EVOLUTION OF *LIGIA* ISOPODS IN THREE GEOLOGICALLY DYNAMIC REGIONS

A Dissertation

by

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ABSTRACT

The oniscidean genus *Ligia* has a cosmopolitan distribution, occurring mainly in rocky supralittoral habitats, although a few species are found in tropical mountain freshwater habitats. The long-distance dispersal potential of the coastal *Ligia* isopods is very limited, due to a series of biological characteristics, which contributes to a high isolation of their populations. Consistent with this, high levels of allopatric differentiation have been detected for coastal *Ligia* in different parts of the world, with phylogeographic patterns exhibiting signatures of past geological and oceanographic events. In this dissertation, we used mitochondrial and nuclear gene sequences to infer phylogeographic patterns of *Ligia* isopods in the Hawaiian archipelago, and the region comprised by the Caribbean Sea and the Eastern Pacific coast of Colombia and Central America. We also conducted geometric-morphometric analyses to determine whether differences in overall body shape exist between divergent lineages in coastal *Ligia* from the Hawaiian archipelago and from the region between Central California and Central Mexico, including the Gulf of California. We observed that *Ligia* populations from the Caribbean Sea and the Pacific coast of Central America and Colombia, as well as those from the Hawaiian archipelago, harbor highly divergent lineages, suggesting that the *Ligia* species recognized for these regions represent cryptic species complexes. Phylogeographic patterns suggest that passive overwater dispersal has been an important factor shaping the evolutionary history of *Ligia* in these two regions. Geometric morphometric approaches uncovered morphological differences between highly

divergent genetic lineages of *Ligia* isopods in the Hawaiian archipelago and the region between Central California and Central Mexico, including the Gulf of California. Large overlap in body shapes occur, however, suggesting overall body shape evolution is somewhat constrained and this character is unreliable for taxonomic distinction of these lineages.

DEDICATION

To my parents,

for everything you have done for me

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NOMENCLATURE

BS	Bootstrap support value
COI	Cytochrome oxidase I mitochondrial gene
Cytb	Cytochrome b mitochondrial gene
d.f.	Degrees of freedom
DFA	Discriminant function analysis
K2P	Kimura-2-Parameter genetic divergence
LCOOV	Leave-one out cross-validation
My	Million years
Ma	Million years ago
PP	Bayesian posterior probability

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CHAPTER I

INTRODUCTION*

Isopoda represents the most speciose order of crustaceans (~10,500 species), with species inhabiting environments as varied as the deep sea, coastal habitats, and montane habitats at high altitude. Isopods are characterized by high rates of endemism (Brusca 1981; Brusca 1987; Kensley 2001), possibly as a consequence of their biological characteristics, which include direct development (a trait shared by all peracarids). In addition, and perhaps as a result of these biological traits, high levels of previously cryptic biodiversity have been recently reported in several isopod taxa (Eberl et al. 2013; Held and Wagele 2005; Hurtado et al. 2013; Hurtado et al. 2010; Jung 2008; Raupach and Wagele 2006; Taiti et al. 2003; Xavier et al. 2011). Of these, intertidal isopods in the genus *Ligia* are of particular interest. This genus consists of ~37 nominal species (Schmalfuss 2003), most of which are found in a very narrow vertical range of upper rocky intertidal habitats throughout the world (Jackson 1922; Schotte et al. 1995). Intertidal species remain most of the day hiding under rocks and inside crevices in the upper and dry intertidal, and eventually can be found in the rocky intertidal spray area or supralittoral. At night they are more active, feeding mainly on algae and detritus found on the shore. Despite the retention of an ancestral ability to swim and breathe

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underwater, they avoid immersing into open water (Barnes 1932; Barnes 1935). Movement outside the upper rocky intertidal is also prevented by their low desiccation resistance (Barnes 1932; Barnes 1934; Barnes 1935). Because of this, they do not venture inland and also avoid dispersal through large stretches of sandy beaches devoid of rocks. Therefore, *Ligia* populations appear to remain highly isolated on discrete rocky intertidal beaches. Not surprisingly, high levels of allopatric cryptic biodiversity are reported for *Ligia* from different regions of the world (Eberl et al. 2013; Hurtado et al. 2010; Jung 2008; Taiti et al. 2003). In addition, *Ligia* isopods appear to have high potential as biogeographic indicators, as they are known to retain signatures of past geological and oceanographic events in their phylogeographic patterns (Eberl et al. 2013; Hurtado et al. 2010; Jung 2008; Taiti et al. 2003). Therefore, studies of *Ligia* in highly dynamic geological dynamic regions may not only uncover high levels of cryptic biodiversity, but also be informative on the factors contributing to their diversification.

This main goal of this dissertation is to increase our understanding of the biodiversity of *Ligia* isopods in three geologically dynamic regions: (1) the Caribbean islands including the Atlantic and Pacific coasts of Central America (hereafter Caribbean region); (2) the Hawaiian archipelago; and (3) the region between Central California and Central Pacific Mexico, including the Gulf of California. We conduct phylogeographic analyses in populations of *Ligia* isopods in the Hawaiian archipelago and the Caribbean region. This may allow for the detection of previously unknown genetic biodiversity. We also apply geometric-morphometric analyses to determine whether *Ligia* lineages reported from the region between Central California and Central Pacific Mexico,

including the Gulf of California, and the Hawaiian archipelago exhibit morphological differences. These analyses may be useful for taxonomic revision of *Ligia* species from these regions.

I.1 The Caribbean region

The Caribbean region, a biodiversity hotspot (Myers et al. 2000), includes the Caribbean Sea, its islands, and the surrounding continental coasts of the Americas. The region's value to evolutionary research is highlighted by its complex geological history and the striking adaptive radiations observed for some poorly dispersing endemics (e.g. *Anolis* lizards) (Ricklefs and Bermingham 2008). The Caribbean Plate is suggested to have formed in the eastern Pacific during the Late Jurassic to Mid-Cretaceous (~90–160 Ma) (Burke et al. 1978; Malfait and Dinkelman 1972; Pindell 1994; Wilson 1965), reaching its current position after an east-northeast displacement relative to the American Plate (Dengo and Case 1990; Donovan and Jackson 1994; Pindell 1994). Although alternative hypotheses exist (James 2009a; James 2009b; Meschede and Frisch 1998), a Pacific origin is considered the most likely explanation (Pindell et al. 2006). Magmatism associated with the subduction of the American plate under the Caribbean plate during the early Cretaceous (Pindell 1994) is thought to have given rise to a proto-Antillean archipelago between North and South America. Whether this archipelago formed a solitary landmass at some point is unsettled (Hedges 2006). In the late Cretaceous, the proto-Antilles began to drift eastwardly from the continental mainland (Burke 1988; Ross and Scotese 1988). The collision of the proto-Antillean archipelago with the

Bahamas Platform in the Paleogene caused subduction of the North American Plate under the Caribbean Plate and associated volcanic activity to cease (Hedges 2006), giving rise to the Cayman Trough. These geological events led to the fixation of, in chronological order, western Cuba (Bralower and Iturralde-Vinent 1997), Central Cuba (Hempton and Barros 1992; Pardo 1975), Hispaniola (Mann et al. 1991), and Puerto Rico (Dolan et al. 1991) to the North American Plate. Alternate timing for these events have been proposed (see references in Crother and Guyer 1996); and it is unclear whether these landmasses were emergent prior to 45 Ma (Iturralde-Vinent and MacPhee 1999; MacPhee and Grimaldi 1996; MacPhee and Iturralde-Vinent 1994). Subsequent volcanic activity associated with subduction along the eastern edge of the Caribbean Plate during the Cenozoic is thought to have given rise to the islands of the Lesser Antilles, with the exception of Barbados (Briden et al. 1979).

The long-standing isolation of the Greater Antilles, coupled with their peculiar fauna and high rate of endemism (Baker and Genoways 1978; Borhidi 1996; Hedges 2006), has long attracted the attention of biogeographers (Barbour 1914; Darwin 1859; Gosse and Hill 1851; Sloane 1707; Wallace 1880). Although the Caribbean Sea harbors the highest marine biodiversity of the Atlantic basin (Miloslavich et al. 2010; Roberts et al. 2002), organisms inhabiting rocky intertidal habitats throughout the ~20,000 Km of Caribbean coastline remain surprisingly under studied (Miloslavich et al. 2010). Considering the high levels of cryptic biodiversity reported for *Ligia* isopods from other geologically dynamic regions (Eberl et al. 2013; Hurtado et al. 2010; Jung 2008; Taiti et al. 2003) and their presence throughout the Greater Caribbean region (Kensley and

Schotte 1989), it is likely that *Ligia* in this region harbors high levels of cryptic biodiversity. In addition, phylogenetic studies of *Ligia* from the Caribbean may also shed light on the biogeography of the region, as these isopods can reveal high levels of allopatric genetic differentiation at a fine scale (Hurtado et al. 2010), and are known to retain signatures of past geological and oceanographic events in different parts of the world (Eberl et al. 2013; Hurtado et al. 2010). In Chapter II of this dissertation, we conduct phylogeographic analyses of *Ligia* from the Caribbean region based on both mitochondrial and nuclear gene data, to uncover cryptic diversity and understand the evolution of these isopods in this region.

I.2 The Hawaiian archipelago

The main Hawaiian Islands consist of eight remote islands belonging to a chain of atolls, seamounts, and islets extending for ~5,700km in the North Pacific Ocean. These islands are thought to have arisen by a relatively stationary hotspot on the Earth's mantle (Wilson 1963). As the Pacific Plate drifted in a northwestern direction over it, magma from the hotspot punctured through the Pacific plate, with deposited magma forming seamounts. If enough volcanic activity occurred, these seamounts became aerially positive, leading to the formation of islands as the Pacific plate continued its westward movement. This process is thought to have occurred over the last 80 My, with a change in direction of the Pacific plate about 50–42 Ma accounting for a corresponding change in direction in the island chain known as the Hawaiian-Emperor Bend (Sharp and Clague 2006), and with older islands disappearing due to erosion and subsidence. The Hawaiian

Main Islands lie at the eastern end of the chain and are the youngest islands in the chain, ranging in age from the 0.6–0 My for the youngest island (Hawai'i) to 5.0 My for the oldest (Ni'ihau) (Price and Clague 2002).

