Natl. Mus.* 292: 1–258.

Clement, M., Posada, D. & Crandall, K.A. 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9: 1657–1659.

Cook, B.D., Pringle, C.M. & Hughes, J.M. 2008. Phylogeography of an island endemic, the Puerto

Rican freshwater crab (*Epilobocera sinuatifrons*). *J. Hered.* 99: 157–164.

Covich, A. & McDowell, W. 1996. The stream community. In: Reagan, D.P. & Waide, R.B. (eds.), *The food web of a tropical rainforest*: 433–459. Chicago, IL: University of Chicago Press.

Crandall, K.A. & Templeton, A.R. 1996. Applications of intraspecific phylogenetics. In: Harvey, P.H., Brown, A.J.L., Smith, J.M. & Nee, S. (eds.), *New Uses for New Phylogenies*: 81–99. New York, Oxford: Oxford University Press.

Daniels, S.R., Gouws, G. & Crandall, K.A. 2006. Phylogeographic patterning in a freshwater crab species (Decapoda: Potamonautidae: *Potamonautes*) reveals the signature of historical climatic oscillations. *J. Biogeogr.* 33: 1538–1549.

Daniels, S.R., Stewart, B.A. & Burmeister, L. 2001. Geographic patterns of genetic and morphological divergence amongst populations of a river crab (Decapoda: Potamonautidae) with the description of a new species from mountain streams in the Western Cape, South Africa. *Zool. Scripta* 30: 181–197.

Excoffier, L., Laval., G. & Schneider, S. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol. Bioinform. Online* 1: 47–50.

Fetzner Jr., J.W. & Crandall, K.A., 2003. Linear habitats and the nested clade analysis: an empirical evaluation of geographic versus river distances using an Ozark crayfish (Decapoda: Cambaridae). *Evolution* 57: 2101–2118.

González-Gordillo, J.I., Anger, K., Schubart, C.D. 2010. Morphology of the larval and first juvenile stages of two Jamaican endemic crab species with abbreviated development, *Sesarma windsor* and *Metopaulias depressus* (Decapoda: Brachyura: Sesarmidae). *J. Crust. Biol.* 30: 101–121.

Graham, A. 2003. Geohistory models and Ceonozoic paleoenvironments of the Caribbean region. *Syst. Bot.* 28: 378–386.

Grant, W.A.S. & Bowen, B.W. 1998. Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. *J. Hered.* 89: 415–426.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41: 95–98.

Harris, D.J. & Crandall, K.A. 2000. Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies. *Mol. Biol. Evol.* 17: 284–291.

Hedges, S.B. 1996. Historical biogeography of West Indian vertebrates. *Annu. Rev. Ecol. Syst.* 27: 163–196.

Hedges, S.B. 2001. Caribbean biogeography: an overview. In: Woods, C.A. & Sergile, F.E. (eds.), *Biogeography of the West Indies: Patterns and Perspectives*: 15–33. Boca Raton, FL: CRC Press.

Hedges, S.B. 2006. Paleogeography of the Antilles and origin of West Indian Terrestrial Vertebrates. *Ann. Missouri Bot. Gard.* 93: 231–244.

Hedges, S.B. 2010. Molecular clocks, flotsam, and Caribbean islands. In: Cox, C.B. & P.D. Moore (eds.), *Biogeography: An Ecological and Evolutionary Approach. 8th Edition*: 353–354. Hoboken, NJ: John Wiley & Sons, Inc.

Henry, J.K., Covich, A.P., Bowden, T.S. & Crowl, T.A. 2000. Mayfly predation by juvenile freshwater crabs: implications for crab habitat selection. *Bull. North Amer. Benthol. Soc.* 17: 123.

Huson, D.H. & Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23: 254–267.

Iturralde-Vinent, M.A. & MacPhee, R.D.E. 1999. Paleogeography of the Caribbean region: implications for Cenozoic biogeography. *Bull. Amer. Mus. Nat. Hist.* 238: 1–95.