The remoteness of the islands, coupled with the progressive nature of island formation is thought to have given rise to several striking examples of adaptive radiations in terrestrial organisms (Carson and Kaneshiro 1976; Gillespie et al. 1994; Otte 1994; Zimmerman 1958). Diversification, however, was not thought to have occurred in marine organisms within the archipelago (Kay and Palumbi 1987). Recent studies, however, have identified three notable exceptions in coastal organisms from patchy habitats: *Cellana* limpets (Bird et al. 2011), the anchialine shrimp *Halocaridina rubra* (Craft et al. 2008), and *Ligia* isopods (2003). Currently, three *Ligia* species are reported from the Hawaiian archipelago: the endemic *Ligia hawaiensis* (Dana 1853) and *Ligia perkinsi* (Dollfus 1900), and the introduced *Ligia exotica* (Roux 1828). Both *L. hawaiensis* and *L. exotica* are found in rocky intertidal habitats, but *L. exotica* is a cosmopolitan species only found on artificial substrate, such as piers and harbors (Taiti and Howarth 1996). The terrestrial *L. perkinsi* is found at high altitude on the islands of Kaua'i, O'ahu, and Hawai'i, and is one of the only seven terrestrial species of *Ligia* described to date (Taiti and Howarth 1996). The monophyly of *L. hawaiensis* with *L. perkinsi* indicate that these species diversified within the Hawaiian archipelago (Hurtado et al. 2010; Taiti et al. 2003). Furthermore, high levels of allopatric genetic divergence amongst *L. hawaiensis* and *L. perkinsi* populations from Kaua'i and O'ahu are reported (Taiti et al. 2003).

The molecular characterization of *Ligia* isopods from previously unsampled main Hawaiian Islands may uncover additional divergent allopatric lineages, and thus further our understanding of diversification in coastal environments of the Hawaiian archipelago. In Chapter III of this dissertation, we apply robust phylogenetic approaches to a molecular dataset, both mitochondrial and nuclear markers, from previously unsampled *Ligia* populations from the main Hawaiian Islands. Given the biology of *Ligia* isopods, we expect to find additional highly divergent lineages and evidence of allopatric isolation between populations. We interpret phylogenetic relationships in light of the geological history of the islands. Furthermore, we also apply geometric-morphometric analyses to determine whether *L. hawaiensis* lineages exhibit any morphological differences that may aid in the taxonomy of these coastal isopods.

I.3 The Gulf of California and the Eastern Pacific

The Gulf of California, also known as the Sea of Cortez, is a 160,000-km^2 marine basin, situated between the Baja California Peninsula and the west coast of mainland Mexico. With about 4,000 km of coastline, it is considered a marine biodiversity hotspot. Despite a long-standing interest in the biogeography of the region, the geological origins of this region remain controversial (Carreño and Helenes 2002; Durham and Allison 1960; Grismer 1994; Helenes and Carreño 1999; Murphy and Aguirre-Leon 2002; Riddle et al. 2000). Traditionally, phylogeographic studies have followed the geological framework of Riddle et al. (2000). Recently, however, Hurtado et al. (2010) studied phylogeographic patterns of *Ligia* isopods from Central California to Central Mexico,

including the Gulf of California, and found that their patterns were not congruent with the framework in Riddle et al. (2000), but with alternative geological hypotheses.

The study of Hurtado et al. (2010) found remarkable levels of cryptic allopatric genetic diversity in *Ligia* (Figures I.1 and I.2), with most of the rocky beaches surveyed corresponding to unique evolutionary lineages, many of which are highly divergent. Hurtado et al. (2010) identified four highly divergent lineages (Figure I.1): (1) a Central California clade (*Clade A*); (2) a Baja Pacific-Southern California clade (*Clade BCDE*); (3) a Gulf clade (*Clade NS*); and a Careyes clade (*Clade F*). The Gulf clade was composed of two highly divergent lineages (Figure I.2): the Gulf North (*Clade N*) and the Gulf South clade (*Clade S*) with among clade divergences, as measured by Kimura-2-Parameter distances (K2P) for the mitochondrial Cytochrome Oxidase I (COI) gene, ranging between 15.16–27.47%. Maximum within clade divergences were 25.30% and 21.55% for clades *N* and *S* respectively, indicating a long history for *Ligia* isopods within the Gulf of California region. The *Clade BCDE* was in turn composed of four divergent lineages: (1) a Southern California clade (*Clade B*); (2) a California clade (*Clade C*); (3) a Baja Pacific North clade (*Clade D*); (4) and a Baja Pacific South clade (*E*). COI K2P divergences among these clades ranged between 7.28–19.6%, whereas within clade divergences were lower than those observed in the Gulf clades (maximum divergences: *Clade A*: 1.55%; *Clade B*: 8.60%, *Clade C*: 2.10%; *Clade D*: 2.08%; *Clade E*: 8.77%). COI K2P divergence values among clades in the region ranged between 7.28–29.89%, whereas within clade divergences range between 1.14–25.30%.

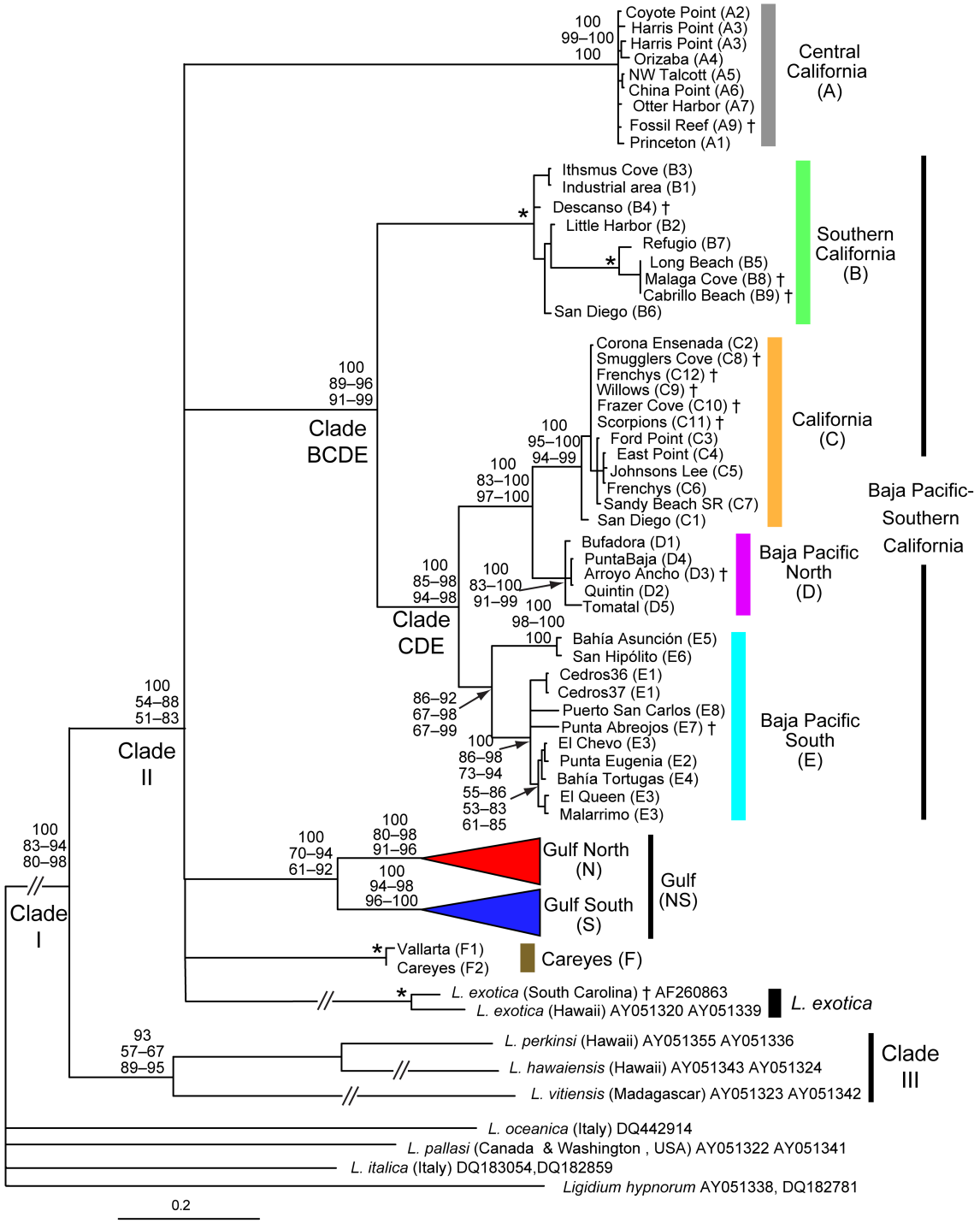

Figure I.1: Maximum likelihood tree of Ligia samples from Central California to Central Mexico, including the Gulf of California reported by Hurtado et al. (2010). The tree was obtained by RaxML for the 16S rDNA and COI genes (model GTR + G), and rooted with Ligidium. Numbers by nodes indicate the corresponding range of node support values obtained for each method: Top-Bayesian Posterior Probabilities; Middle-GARLI bootstrap support; and Bottom-RaxML bootstrap support. * denotes nodes that received 100% support for all methods. For more information, see original reference.

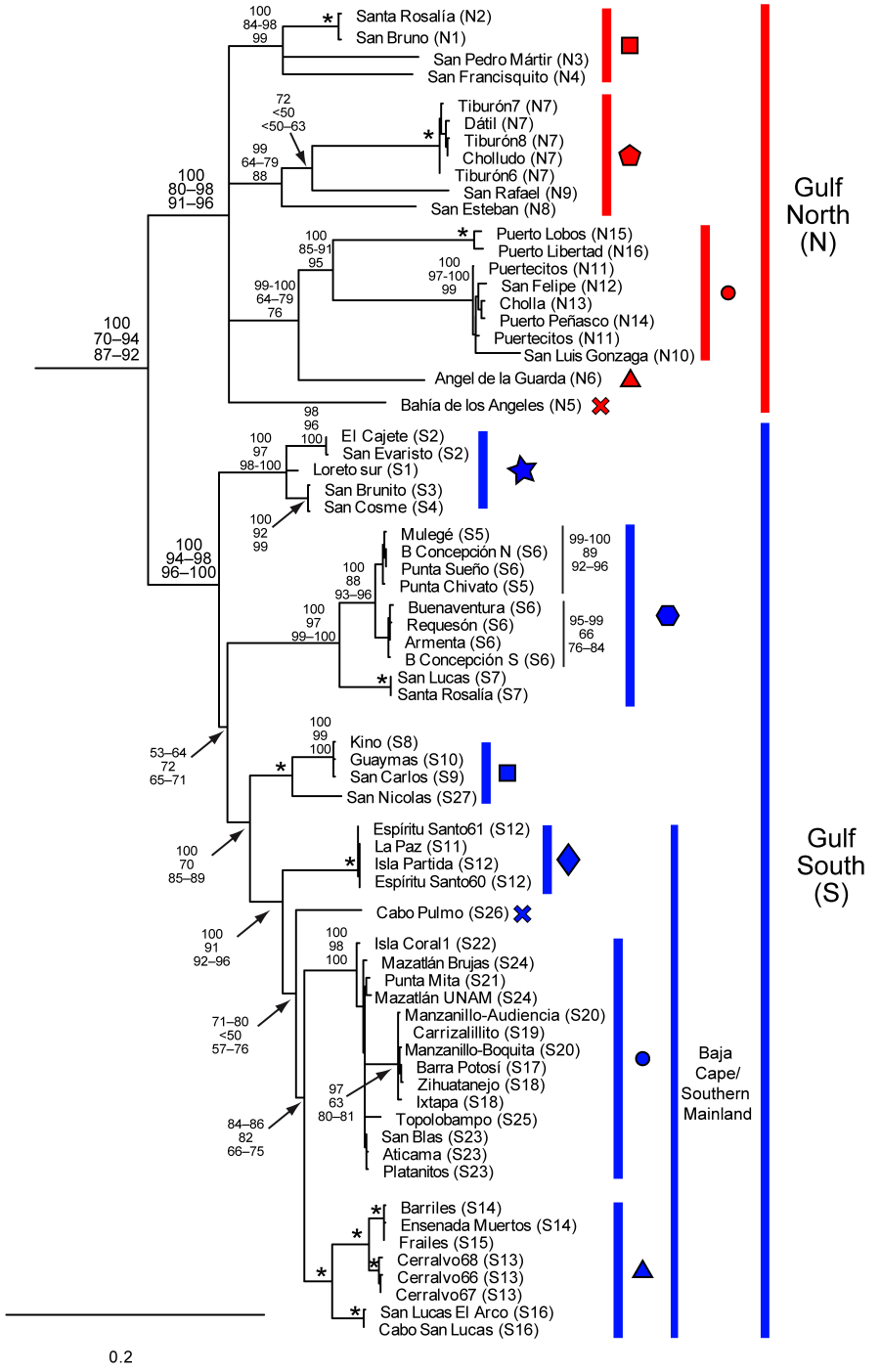

Figure I.2: Maximum likelihood tree of *Ligia* Gulf samples (expansion of the Gulf clade in Figure I.1) reported by Hurtado et al. (2010). Numbers by nodes indicate the corresponding range of node support values obtained for each method: Top-Bayesian Posterior Probabilities; Middle-GARLI bootstrap support; and Bottom-RaxML bootstrap support. * denotes nodes that received 100% support for all methods. Nodes receiving less than 50% support for all methods were collapsed. Nodes with no corresponding support values were of little relevance or had low support values. For further information, see original reference.

Given the extraordinary levels of allopatric genetic divergence among *Ligia* populations in this region, it is important to assess whether distinct clades can be differentiated morphologically. Doing so may aid with the taxonomy of *Ligia* isopods in this region, while also being informative on the processes driving morphological evolution. In Chapter IV of this dissertation, we compare overall body shapes across the lineages identified by Hurtado et al. (2010) using landmark-based geometric-morphometric approaches. These methods have been used to detect morphological differences between previously cryptic species in several invertebrate groups (Carvajal-Rodríguez et al. 2006; Francuski et al. 2009; Milankov et al. 2009; Mitrovski-Bogdanovic et al. 2013), including crustaceans (Bertocchi et al. 2008; Zuykova et al. 2012).

CHAPTER II

CRYPTIC DIVERSITY AND EVIDENCE OF PASSIVE OVERWATER DISPERSAL IN *LIGIA* ISOPODS FROM THE CARIBBEAN

II.1 Introduction

The Caribbean Sea, including its thousands of islands, has served as incubator for an exceptional marine and terrestrial biodiversity (Brummitt and Lughadha 2003; Kerswell 2006; Miloslavich et al. 2010; Myers et al. 2000), making this region a natural laboratory for research on evolution, biogeography, phylogeography, and biodiversity (Ricklefs and Bermingham 2008). A long and complex geological history, as well as the potential for isolation of populations among and within islands, provided numerous opportunities for allopatric differentiation (Ricklefs and Bermingham 2008). Striking radiations observed in several terrestrial taxa, such as the Caribbean *Anolis* lizards, a classic case of adaptive radiation (Losos 2009), exemplify the region's value for evolutionary research (Alonso et al. 2012; Dávalos 2007; Francisco-Ortega et al. 2008; Rodriguez et al. 2010). Research on biogeography and phylogeography has generated enduring controversies concerning the contributions of vicariance and over-water dispersal in shaping the region's biodiversity (Barbour 1914; Crother and Guyer 1996; Darlington 1938; Guyer and Crother 1996; Hedges 1996a; Myers 1937). These studies, however, exhibit a strong bias towards terrestrial vertebrates (Alonso et al. 2012; Dávalos 2004; Dávalos 2007; Hedges 2006; Heinicke et al. 2007; Hower and Hedges 2003), and to a lesser extent, terrestrial invertebrates (Crews and Gillespie 2010; Felix and Mejdalani 2011;

Oneal et al. 2010), despite the high marine diversity of the Caribbean Sea, which is the highest of the Atlantic basin (Miloslavich et al. 2010; Roberts et al. 2002). Studies of marine biodiversity and phylogeography have been strongly biased toward members of coral reefs (e.g. Baums et al. 2005; Eytan and Hellberg 2010; Taylor and Hellberg 2006), whereas members of other marine habitats have been poorly studied. Organisms inhabiting the transition between sea and land (e.g. the supralittoral zone), tightly connected to specific patchy habitats (e.g. rocky or sandy), and with low dispersal potential, likely harbor high levels of population genetic differentiation (e.g. Hurtado et al. 2013). Therefore, phylogeographic studies of such taxa in the Caribbean are expected to reveal high levels of cryptic diversity and phylogeographic structure, thus enhancing our understanding on marine diversification, evolution, and biogeography in this region.

Coastal isopods of the genus *Ligia*, tightly associated to the rocky supralittoral of intertidal shores, exhibit high levels of allopatric genetic differentiation in different parts of the world; with highly structured phylogenetic patterns including highly differentiated cryptic lineages that indicate geographically localized radiations (Eberl et al. 2013; Hurtado et al. 2010; Jung 2008; Taiti et al. 2003; Santamaria et al. in preparation). In some cases, phylogenetic patterns are congruent with past tectonic events at the continental margin (Hurtado et al. 2010), with oceanic environmental factors, such as sea surface temperature (Eberl et al. 2013), or suggestive of past oceanic dispersal events (Santamaria et al. in preparation). Biological characteristics of coastal *Ligia* isopods, including direct development, restrict these animals to a very narrow vertical portion of

rocky intertidal shores that extends from the splash to the supralittoral zone, limiting gene flow among isolated populations (see Carefoot and Taylor 1995; Hurtado et al. 2010).

Ligia baudiniana Milne-Edwards 1840 is the only valid native species of *Ligia* in the Caribbean Sea region (Schmalfuss 2003). This species was originally described from specimens collected in the San Juan de Ulúa Fort in Veracruz, Mexico, in the Gulf of Mexico (Milne-Edwards 1840). The distribution of *L. baudiniana* is considered to include the Atlantic coast of the Americas from Florida to Brazil, the Caribbean Sea Islands, Barbados, Bermuda, and the Eastern Pacific coast from California to Ecuador including the Gulf of California and the Galapagos Islands (Brusca 1980; Espinosa-Pérez and Hendrickx 2001; Kensley and Schotte 1989; Mulaik 1960; Schmalfuss 2003; Schultz 1972; Schultz 1974; Van Name 1936). Other *Ligia* species have been described in the Caribbean region (Brandt 1833; Budde-Lund 1893; Dahl 1892; Moore 1901; Perty 1834), but are no longer valid. *Ligia gracilis* Moore 1901 and *Ligia hirtitarsis* Dahl 1892 have been synonymized with *L. baudiniana* (Schmalfuss 2003); whereas *Ligia filicornis* Budde-Lund 1893, *Ligia grandis* Perty 1834, and *Ligia olfersii* Brandt 1833 have been synonymized with *Ligia exotica* Roux 1828 (Schmalfuss 2003), which is considered a cosmopolitan invasive species common in harbors around the world. Adding to the taxonomic confusion, *Ligia baudiniana* was suggested to be a synonym of *L. exotica* by Budde-Lund (1885). Phylogeographic analyses of *Ligia* in the Caribbean Sea region can help clarify the confusing taxonomy of this isopod in this region, providing information on the number of lineages present, their phylogenetic

relationships, and their affinities to other *Ligia*.

To obtain a better understanding on the evolution of *Ligia* isopods in the Caribbean, we studied phylogeographic patterns of these isopods in this region, including also samples from Bermuda, the Bahamas, the Gulf of Mexico and the Pacific coasts of Central America and Colombia. In addition, we examined male gonopodia to identify specimens possessing a diagnostic character attributed to *L. baudiniana* (Schultz 1972; Schultz and Johnson 1984). We expected to find high levels of cryptic diversity as observed in other regions. We examined whether the resulting phylogeographic patterns of *Ligia* in the Caribbean Sea are consistent with: (1) suggested biogeographic patterns based on contemporaneous population connectivity of marine organisms via larval dispersal (Cowen et al. 2006); (2) a colonization pattern from the southeast to the northwest suggested for the colonization of terrestrial animals in the Caribbean (Hedges 1996b); or (3) heterogeneity and stochasticity of past oceanographic patterns (Iturralde-Vinent and MacPhee 1999). In addition, we examined whether phylogeographic patterns are consistent with GAARlandia, a temporary land bridge hypothesized to exist 33–35 Ma, connecting the northern South American coast with the Greater Antilles (Iturralde-Vinent and MacPhee 1999).

II.2 Material and methods

II.2.1 Sampling

We examined samples of *Ligia* from 35 localities in the Caribbean Sea (including the West Indies and the mainland Caribbean coast of Central America and northern South

America), Bermuda, and on the Pacific coasts of Central America and Colombia; hereafter, the study area (Figure II.1; Table II.1). We also examined individuals from the San Juan de Ulúa Fort (Gulf of Mexico, Veracruz, Mexico), the type locality of *L. baudiniana* (star in Figure II.1). Most populations were sampled by hand and preserved in 70–100% Ethanol or 20% DMSO upon collection; others were obtained from museums and collaborators.