Jesse, R., Pfenninger, M., Fratini, S., Scalici, M., Streit, B. & Schubart, C.D. 2009. Disjunct distribution of the freshwater crab *Potamon fluviatile*—natural expansion or human introduction?

Biol. Invas. 11: 2209–2221.

Levins, R. 1969. Some demographic and genetic consequences of environmental heterogeneity for biological control. *Bull. Entomol. Soc. America* 15: 237–240.

Lewis, J.F. & Draper, G. 1990. Geological and tectonic evolution of the northern Caribbean margin. In: Dengo, G. & Case, J.E. (eds.), *Decade of North American Geology. The Caribbean. Volume H*: 77–140. Boulder, CO: Geological Society of America.

Little, T. & Hebert, P. 1996. Endemism and ecological islands: the ostracods from Jamaican bromeliads. *Freshw. Biol.* 36: 327–338.

March, J.G. & Pringle, C.M. 2003. Food web structure and basal resource utilization along a tropical island stream continuum, Puerto Rico. *Biotropica* 35: 84–93.

Milne Edwards, A. 1866. Description de trois nouvelles especes du genre *Boscia*, Crustaces Brachyures de la tribu des Telpheusiens. *Ann. Soc. Entomol. France* (4)6: 203–205.

Mittermeier, R.A., Gil, P.R., Hoffman, M., Pilgrim, J., Brooks, T., Goettsch Mittermeier, C. & Lamoreux, J. 2004. *Hotspots Revisited: Earth's Biologically Richest and Most Endangered Terrestrial Ecoregions.* Mexico City: CEMEX.

Neigel, J.E. & Avise, J.C. 1986. Phylogenetic relationships of mitochondrial DNA under various models of speciation. In: Nevo, E. & Karlin, S. (eds.), *Evolutionary Processes and Theory*: 515–534. New York, NY: Academic Press.

Orvis, K.H. 2003. The highest mountain in the Caribbean: controversy and resolution via GPS. *Carib. J. Sci.* 39: 378–380.

Pindell, J.L. 1994. Evolution of the Gulf of Mexico and the Caribbean. In: Donovan, S.K. & Jackson, T.A. (eds.), *Caribbean Geology: An Introduction*: 13–39. Kingston, Jamaica: The University of the West Indies Publishers Association.

Posada, D., Crandall, K.A. & Templeton, A.R. 2000. GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Mol. Ecol.* 9: 487–488.

Posada, D., Crandall, K.A. & Templeton, A.R. 2006. Nested clade analysis statistics. *Mol. Ecol. Notes* 6: 590–593.

Pretzmann, G. 1974. Zur Systematik der Pseudothelphusidae (Decapoda, Brachyura). *Crustaceana* 27: 294–304.

Rathbun, M.J. 1893. Descriptions of new species of American fresh-water crabs. *Proc. U. S. Natl. Mus.* 16: 649–661, pls. LXXIII–LXXVII.

Reimer, J., Schubart, C.D. & Diesel, R. 1998. Description of a new freshwater crab of the genus *Sesarma* Say, 1817 (Brachyura: Grapsidae: Sesarminae) from western Jamaica. *Crustaceana* 71: 186–196.

Reuschel, S. & Schubart C.D. 2006. Phylogeny and geographic differentiation of two Atlanto-Mediterranean species of the genus *Xantho* (Crustacea: Brachyura: Xanthidae) based on genetic and morphometric analyses. *Mar. Biol.* 148: 853–866.

Rivera, N.T. & Schubart C.D. (in preparation). Phylogeography of the freshwater crab *Epilobocera haytensis* (Brachyura: Pseudothelphusidae) from Hispaniola reveals partly restricted gene flow among different river systems.

Robinson, E.J. 1994. Jamaica. In: Donovan, S.K. & Jackson, T.A. (eds.), *Caribbean Geology: An Introduction*: 111–127. Kingston, Jamaica: The University of the West Indies Publishers Association.

Rosenberg, G. & Muratov, I.V. 2006. Status report on the terrestrial Mollusca of Jamaica. *Proc. Acad. Nat. Sci. Philadelphia* 155: 117–161.

Roughgarden, J. 1995. *Anolis Lizards of the Caribbean: Ecology, Evolution and Plate Tectonics*. New York, Oxford: Oxford University Press.

Santl, T. 2009. Comparative diversification potential of an old and a young lineage of freshwater crabs on two Caribbean islands explained at the population level. Electronically published Ph.D. dissertation, Universität Regensburg, http://epub.uni-regensburg.de/13391/.

Schubart, C.D. 2009. Mitochondrial DNA and decapod phylogenies; the importance of pseudogenes and primer optimization. In: Martin, J.W., Crandall, K.A. & Felder, D.L. (eds.), *Crustacean Issues 18: Decapod Crustacean Phylogenetics*: 47–65. Boca Raton, FL: Taylor & Francis/CRC Press.

Schubart, C.D., Diesel, R. & Hedges, S.B. 1998. Rapid evolution to terrestrial life in Jamaican crabs. *Nature* 393: 363–365.

Schubart, C.D. & Huber, M.G.J. 2006. Genetic comparisons of German populations of the stone crayfish, *Austropotamobius torrentium* (Crustacea: Astacidae). *Bull. Franç. Pêche Piscic.* 380–381: 1019–1028.

Schubart, C.D., Koller, P. 2005. Genetic diversity of freshwater crabs (Brachyura: Sesarmidae) from central Jamaica with description of a new species. *J. Nat. Hist.* 39: 469–481.

Schubart, C.D., Weil, T., Stenderup, J.T., Crandall, K.A. & Santl, T. 2010. Ongoing phenotypic and genotypic diversification in adaptively radiated freshwater crabs from Jamaica. In: Glaubrecht, M. (ed.), *Evolution in Action*: 323–349. Berlin, Heidelberg: Springer-Verlag.

Schulenburg, J.H.G.v.d., Hancock, J.M., Pagnamenta, A., Sloggett, J.J., Majerus, M.E.N. & Hurst, G.D.D. 2001. Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). *Mol. Biol. Evol.* 18: 648–660.

Shih, H.-T., Hung, H.-C., Schubart, C.D., Chen, C.A. & Chang, H.-W. 2006. Intraspecific genetic diversity of the endemic freshwater crab *Candidiopotamon rathbunae* (Crustacea: Decapoda, Brachyura, Potamidae) reflects five million years of geological history of Taiwan. *J. Biogeogr.* 33: 980–989.

Simmons, M.P. & Ochoterena, H. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Syst. Biol.* 49: 369–381.

Simmons, M.P., Ochoterena, H. & Carr, T.G. 2001. Incorporation, relative homoplasy, and effect of gap characters in sequence-based phylogenetic analysis. *Syst. Biol.* 50: 454–462.

Tang, B., Zhou, K., Song, D., Yang, G. & Dai, A., 2003. Molecular systematics of the Asian mitten crabs, genus *Eriocheir* (Crustacea: Brachyura). *Mol. Pylogenet. Evol.* 29: 309–316.

Templeton, A.R. 2004. Statistical phylogeography: methods of evaluating and minimizing inference errors. *Mol. Ecol.* 13: 789–809.

Templeton, A.R., Crandall, K.A. & Sing, C.F. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* 132: 619–633.

Templeton, A.R., Routman, E. & Phillips, C.A. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics* 140: 767–782.

Templeton, A.R. & Sing, C.F. 1993. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics* 134: 659–669.

Vogler, A.P. & DeSalle, R. 1994. Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*. *Mol. Biol. Evol.* 11: 393-405.

Villalobos-Figueroa, A. 1982. Decapoda. In: Hurlbert, S.H. & Villalobos-Figueroa, A. (eds.), *Aquatic Biota of Mexico, Central America and the West Indies*: 215–239. San Diego, CA: San Diego State University Press.

Woods, C.A. & Sergile, F.E. 2001. *Biogeography of the West Indies: Patterns and Perspectives*. Boca Raton, FL: CRC Press.

Young, N.D. & Healy, J. 2003. GapCoder automates the use of indel characters in phylogenetic analysis. *BMC Bioinform.* 4: 6.