II.2.2. Molecular methods

We extracted total genomic DNA from pleopods/legs of *Ligia* individuals with the DNEasy Blood & Tissue kit (Qiagen Inc., Valencia, CA), following standard protocol manufacturer's instructions. We PCR-amplified and sequenced a 361-bp fragment of the Cytochrome-b mitochondrial (mt) gene (Cytb) from 1–5 individuals per locality, using primers (144F/151F and 270R/272R) and conditions described by Merritt et al. (1998). A subset of these individuals (essentially one individual per locality; see Supplementary Figure S.II.1) was then amplified and sequenced for three additional mitochondrial gene fragments: a ~490-bp fragment of the 16S rDNA (primers 16Sar/16Sbr; Palumbi 1996); ~495-bp of the 12S rDNA (primers crust-12Sf/crust-12Sr; Podsiadlowski and Bartolomaeus 2005); and 658-bp of the Cytochrome Oxidase I gene (COI, primers LCO1490/HCO2198; Folmer et al. 1994). For several individuals (Table 1), we also amplified and sequenced a 661-bp segment of the nuclear gene Sodium Potassium ATPase alpha-subunit (NaK, primers NaK for-b/NaK rev2; Tsang et al. 2008). We cleaned PCR products with a mixture of Exonuclease I (New England

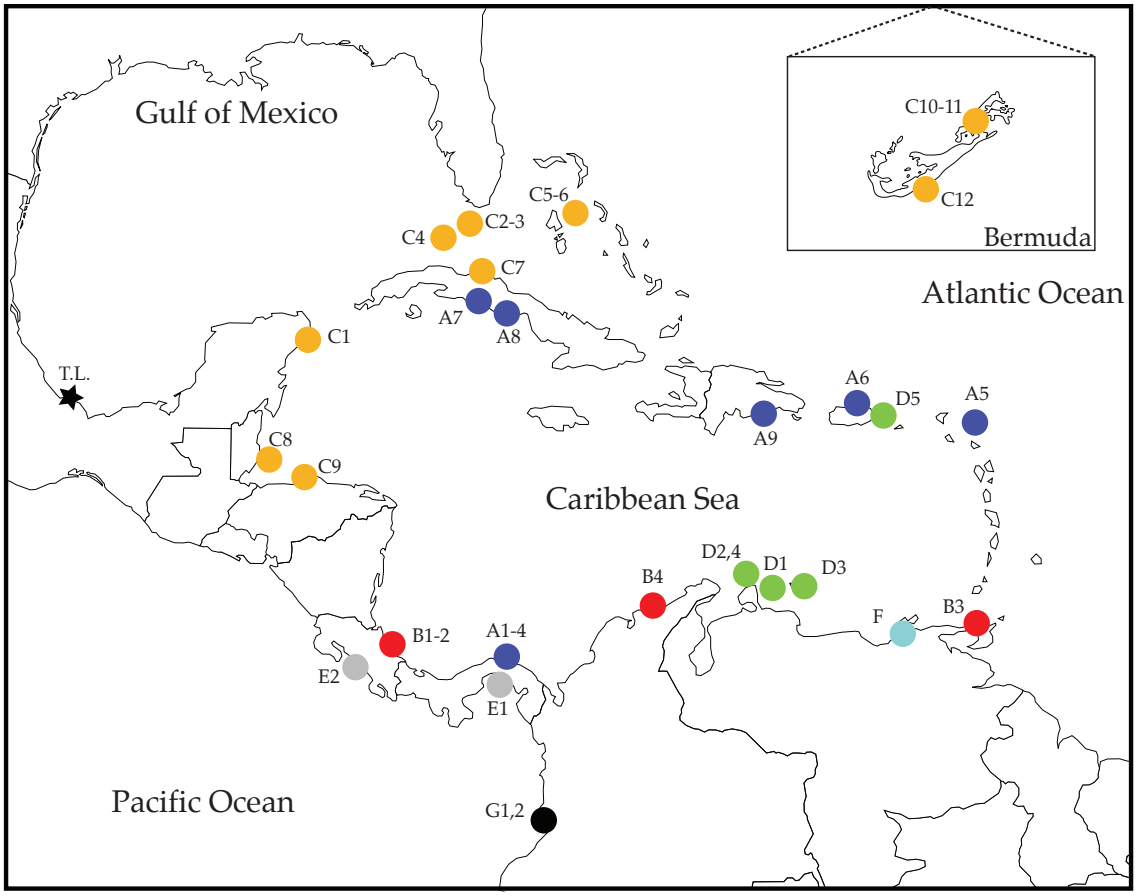

Figure II.1. Sampled localities in the Caribbean region Color and shapes correspond to clades in other figures in the chapter and labels correspond to those in Table II.1. A1–4-Portobelo and Fort Sherman, Panama; A5-Marigot, St. Martin; A6-Condado Beach, Puerto Rico; A7-Yaguanabo, Cuba; A8-Playa Ancon, Cuba; A9-Boca Chica, Dominican Republic; B1-El Limon, Costa Rica; B2-Piuta, Costa Rica; B3-Maracas Bay, Trinidad and Tobago; B4-Santa Marta, Colombia; C1-Cozumel, Mexico; C2-Duck Key, FL, USA; C3-Indian Key, FL, USA; C4-Summerland Key, FL, USA; C5-Nassau, The Bahamas; C6-Jaws Beach, The Bahamas; C7-Habana, Cuba; C8-Carrie Bow Cay, Belize; C9-La Ensenada, Tela, Honduras; C10-Long Bird Bridge, Bermuda; C11-Cricket Field, Bermuda; C12-Stonehole Bay; Bermuda; D1-Piscaderabaai Bay, Curaçao; D2-Spaans Lagoen, Aruba; D3-Donkey Beach, Bonaire; D4-East Coast of Aruba; D5-Fajardo, Puerto Rico; E1-Veracruz, Panama; E2-Caldera, Costa Rica; F-El Morro, Venezuela; G1-Maguipi, Colombia; G2-Isla Palma, Colombia. The type locality (T.L.) of *Ligia baudiniana* is marked by a star. Boldfaced locality names indicate those examined in nuclear gene analyses.

Table II.1: Included localities and corresponding GenBank accession numbers, Latitude and Longitude

Species	Locality Name	Map Label	16S. Acc. No	12S Acc. No.	COI Acc. No.	Cyt-b Acc. No.	NaK Acc. No.	Lat.	Long.
L. baudiniana	Nassau, The Bahamas	C5	KF555785	KF555827	KF555858	KF555658-63	KF555880	25°04'47.22"N	77°22'11.52"W
L. baudiniana	Jaws Beach, The Bahamas	C6	KF555787	KF555821	KF555862	KF555683	KF555882	25°01'05.05"N	77°32'49.00"W
L. baudiniana	Maracas Bay, Trinidad and Tobago	B3	KF555773	KF555820	N/A	KF555723-26	KF555879	10°45'28.98"N	61°26'07.38"W
L. baudiniana	Long Bird Bridge, Bermuda	C10	KF555782	KF555829	KF555856	KF555727-28	KF555873	32°21'05.34"N	64°42'35.16"W
L. baudiniana	Cricket Field, Bermuda	C11	KF555781	KF555830	N/A	KF555729-30	N/A	32°21'21.00"N	64°42'58.44"W
L. baudiniana	Stonehole Bay, Bermuda	C12	KF555783	KF555831	KF555857	KF555731-32	N/A	32°15'19.62"N	64°48'49.68"W
L. baudiniana	Santa Marta, Colombia	B4	KF555776	KF555819	KF555852	KF555733-38	KF555885	11°20'07.74"N	73°58'31.26"W
L. baudiniana	Fajardo, Puerto Rico	D5	KF555793	KF555807	KF555869	KF555664-68	KF555874	18°21'38.84"N	65°37'28.51"W
L. baudiniana	Playa Bonita, Limon, Costa Rica	B1	KF555775	KF555818	KF555850	KF555669-72	N/A	10°00'39.59"N	83°03'46.87"W
L. baudiniana	Piuta, Limon, Costa Rica	B2	KF555774	KF555817	KF555851	KF555673	N/A	10°00'20.70"N	83°02'06.92"W
L. baudiniana	Caldera, Costa Rica	E2	KF555796	KF555836	KF555864	KF555674-79	N/A	09°56'26.96"N	84°44'02.93"W
L. baudiniana	Carrie Bow Cay, Belize	C8	KF555777	KF555832	KF555853	KF555680	KF555889	16°48'09.42"N	88°04'55.59"W
L. baudiniana	Portobelo (A), Panama	A4	KF555767	N/A	KF555845	KF555681	KF555887	09°33'11.70"N	79°39'35.58"W
L. baudiniana	Portobelo (B), Panama	A2	KF555765	KF555810	KF555843	KF555682	KF555886	09°32'14.72"N	79°40'26.30"W
L. baudiniana	Portobelo (C), Panama	A3	KF555766	KF555809	KF555846	KF555684-88	KF555888	09°32'54.24"N	79°40'14.10"W
L. baudiniana	Fort Sherman, Panama	A1	KF555764	KF555811	KF555844	KF555689-93	KF555884	09°21'51.36"N	79°56'55.56"W
L. baudiniana	Veracruz, Panama	E1	KF555795	KF555837	KF555863	KF555694-98	KF555878	08°53'28.30"N	79°35'35.19"W
L. baudiniana	Maguipi, Valle del Cauca, Colombia	G1	KF555798	KF555835	KF555871	KF555699	KF555876	N/A	N/A
L. baudiniana	Buenaventura, Isla Palma, Colombia	G2	KF555799	KF555808	KF555870	KF555700	N/A	N/A	N/A
L. baudiniana	Buenaventura, Isla Palma, Colombia	G2	KF555797	KF555834	KF555872	KF555701	KF555877	N/A	N/A
L. baudiniana	La Ensenada, Tela, Honduras	C9	KF555788	KF555825	KF555854	KF555702-06	N/A	15°48'24.03"N	87°25'50.46"W
L. baudiniana	El Morro, Anzoategui, Venezuela	F	KF555789	KF555833	N/A	KF555707	KF555875	N/A	N/A
L. baudiniana	El Condado Beach, Puerto Rico	A6	KF555770	KF555813	N/A	KF555708-12	KF555883	18°27'41.28"N	66°04'55.62"W
L. baudiniana	Marigot, St. Martin	A5	KF555769	KF555812	N/A	KF555713	N/A	18°04'26.82"N	63°04'59.22"W
L. baudiniana	Piscaderabaai, Curacao	D1	KF555790	KF555804	KF555866	KF555714	N/A	12°07'25.38"N	68°58'09.30"W
L. baudiniana	Spaans Lagoen, Aruba	D2	KF555791	KF555805	KF555865	KF555715	N/A	12°27'45.18"N	69°58'00.42"W
L. baudiniana	East Coast, Aruba	D4	KF555794	KF555803	KF555868	KF555716	N/A	12°32'44.58"N	69°57'46.68"W
L. baudiniana	Donkey Beach, Bonaire	D3	KF555792	KF555806	KF555867	KF555717	KF555890	12°07'50.10"N	68°17'04.44"W
L. baudiniana	Boca Chica, Dominican Republic	A9	KF555768	KF555816	KF555847	KF555718-22	N/A	18°26'37.02"N	69°36'37.98"W
L. baudiniana	Cozumel, Mexico	C1	KF555780	KF555828	KF555855	KF555739-44	N/A	20°25'13.64"N	86°50'42.26"W
L. baudiniana	Duck Key, Florida, U.S.A	C2	KF555784	KF555826	N/A	KF555745	N/A	24°46'36.90"N	80°55'26.40"W
L. baudiniana	Indian Key, Florida, U.S.A	C3	KF555779	KF555823	KF555859	KF555752	N/A	24°53'23.70"N	80°40'31.38"W
L. baudiniana	Summerland Key, Florida, U.S.A.	C4	KF555778	KF555822	KF555860	KF555755-60	N/A	24°39'7.62"N	81°26'09.48"W
L. baudiniana	Playa Ancon, Cuba	A8	KF555772	KF555814	KF555848	KF555656	N/A	N/A	N/A
L. baudiniana	Habana, Cuba	C7	KF555786	KF555824	KF555861	KF555746-51	N/A	N/A	N/A
L. baudiniana	Yaguanabo, Cuba	A7	KF555771	KF555815	KF555849	KF555761-63	N/A	N/A	N/A
L. vitiensis	Parangtritis, Java, Indonesia		KF546554	KF546582	KF546665	KF546727	N/A	08°01'46.44"S	110°20'29.81"E
L. hawaiensis	North of Puko'o, Moloka'i, U.S.A.		KF546540	KF546565	KF546608	KF546713	N/A	21°06'06.84"N	156°45'06.66"W
L. hawaiensis	Manele Bay, Lana'i, U.S.A.		KF546538	KF546564	KF546644	N/A	N/A	20°44'37.37"N	156°53'12.47"W
L. hawaiensis	Spreckelsville, Maui, U.S.A.		KF546539	KF546567	KF546650	KF546712	N/A	20°54'31.38"N	156°24'40.26"W
L. hawaiensis	Pupukea, O'ahu, U.S.A.		KF546531	KF546562	KF546622	KF546709	N/A	21°38'59.70"N	158°03'45.48"W
L. hawaiensis	Onekahakaha Beach, Hawai'i, U.S.A.		KF546534	KF546561	KF546629	KF546705	N/A	19°44'16.05"N	155°02'20.15"W
L. hawaiensis	Kapua'a Beach Park, Kaua'i, U.S.A.		KF546544	KF546571	KF546598	KF546721	N/A	22°13'05.30"N	159°25'31.15"W
L. perkinsi	Haupu Mt. Range, Kaua'i, U.S.A.		KF546547	KF546579	KF546655	KF546722	N/A	N/A	N/A
L. occidentalis	Guaymas, Sonora, Mexico		KF546553	KF546583	KF546666	KF546728	N/A	27°54'44.33"N	110°56'49.56"W
L. exotica	Veracruz Harbor	T.L.	KF546552	KF546584	KF546664	KF546726	N/A	19°11'40.19"N	96°07'24.41"W
Ligia sp.	Patong Beach, Phuket, Thailand		KF555801	KF555838	KF555841	KF555754	N/A	07°53'10.95"N	98°17'10.31"E
L. oceanica	Appledore Is., Maine, U.S.A.		KF555802	KF555839	N/A	KF555657	N/A	N/A	N/A
L. italica	Trieste, Italy		KF555800	KF555840	KF555842	KF555753	N/A	N/A	N/A