Zimmerman, J.K.H. & Covich, A.P. 2003. Distribution of juvenile crabs (*Epilobocera sinuatifrons*) in two Puerto Rican headwater streams: effects of pool morphology and past land-use legacies. *Archiv Hydrobiol.* 158: 343–357.

APPENDIX

Table A1. List of localities and specimens of *Epilobocera sinuatifrons* used for genetic and morphometric comparisons (sample numbers according to map in Figure 1).

Assignment	Sample No.	Drainage System	Locality	Sampling Date	Coordinates	*N* (Morpho-metrics)	*N* (Genetics)	Haplotype No. (see Figure 3)	Museum No.
Southwest (Blue)	1	Río Tallaboa	Convento Cave	07.03.2008	18°02.631′N 66°44.903′W	0	3	8, 14, 22	ZRC 2011-0204
	2	Río Tallaboa	Guayanés 2	18.10.2004	18°05.773′N 66°44.196′W	5	5	1, 4, 16, 22, 27	SMF 38907
	3	Río Guanajibo	Nueve Pasos	17.10.2004	18°09.467′N 67°04.534′W	14	11	8 (2×), 13, 15 (2×), 21 (3×), 22, 25, 29	SMF 38905
	4	Río Guanajibo	Pico Fraile	17.10.2004	18°07.524′N 66°54.975′W	6	4	17, 25, 26, 34	BMNH
	5	Río Guanajibo	Quebrada Flora	05.03.2008	18°10.045′N 67°04.135′W	0	3	16, 22, 30	
South-Center (Orange)	7	Río Coamo	Cuyon	09.05.2006	18°05.253′N 66°16.266′W	0	2	5, 37	
	8	Río Coamo	Coamo	10.05.2006	18°07.001′N 66°21.901′W	0	1	3	RMNH Crust.D.53425
	9	Río Salinas	Jajome	09.05.2006	18°02.856′N 66°11.950′W	0	2	12, 38	SMF 38909
	10	Río Jacaguas	Toa Vaca	10.05.2006	18°09.320′N 66°23.621′W	0	1	1	
	11	Río Bucaná	Cerillo	15.05.2006	18°08.753′N 66°36.472′W	0	1	1	
Southeast (Darkred)	12	Río Guayanés	Arenas	02.03.2008	18°03.581′N 65°57.896′W	0	3	31, 38 (2×)	MNHN U-2011-861
	13	Río Guayanés	Guayanés 1	12.10.2004	18°17.717′N 65°71.117′W	4	0		SMF 38911
	14	Río Guamaní	Guamaní	13.10.2004	18°02.310′N 66°06.110′W	4	2	21, 32	
	15	Río Jacaboa	Jacaboa	13.10.2004	18°01.149′N 65°57.704′W	4	2	38, 40	ZSM A 20110102
Northeast (Red)	16	Río Grande de Loiza	Cuevas Aguas Buenas	03.03.2008	18°13.950′N 66°06.490′W	0	3	11, 32, 38	SMF 38908
	17	Río Grande de Loiza	Grande de Loiza	13.10.2004	18°05.072′N 65°59.936′W	4	2	38 (2×)	
	18	Río Herrera	Herrera	10.03.2008	18°19.507′N 65°51.546′W	0	3	32, 39, 41	ZRC 2011-0206
	19	Río Espíritu Santo	El Verde	09.03.2008	18°19.264′N 65°49.185′W	0	1	38	
	20	Río Espíritu Santo	Espíritu Santo	23.10.2004	18°19.464′N 65°49.140′W	4	1	37	RMNH Crust.D.53426
	21	Río Fajardo	Fajardo	16.10.2004	18°16.904′N 65°43.896′W	8	4	9, 36, 38 (2×)	NHMW 25234
	22	Río Blanco	Blanco	16.10.2004	18°14.407′N 65°45.329′W	8	1	37	SMF 38910

Table A1. Continuation.

Assignment	Sample No.	Drainage System	Locality	Sampling Date	Coordinates	*N* (Morpho-metrics)	*N* (Genetics)	Haplotype No. (see Figure 3)	Museum No.
North-Center (Yellow)	23	Río Manatí	Manatí	09.03.2008	18°15.640′N 66°17.880′W	0	3	1, 2, 7	BMNH
	24	Río Manatí	Canabon	09.05.2006	18°13.685′N 66°20.488′W	0	2	3, 4	
	25	Río Manatí	Bauta	10.05.2006	18°10.444′N 66°24.439′W	0	2	3 (2×)	NHMW 25233
	26	Río Bayamón	Bayamón 1	04.03.2008	18°12.323′N 66°08.365′W	0	3	1, 31, 37	
	27	Río Bayamón	Bayamón 2	09.05.2006	18°12.328′N 66°08.352′W	0	1	37	
	28	Río Cibuco	Mavilla	09.03.2008	18°16.106′N 66°16.424′W	0	3	3, 6, 10	ZSM A 20110101
	29	Río Cibuco	Cueva Buruquena	25.07.2008	18°21.761′N 66°22.649′W	0	3	1, 3, 4	
	30	Río de la Plata	Plata	13.10.2004	18°05.710′N 66°04.854′W	4	2	37 (2×)	ZRC 2011-0205
	31	Río de la Plata	Arroyata	09.05.2006	18°11.966′N 66°12.528′W	0	1	37	MNHN U-2011-860
Northwest (Green)	32	Río Grande de Arecibo	Río Vacas	17.10.2004	18°08.624′N 66°44.952′W	2	2	19, 28	SMF 38906
	33	Río Grande de Arecibo	Río Tanama	21.10.2004	18°13.150′N 66°45.471′W	3	2	29, 33	MNHN U-2011-859
	34	Río Grande de Arecibo	Río Jauca	15.05.2006	18°11.158′N 66°38.365′W	0	2	4, 21	
	35	Río Camuy	Cueva Represa	08.03.2008	18°24.031′N 66°47.609′W	0	3	14, 20, 21	
	36	Río Guajataca	Guajataca	20.10.2004	18°19.822′N 66°54.955′W	7	5	1, 16, 18, 22, 35	RMNH Crust.D.53424
	37	Río Guajataca	Busque Estatal	20.10.2004	18°24.791′N 66°57.980′W	7	1	35	SMF 38917
	38	Río Culebrinas	Culebrinas	12.10.2004	18°22.105′N 66°57.185′W	6	1	22	SMF 38904
	39	Río Grande de Añasco	Guilarte 11	17.10.2004	18°10.300′N 66°46.292′W	2	2	1, 24	NHMW 25232
	40	Río Grande de Añasco	Guilarte 12	15.05.2006	18°08.550′N 66°45.951′W	3	8	1 (2×), 16 (3×), 19, 22, 23	
	41	Río Grande de Añasco	Limani	17.10.2004	18°10.392′N 66°48.217′W	5	2	22, 24	ZSM A 20110100
						Total: 100	Total: 103		