Biolabs) and Shrimp Alkaline Phosphatase (USB Scientific) prior to cycle sequencing at the University of Arizona Genetics Core (UAGC). We edited sequences and removed the corresponding primer regions with Sequencher 4.8 (Genecodes, Ann Arbor, MI). We did not observe premature stop codons in the protein-coding gene sequences.

II.2.3 Phylogenetic analyses

Preliminary phylogenetic analyses of mitochondrial and nuclear datasets that included a broad representation of *Ligia* lineages from around the world (not shown), indicated that all the specimens from the study area correspond to a well-supported monophyletic group that is highly divergent from all other lineages (Hurtado et al. unpublished). In contrast, the Gulf of Mexico specimens (i.e., those from the type locality of *L. baudiniana* in Veracruz, Mexico) were highly divergent from the study area specimens (Figure II.2), and were more closely related to members of the *L. exotica* clade; a finding that was corroborated by morphological comparisons (see Results). Therefore, we used the putative *L. exotica* from this locality, as well as twelve additional *Ligia* lineages from around the world, as outgroups (Table II.1).

We aligned the 16S rDNA and 12S rDNA gene fragments with the MAFFT algorithm (Katoh et al. 2005) assuming the Q-INS-I strategy as implemented in the GUIDANCE server (Penn et al. 2010a). The high divergence among lineages of *Ligia* (see Results) led to several regions of ambiguous alignment. We therefore estimated confidence scores for each nucleotide position in the alignment by conducting 100 independent alignments based on different bootstrap guide trees as implemented by the

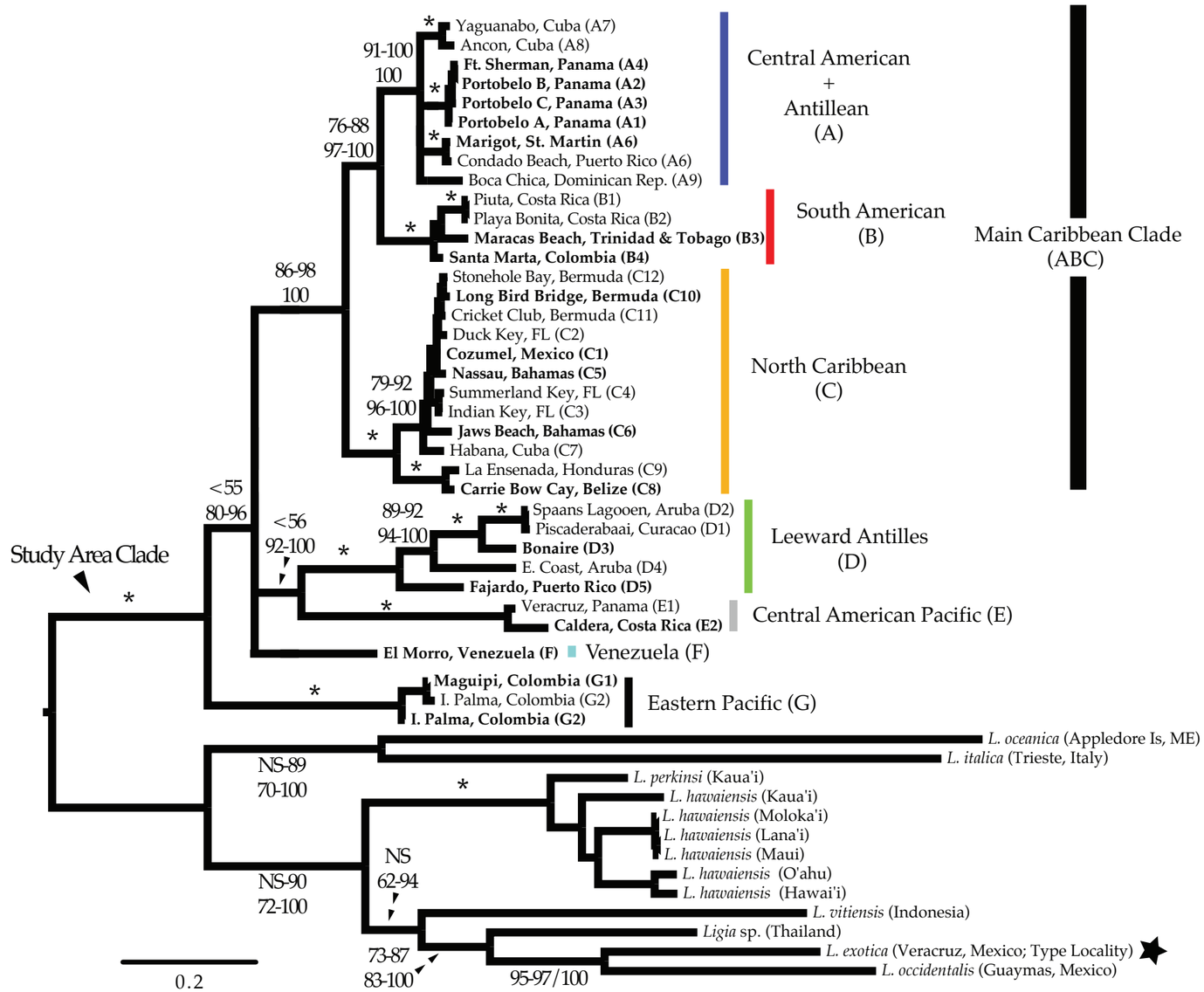

Figure II.2: Maximum likelihood tree of *Ligia* samples from the Caribbean region. The tree was obtained by analysis of the concatenated mitochondrial dataset under the GTR +Γ model in RAxML, and is rooted with all included outgroups. Clade colors and locality names correspond with those in other figures and tables in this chapter. Numbers indicate node support. Top: bootstrap support for ML methods; Bottom: Bayesian Posterior Probabilities. Nodes receiving 100% for all methods are denoted with an *.

GUIDANCE server (Penn et al. 2010b). All positions with a confidence score below 1.00, and those for which alignments could be considered ambiguous, were removed from all analyses. We estimated pairwise genetic distances with the Kimura-2-Parameter (K2P) correction in MEGA v5.05 (Tamura et al. 2011) for the 16S rDNA and the COI gene fragments separately, excluding ambiguous sites for each comparison.

We used jModeltest v2.1.1 (Darriba et al. 2012) to determine the most appropriate model of DNA substitution from among 1,624 candidate models for each gene fragment and concatenated dataset by evaluating their corresponding likelihood scores on a fixed BioNJ-JC tree under the Akaike Information Criterion (AIC), corrected AIC (AICc), and the Bayesian Information Criterion (BIC) (Table II.2). The selected model was applied in phylogenetic searches, with two general exceptions. First, if the software did not implement the selected model, we applied the next most complex model available (Table II.2). Second, as the joint estimation of Γ and I parameters can be problematic (see RAxML manual; pages 113-114 of Yang 2006), we carried out all analyses under the simpler +Γ model in those instances where the selected model included both I and Γ parameters. For each dataset, we implemented several partitioning schemes: (a) all positions within a single partition; (b) partitioned by gene; (c) the best partitioning scheme according to the BIC implemented in PartitionFinder v1.0.0 (Lanfear et al. 2012); and (d) 1–4 partitions not specified *a priori* (i.e., BayesPhylogenies). We used the following parameters in PartitionFinder searches: branch lengths = linked; models = all; model selection = BIC; search = greedy; and *a priori* partitioning combining each gene and codon position.

Table II.2: Number of included and excluded characters, per gene, in Caribbean phylogenetic analyses.

Gene	Samples	Total Chars.	Exc. Chars.	Inc. Chars.	Pars. Inf.	AIC (weight)	AICc (weight)	BIC (weight)
16S rDNA	49	501	167	334	114	010234+I+G+F (0.2881)	TIM2+I+G (0.5250)	TIM2+I+G (0.7120)
12S rDNA	49	509	165	344	135	012343+I+G+F (0.2164)	TIM2+I+G (0.3224)	TIM2+I+G (0.3256)
COI	42	658	0	658	258	TIM1+I+G (0.4279)	TIM1+I+G (0.4947)	TPM1uf+I+G (0.4514)
Cyt-b	49	361	0	361	173	TrN+I+G (0.2948)	TrN+I+G (0.6053)	TrN+I+G (0.8220)
mtDNA	49	2029	332	1697	680	012313+I+G+F (0.2754)	012313+I+G+F (0.2916)	012010+I+G+F (0.6191)

Table II.3: Settings for maximum likelihood and Bayesian analyses for the concatenated mitochondrial (MT) dataset.