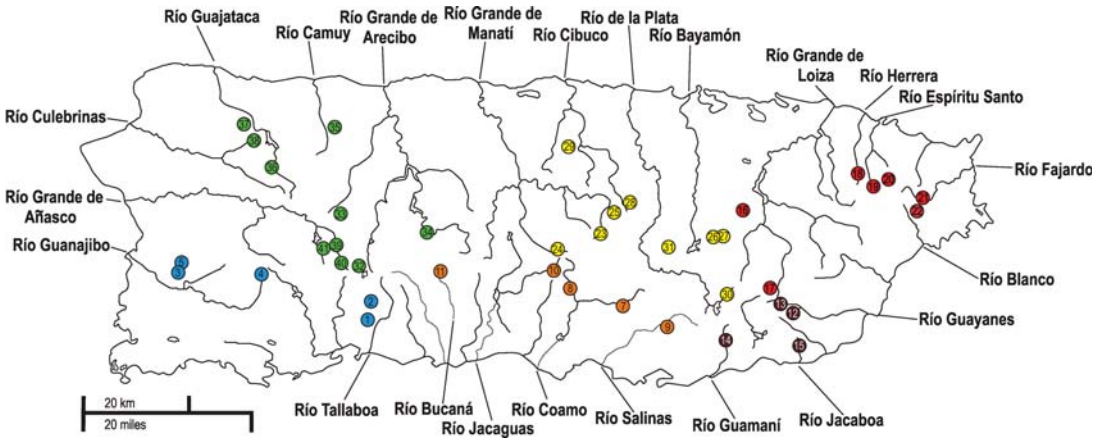

Figure 7 (Figure 1 in Schubart et al.). Map of Puerto Rico with 40 collection points, 23 rivers systems with corresponding names, and the colour coding for metapopulations of the freshwater crab *Epilobocera sinuatifrons* as used in the mitochondrial DNA phylogeographic comparisons based on mitochondrial DNA. Green: North-West, blue: South-West, yellow: North-Center, orange: South-Center, red: North-East, dark red: South-East.

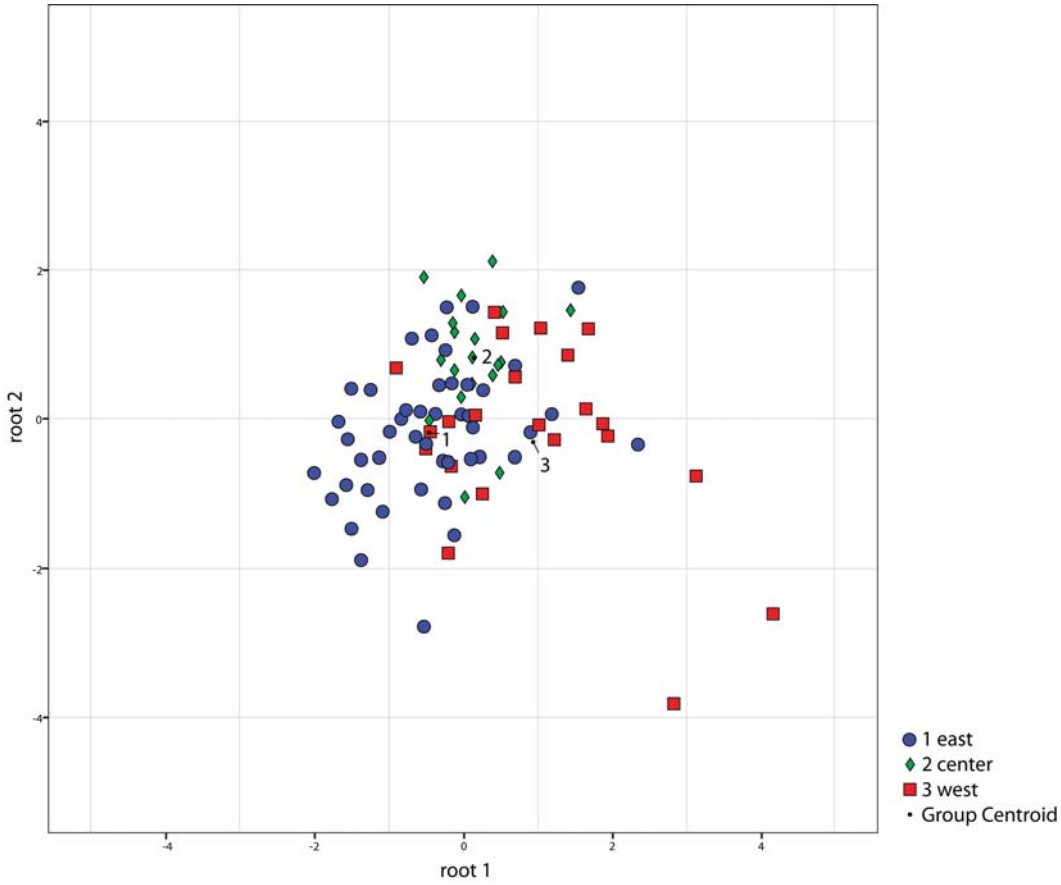

Figure 8 (Figure 2 in Schubart et al.). Morphometric analysis of *Epilobocera sinuatifrons* throughout Puerto Rico. Canonical analysis showing plot of the first two discriminant functions. Discrimination based on 10 normally distributed measurements among three metapopulations: West, Center, East. Coloration explained in legend.

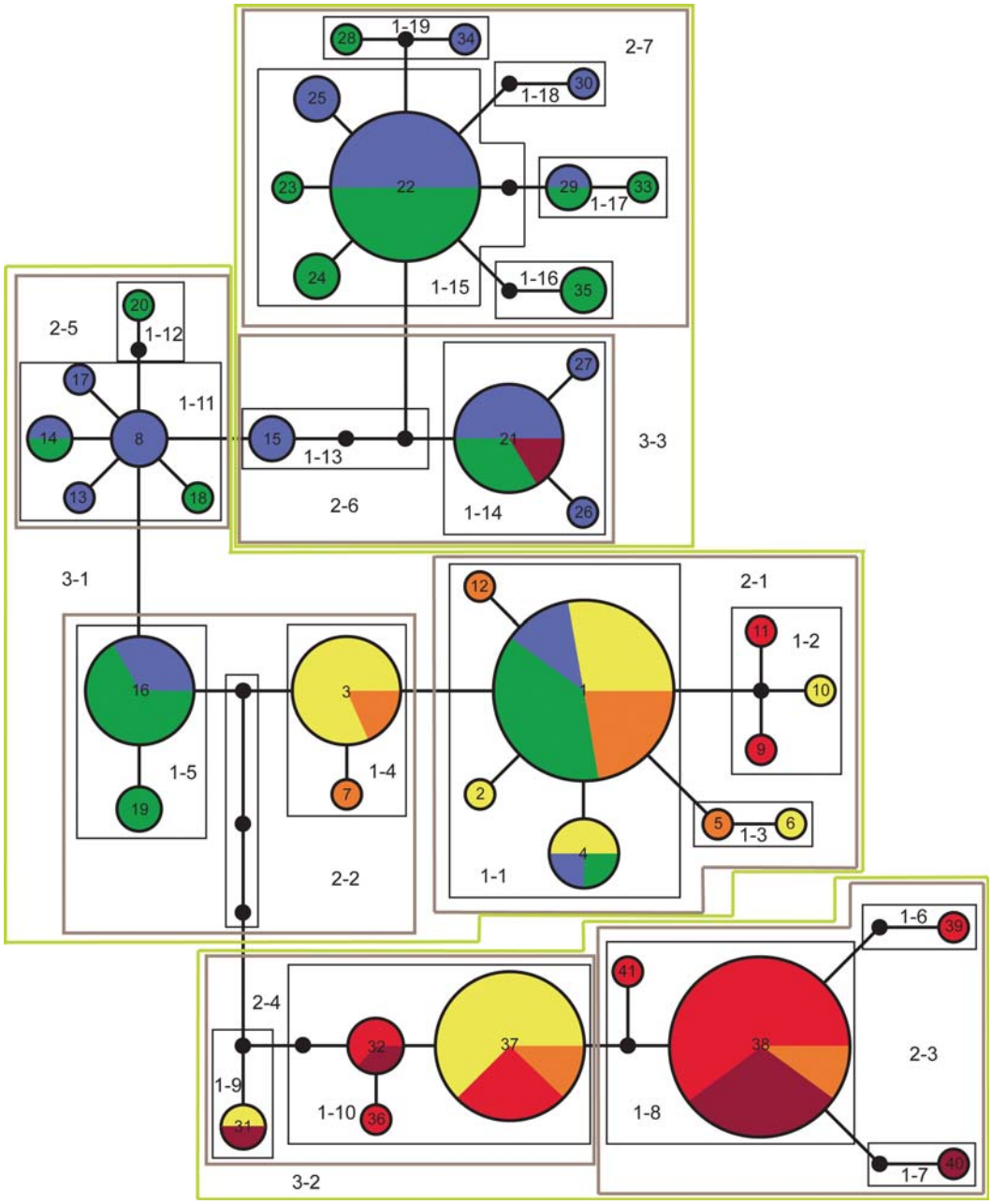

Figure 9 (Figure 3 in Schubart et al.). Statistical parsimony network of ND1 haplotypes of *Epilobocera sinuatifrons* ($N = 103$) from Puerto Rico constructed with TCS and the corresponding nesting design for the Nested Clade Analysis. Each black line represents one substitution, dots on lines indicate additional substitutions between haplotypes. The size of a circle represents the frequency of the corresponding haplotype. Coloration according to Figure 1 in Schubart et al.