Method	Model and Priors [A]	Part. Scheme [B]	iterations gen./ bootstrap replicates	Sample Freq.	Runs/ Chains	Burnin	ASDSF [C]	Bayes Factors/ ML Scores (-lLn) [D]	ESS >200 [E]	PSRF [F]
RAxML	GTR +Γ	1	1,000	n/a	n/a	n/a	n/a	-17192.378	n/a	n/a
RAxML	GTR +Γ	by gene: 4	1,000	n/a	n/a	n/a	n/a	-17038.368	n/a	n/a
RAxML	GTR +Γ	5*	1,000	n/a	n/a	n/a	n/a	-16276.941	n/a	n/a
Garli	010210+I+F	1	1,000	n/a	n/a	n/a	n/a	-17137.776	n/a	n/a
Garli	Mixed Model	by gene: 4	1,000	n/a	n/a	n/a	n/a	-16974.566	n/a	n/a
Garli	Mixed Model	5*	1,000	n/a	n/a	n/a	n/a	-16264.791	n/a	n/a
MrBayes	GTR +Γ	1	200,000,000	5,000	4	25%	0.000988	-17207.223	Yes	1
MrBayes	GTR +Γ	by gene: 4	200,000,000	5,000	4	25%	0.001006	-17100.289	Yes	1
MrBayes	GTR +Γ	5*	200,000,000	5,000	4	25%	0.000852	-16998.299	Yes	1
BayesPhyl.	GTR +Γ	1	100,000,000	5,000	8/1	25%	n/a	-17212.831	Yes	n/a
BayesPhyl.	GTR +Γ	4	100,000,000	5,000	8/1	25%	n/a	-16865.321	Yes	n/a
BayesPhyl.	GTR +Γ	5	100,000,000	5,000	8/1	25%	n/a	-16786.232	Yes	n/a
Phycas	GTR +Γ	1	1,000,000	50	n/a	25%	n/a	-17211.220	Yes	n/a
Phycas	GTR +Γ	by gene: 4	1,000,000	50	n/a	25%	n/a	-17060.861	Yes	n/a
Phycas	GTR +Γ	best partition: 5*	1,000,000	50	n/a	25%	n/a	-16437.106	Yes	n/a

[A] All others default; [B] different partitions separated by comma; [C] Average standard deviation of split frequencies; [D] estimated in Tracer v.1.5; [E] Effective Sample Size; [F] Potential Scale Reduction Factor for all parameters. * PartitionFinder 1.0: (A) 12S+16S+Cyt-b1 (GTR+I+G); (B) Cyt-b3 (TrN+G); (C) COI2+Cyt-b2 (HKY+I); (D) COI3 (TIM+G); (E) COI1 (TrNef+I+G).

We conducted maximum likelihood (ML) searches in both RAxML v7.2.6 (Stamatakis 2006a; Stamatakis 2006b; Stamatakis et al. 2008) and GARLI v2.0 (Zwickl 2006). RAxML analyses were run under the Rapid Bootstrap Algorithm and consisted of 1,000 bootstrap replicates followed by a thorough ML search under the GTR+Γ model, with all other settings as default. GARLI analyses consisted of 1,000 bootstrap replicates under the appropriate model of evolution identified by jModeltest. All other settings were used as default. For each analysis, we calculated a majority-rule consensus tree with the SumTrees command of DendroPy v3.10.1 (Sukumaran and Holder 2010).

We conducted Bayesian phylogenetic reconstructions with three different software packages: MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003); Phycas v1.2.0 (Lewis et al. 2008) with a polytomy prior (Lewis et al. 2005), which is aimed at alleviating potential overestimation of clade confidence by Bayesian methods (Suzuki et al. 2002); and BayesPhylogenies parallel v2.0.2 (Pagel and Meade 2004). BayesPhylogenies was used to fit more than one substitution model to different positions in the dataset without the need to identify these partitions *a priori* (Pagel and Meade 2004). The number of independent MCMC runs, chains, and generations is presented in Table II.3. All other parameters were as default. We evaluated if Bayesian analyses had reached stationarity based on the following criteria: (a) stable posterior probability values; (b) high correlation between the split frequencies of independent runs as implemented in AWTY (Nylander et al. 2008); (c) small and stable average standard deviation of the split frequencies of independent runs; (d) Potential Scale Reduction Factor close to 1; and (e) an Effective Sample Size (ESS) > 200 for the posterior

probabilities, as evaluated in Tracer v1.5 (Rambaut and Drummond 2009). We discarded samples prior to reaching a stationary posterior distribution (i.e., "burnin"; Table II.3). To estimate the posterior probability of each node, we used the SumTrees command (Sukumaran and Holder 2010) to compute a majority-rule consensus tree of the stationary stage for each run.

II.2.4 Nuclear phylogenetic analyses

Given the low levels of variation in the amplified nuclear gene (NaK), we visualized relationships among alleles on a network constructed with the cladogram estimation algorithm of Templeton et al. (1992), as implemented by TCS v1.21 (Clement et al. 2000). We calculated the 95% most parsimoniously plausible branch connections between alleles with all other settings as default.

II.2.5 Morphology of the male gonopodia

We dissected and examined the appendix masculina of the left 2nd pleopod of mature male specimens from populations within the study area. For each specimen, we photographed this structure, using a Zeiss AxioCam MRc5 (Thornwood, NY) mounted on a Zeiss Stereo Discovery.V20 (Thornwood, NY) microscope, and compared it with that of specimens from the type locality of *L. baudiniana*, and with those reported for other *Ligia* species (Khalaji-Pirbalouty and Wägele 2010; Lee 1994; Schultz 1972; Schultz and Johnson 1984; Taiti et al. 2003).

II.3 Results

All new sequences were deposited in GenBank under accession numbers KF555656–KF555890 (Table II.1).

II.3.1 Mitochondrial phylogenetic results

Cytochrome b (Cytb) sequences were obtained from a total of one hundred and five individuals representing thirty-six localities. No sharing of haplotypes was observed among localities. As Cytb variability within localities was very low (Supplementary Figure S.II.1), we chose one individual per locality for amplification of the other mitochondrial genes, ensuring appropriate representation of all main lineages observed. The final mitochondrial concatenated dataset included thirty-six ingroup individuals from thirty-five localities and thirteen *Ligia* outgroup taxa. A total of 332 characters that could not be confidently aligned were excluded for the phylogenetic analyses (16S rDNA: 167; 12S rDNA: 165), producing a final alignment of 1,697 nucleotide bases, 680 of which were parsimony informative. For the resulting mitochondrial concatenated dataset, different models were chosen under the Aikake Information Criteria (AIC and AICc) and Bayesian Information Criterion (BIC). Under both AIC and AICc a complex model having four substitution parameters (rate matrix: 012313; see jModeltest manual), +F, +I, and +Γ was selected (Table II.2). Under the BIC, a relatively simpler model having three substitution parameters (rate matrix: 012010; see jModeltest manual), +F, +I, and +Γ was selected (Table II.2). Considering the low weights observed for the chosen model under the AIC and AICc (Table II.2), and that the 95% confidence interval

for these analyses included the model chosen under the BIC fell, we applied the BIC model in GARLI analyses. Because this model is not available in the other software packages (e.g. RAxML, Phycas), we used the GTR+Γ model, which was included in the 99% cumulative weight interval under the three selection criteria.

Mitochondrial phylogenetic reconstructions (Figure II.2) recovered a highly supported split [100 Bootstrap Support (BS) and Posterior Probability (PP)] between the *Ligia* specimens from the study area and the outgroup taxa, from which the *Study Area Clade* is highly divergent (COI K2P: 20.40–30.08%). Five highly divergent lineages were observed within the *Study Area Clade*: (1) a 'Main Caribbean' lineage (*Clade ABC*; blue, red, and orange in Figures II.1 and II.2); (2) a 'Leeward Antilles' lineage (*Clade D*; light green in Figures II.1 and II.2); (3) a 'Central American Pacific' lineage (*Clade E*; grey in Figures II.1 and II.2); (4) a lineage from the eastern coast of Venezuela (*F*; light blue in Figures II.1 and II.2) and (5) a 'Colombian Pacific' lineage (*Clade G*; black in Figures II.1 and II.2). Maximum likelihood analyses were unable to resolve with confidence the relationships among these lineages, resulting in a basal polytomy. Bayesian results, however, suggest the following: *Clade G* is the most basal; clades *D* and *E* are sister lineages; and the relationships among *Clade ABC*, *Clade DE*, and *Clade F*, are not well resolved, resulting in a polytomy.

The 'Main Caribbean' lineage (*Clade ABC*), to which most of the Caribbean basin samples belonged (BS: 86–98; PP: 100), was divided into three main clades (*A*, *B*, and *C*). Most analyses supported a sister relationship between clades *A* and *B* (BS: 76–88; PP: 98–100), which were in turn sister to *Clade C*. Within *Clade A* (blue in Figures II.1

and II.2), four main lineages were observed, but the relationships among them were not resolved: a lineage containing all samples from the Caribbean coast of Panama (A1–4; BS: 100; PP: 98–100); a St. Martin + Puerto Rico lineage (A5–6; BS: 100; PP: 100); a southern Cuba lineage (A7–8; BS: 100; PP: 100); and a lineage from Hispaniola (A9). Minimum and maximum COI K2P divergences among these four *Clade A* lineages were 3.67 and 7.88%, respectively (Table III.4). *Clade B* (red in Figures II.1 and II.2; BS: 100; PP: 100) included samples from the Caribbean coasts of Colombia (B4) and Costa Rica (B1–2), and from Trinidad (B3). Relationships among these three lineages were not well resolved, and maximum K2P COI divergence was 5.10%, (Table II.4). Within *Clade C* (orange in Figures II.1 and II.2), two monophyletic lineages were detected: a lineage containing samples from the Caribbean coast of Honduras and Belize (C8–C9; BS: 100; PP: 100); and a lineage containing samples from the Florida Keys, Bahamas, Bermuda, northern Cuba, and Cozumel (C1–C7; BP: 70–86; PP: 100). Within-clade COI K2P divergences were similar to those observed in clades *A* and *B* (maximum = 6.82%; Table II.4).

The Leeward Antilles lineage (*Clade D*; light green in Figures II.1 and II.2; BS: 100; PP: 100) contained all samples from the Leeward Antilles (i.e., Aruba, Curaçao, and Bonaire) and a single population from eastern Puerto Rico (Fajardo). The individuals from Aruba (D2, D4), Curaçao (D1), and Bonaire (D3) conformed a well-supported monophyletic group (ArCuBo; BS: 84–100; PP: 100), which was highly divergent (~14–16% COI K2P distance) from its sister lineage found in Fajardo, Puerto Rico (D5). Within the ArCuBo group, a relatively deep split (~14–16% COI K2P

Table II.4: Estimates of evolutionary divergence, as measured by Kimura 2-parameter distances, for main *Ligia* lineages detected from the Caribbean and outgroups. Minima and maxima are given for all pairwise comparisons. Above matrix: COI gene distances. Lower matrix: 16S rDNA gene distances. Diagonals: within-clade divergence (upper values: COI; lower values: 16S rDNA).

	Clade A	*Clade B*	*Clade C*	*Clade D*	*Clade F*	*Clade E*	*Clade G*	*L. hawaiensis*	*L. perkinsi*	*L. exotica*	*L. occidentalis*	*Ligia* sp. (Thailand)	*L. vitiensis*	*L. italica*
Clade A	**0.22-7.88 0.00-1.83**	13.81-15.58	13.18-17.06	18.83-23.24	19.17-23.03	N/A	18.42-21.51	23.35-26.26	21.30-22.90	23.12-25.34	23.08-26.62	23.97-26.08	24.60-27.61	25.37-27.72
Clade B	1.21-2.45	**0.22-5.10 0.30-1.83**	13.24-18.30	19.95-25.13	20.41-23.28	N/A	21.06-23.26	23.71-29.48	24.87-25.24	23.39-24.33	23.42-26.01	23.06-23.61	22.56-24.87	25.64-27.10
Clade C	3.37-5.29	3.06-5.29	**0.67-6.82 0.00-2.14**	20.57-24.50	16.77-19.67	N/A	20.73-23.20	22.78-26.62	21.04-23.23	22.47-24.59	22.49-23.76	24.17-26.69	22.88-24.41	24.63-26.31
Clade D	4.65-7.29	4.65-7.29	5.31-8.34	**0.90-16.34 0.00-4.05**	18.76-23.32	N/A	21.93-23.97	21.91-26.75	22.00-23.55	22.90-24.80	22.72-24.31	21.66-22.88	23.02-24.75	27.68-30.08
Clade F	7.25-8.95	7.58-8.94	6.61-8.26	6.91-7.91	**4.88 1.21**	N/A	21.37-23.26	20.40-23.38	22.74-22.76	23.85-25.13	20.40-21.93	25.39-26.38	21.48-23.71	25.64-26.62
Clade E	4.32-5.62	4.00-4.97	5.29-6.93	5.61-6.60	6.60-7.61	**N/A N/A**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Clade G	9.59-11.71	9.94-11.71	8.58-10.68	9.30-10.01	9.24-10.29	8.59-9.29	**0.22-1.12 0.00-1.21**	21.81-25.57	22.45-22.45	22.45-22.45	24.03-24.36	24.66-24.66	22.17-22.49	29.22-29.22
L. hawaiensis	21.04-24.93	21.87-24.93	22.22-24.89	22.18-25.26	21.45-24.93	23.51-25.67	21.39-25.70	**0.22-14.33 0.00-6.61**	10.30-13.79	21.62-24.20	21.54-23.67	17.50-22.09	22.83-22.83	21.59-25.17
L. perkinsi	22.74-24.93	23.60-24.04	22.32-23.57	23.14-24.00	23.11-24.78	25.22-25.22	22.29-22.71	5.61-6.28	**N/A N/A**	24.06-24.06	22.15-22.15	18.90-18.90	26.79-26.79	25.07-25.07
L. exotica	19.78-21.80	20.16-20.98	20.55-21.78	22.58-25.54	23.48-23.48	21.75-21.75	23.89-24.32	14.63-17.16	15.43-15.43	**N/A N/A**	22.43-22.43	24.50-24.50	21.93-21.93	27.92-27.92
L. occidentalis	23.24-24.12	24.12-24.97	23.57-24.89	23.14-25.78	23.17-23.97	22.81-22.81	20.22-20.63	16.85-19.26	17.28-17.28	12.87-12.87	**N/A N/A**	24.03-24.03	21.88-21.88	28.15-28.15
Ligia sp. (Thailand)	23.89-25.19	24.32-25.16	24.41-25.74	24.75-25.67	25.70-26.08	25.57-25.57	23.86-25.16	15.29-16.64	15.74-15.74	13.79-13.79	14.21-14.21	**N/A N/A**	23.94-23.94	25.31-25.31
L. vitiensis	24.72-26.49	25.16-26.49	25.13-26.46	26.35-28.15	26.95-27.86	26.88-26.88	26.40-27.75	15.74-18.82	19.17-19.17	21.41-21.41	23.76-23.76	22.49-22.49	**N/A N/A**	30.21-30.21
L. italica	21.80-23.91	22.22-23.06	23.51-24.81	20.63-22.29	23.40-24.21	23.91-23.91	21.92-22.35	25.91-28.64	25.07-25.07	28.25-28.25	26.05-26.05	25.20-25.20	23.82-23.82	**N/A N/A**
L. oceanica	24.70-26.01	24.70-26.46	24.34-25.63	24.29-24.70	24.27-24.75	23.42-23.42	26.88-27.79	27.11-28.84	28.10-28.10	28.29-28.29	30.18-30.18	28.25-28.25	28.00-28.00	28.95-28.95

distance was observed between a lineage from the eastern coast of Aruba (D4) and a clade comprised of the remaining taxa (BS and PP: 100). Within this last clade, the lineage from Bonaire (D1) was relatively divergent (~9% COI K2P distance) from its sister clade comprised of western Aruba (D2) and Curaçao (D1). Divergence between western Aruba and Curaçao was 0.90%.

Lineage *F* was another main lineage observed in the Caribbean, and was found at a single locality in the northeastern coast of Venezuela. The two remaining main clades were found in the Pacific Coast of Central America (*Clade E*) and Colombia (*Clade G*). *Clade E* was comprised of samples from Costa Rica (E1) and Panama (E2), which were 4.88% divergent (COI K2P) from each other; whereas *Clade G* contained Pacific samples from Colombia (G1–2), with a maximum COI K2P distance of 1.12% (Table II.4).

II.3.2 Nuclear gene networks

We obtained a 661-bp fragment of the NaK gene for 18 populations representing all major clades identified by the mitochondrial analyses. Several attempts to amplify and sequence additional localities were unsuccessful. Nonetheless, due to the low variation observed at this gene, we likely captured the majority of the diversity present in the localities sampled (localities with NaK data are bolded in Figure II.2). Sequences from the Caribbean individuals (clades *A–D, F*) and the Pacific Central America individuals (*Clade E*) formed a network (Figure II.3); whereas the allele from the Pacific coast of Colombia (*G*) was too divergent to be placed in the same network. The allele from

Clade G was 12–19 mutational steps from clades *A–E* and 13 steps from the *Clade F* allele. Relationships among NaK alleles were highly consistent with the mitochondrial phylogenetic results, indicating a closer relationship between clades *A* and *B* individuals, which are in turn closer to *Clade C* individuals. They also indicate a high divergence of *Clade G* individuals, which occupied a basal position in the mitochondrial results.

II.3.3 Morphology of the male gonopodia

We examined the gonopodia of adult male individuals (N = 68) from twenty-one of the populations, representing the main lineages found in the phylogenetic analyses. The appendix masculina was similar among them, and clearly differentiated from that of other *Ligia* species (Khalaji-Pirbalouty and Wägele 2010; Lee 1994; Schultz 1972; Schultz and Johnson 1984; Taiti et al. 2003) by a large lateral process that bifurcates close to the apex (panels A–D in Figure II.4). Individuals from the San Juan de Ulúa Fort in Veracruz, Mexico, the type locality of *L. baudiniana*, exhibited a different appendix masculina (panel E in Figure II.4), which lacks the large lateral process, and is highly similar to what we observed in *L. exotica* specimens from Asia and the Gulf of Mexico, as well as those reported for *L. exotica* from Florida (Schultz and Johnson 1984).

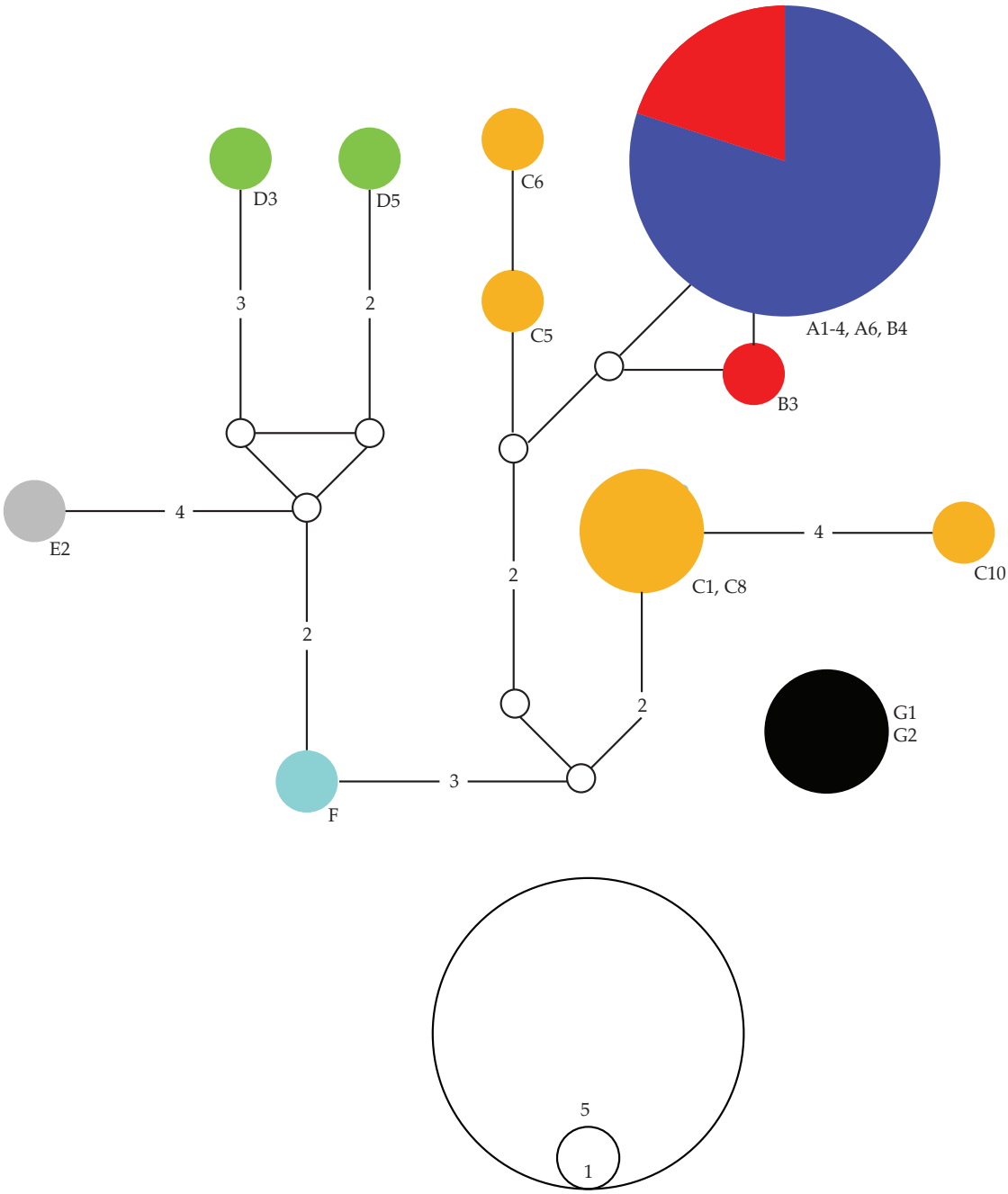

Figure II.3: Haplotype networks for the alpha subunit of the Sodium-Potassium ATPase gene for *Ligia* from the Caribbean. Colors correspond with the main clades in Figures II.1 and II.2. Geographic origin of alleles is indicated by the labels next to the allele, and corresponds with those in Figure II.1 and Table II.1. White circles represent unsampled (i.e., missing) alleles. The size of circles is proportional to the frequency at which an allele was recovered.