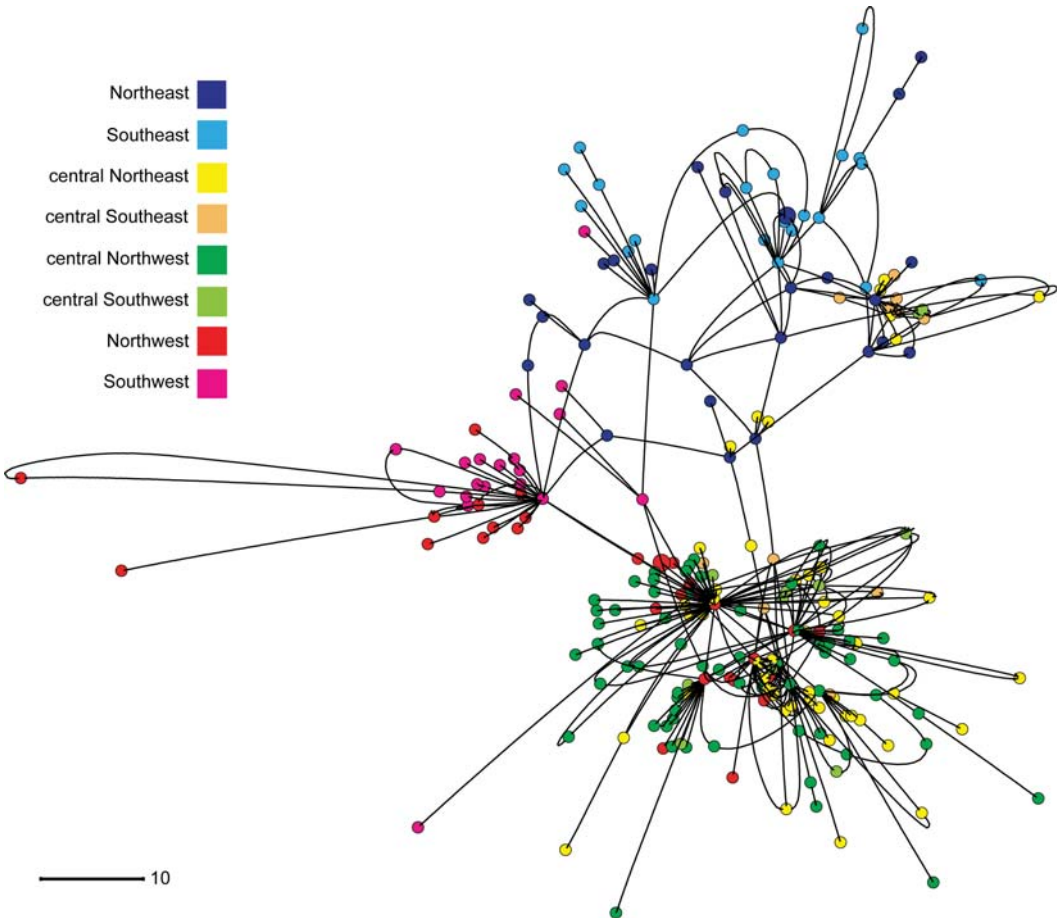

Figure 10 (Figure 4 in Schubart et al.). Minimum spanning network based on 238 clones of 1765 gap-coded basepairs of the ITS1-5.8S-ITS2 nuclear DNA region of *Epilobocera sinuatifrons* ($N = 40$) from Puerto Rico. Coloration explained in legend.