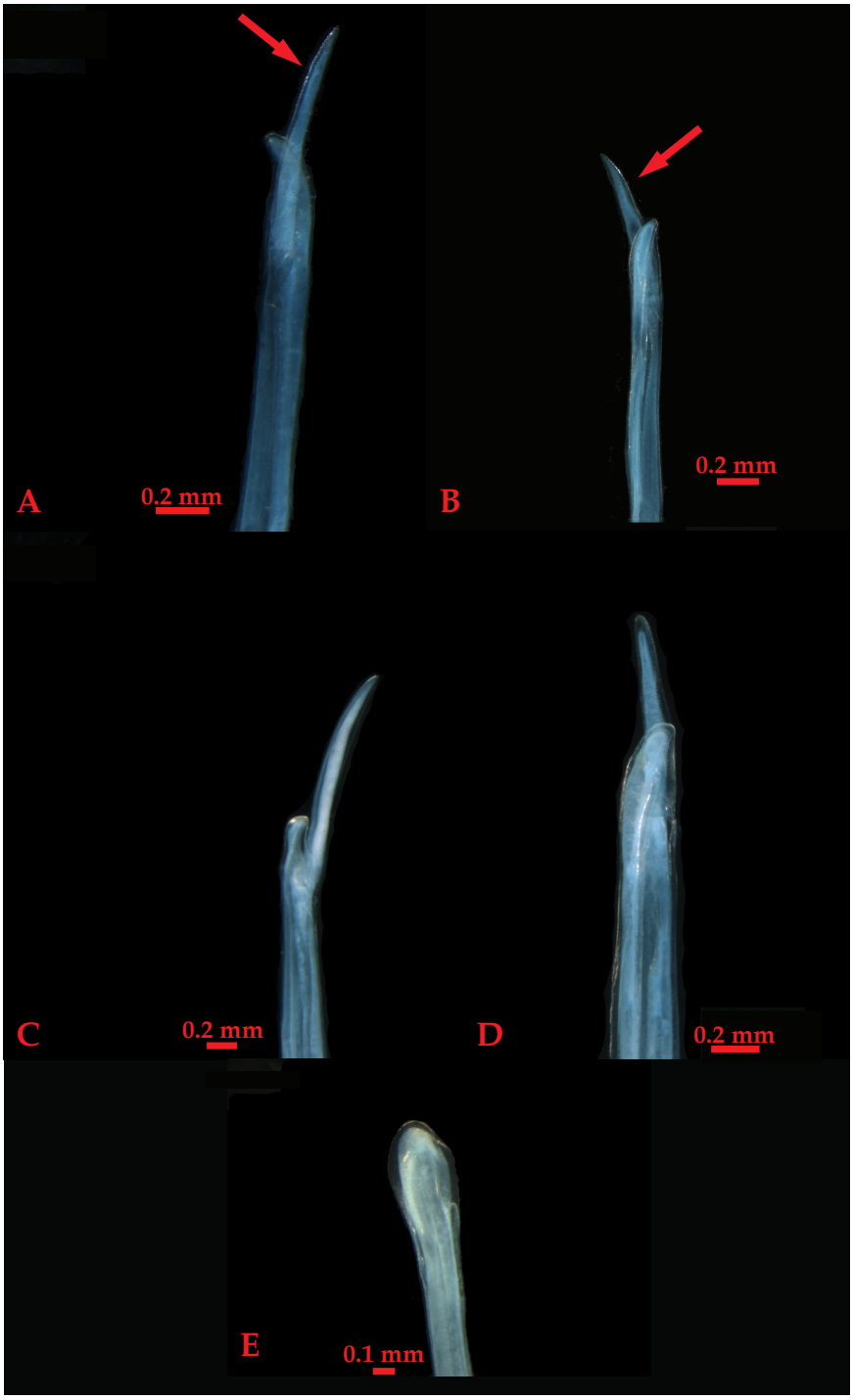

Figure II.4: Appendix masculina photographs from representative individuals (locality information is indicated in parenthesis). A *Clade C* (Nassau, The Bahamas; C5). B. *Clade A* (Marigot, St. Martin; A5). C (*Clade E*) (Maguipi, Colombia; E1). D. *Clade G* (El Morro, Venezuela; G). E. *Ligia exotica* (type locality, Veracruz, Mexico; T.L.). Arrows indicate the large lateral process unique to *Ligia* from the Caribbean.

II.4 Discussion

II.4.1 Cryptic biodiversity and taxonomic status of L. baudiniana

Due to their wide distribution, cosmopolitan in the case of *Ligia*, supralittoral isopods were suggested to be highly dispersive species (Vandel 1960). Challenging this early view, however, high levels of allopatric genetic differentiation have been observed for supralittoral isopods in different regions of the world (Eberl et al. 2013; Hurtado et al. 2013; Hurtado et al. 2010; Jung 2008; Taiti et al. 2003; Xavier et al. 2011), indicating *Ligia* dispersal potential is more limited than previously thought. Consistent with the biological characteristics that confer limited vagility to this isopod and the fragmented nature of its habitat, our results also show elevated levels of cryptic biodiversity for *Ligia* in the study area.

The external morphology of the appendix masculina enabled distinction of the *Study Area Clade* from other *Ligia* specimens examined by us and reported in the literature (Khalaji-Pirbalouty and Wägele 2010; Lee 1994; Schultz 1972; Schultz and Johnson 1984; Taiti et al. 2003), but not from specimens assigned to *L. baudiniana* by Schultz (1972) and Schultz and Johnson (1984) from Florida and Bermuda, in our study area. The appendix masculina of the *Study Area Clade* specimens and those assigned to *L. baudiniana* by Schultz (1972) and Schultz and Johnson (1984) from Florida and Bermuda have a large lateral process that bifurcates close to the apex, which is not observed in other *Ligia* lineages. This observation is congruent with the high divergence of the *Study Area Clade* from other *Ligia* lineages (Figure II.2; Hurtado et al. unpublished results). Within the *Study Area Clade*, we detected several highly divergent

genetic lineages of *Ligia*, in a region where only one native intertidal species of *Ligia* is currently recognized: *L. baudiniana* (Schmalfuss 2003). Seventeen of the lineages in the *Study Area Clade* exceed COI K2P divergences of 3%, and seven exceed 10%. Studies of marine invertebrates based on the same COI fragment used herein have found that intra-specific divergences of marine animals are typically < 3% (Hebert et al. 2003). Therefore, *Ligia* in the study area probably represents a cryptic species complex, as has been observed in other areas of the world (Eberl et al. 2013; Hurtado et al. 2010; Jung 2008; Taiti et al. 2003; Santamaria et al. in preparation). Studies are needed to examine whether these lineages can be distinguished by morphological traits.

Examination of additional localities within the study area may reveal additional divergent lineages belonging (or closely related) to the *Study Area Clade*. Other Caribbean islands may harbor additional lineages of this clade. For example, *Ligia gracilis*, currently considered a synonym of *L. baudiniana* (Schmalfuss 2003), was described from Culebra Island in the Puerto Rican bank (Moore 1901), and is reported from Barbados (Lewis 1960). In addition, the distribution limits of the *Study Area Clade* in the Eastern Pacific are not known. Although Mulaik (1960) reports *L. baudiniana* in the Gulf of California, an extensive phylogeographic study in this basin found lineages very divergent to the *Study Area Clade* (Hurtado et al. 2010). Indeed, Eastern Pacific lineages of *Ligia* found from Oaxaca (southern Mexico) northward (up to Alaska) are highly divergent from the *Study Area Clade*, and correspond to distantly related species (Eberl 2012; Eberl et al. 2013; Hurtado et al. 2010; Hurtado et al. unpublished). To the south, the coast of Chile is also occupied by highly divergent lineages that correspond to

distantly related species (González et al. 2008;Hurtado et al. unpublished). Therefore, the Pacific region between Oaxaca and Chile needs to be further examined to determine the distributional limits of clades *G* and *F*, and whether additional divergent lineages exist. Within this region, Van Name (1936) reports *L. baudiniana* in the Galapagos Islands, with males exhibiting an appendix masculina similar to that of the *Study Area Clade*.

Our phylogenetic and morphological comparisons revealed that the individuals currently occupying the type locality of *L. baudiniana* (i.e., San Juan de Ulúa Fort in Veracruz, Mexico) correspond to *L. exotica*. Therefore, it is possible that *L. baudiniana* was described based on *L. exotica* individuals. *Ligia baudiniana* was originally described in 1840 (Milne-Edwards 1840), but later suggested to be a synonym of *L. exotica* (Budde-Lund 1885). Unfortunately, despite our efforts to locate them, the type specimens of *L. baudiniana* appear to be unavailable or nonexistent. Although several putatively diagnostic traits fail to distinguish between *L. baudiniana* and *L. exotica* (Chilton 1916; Richardson 1905), the morphology of the appendix masculine is a reliable character to distinguish *L. exotica* from members of the *Study Area Clade* and of Florida and Bermuda specimens assigned to *L. baudiniana* by Schultz (1972) and Schultz and Johnson (1984). Besides Veracruz, Mexico, we also found *L. exotica* in our study area in a harbor in Trinidad, but this non-native species may occur at other localities in the study area. In addition, we have sampled *Ligia* throughout the Gulf of Mexico and genetic results indicate they have a haplotype almost identical to the one found in Veracruz. *Ligia filicornis* (Budde-Lund 1893), *L. grandis* (Perty 1834), and *L.*

olfersii (Brandt 1833), which were also described from Caribbean Sea localities, have been synonymized with *L. exotica* (Schmalfuss 2003). Therefore, the taxonomy of *Ligia* in the study area needs to be revised in light of both, our discovery of several highly divergent lineages, and of the historical taxonomic confusion.

II.4.2 Phylogeographic patterns of Ligia in the Study Area

Three main hypotheses have been used to explain the origin and radiations of the terrestrial fauna in the Caribbean: vicariance during the formation of the Caribbean Islands (Buskirk 1985; Rosen 1975; Rosen 1985); passive overwater dispersal (Hedges 1996b; Hedges 2001); and a temporary land bridge that existed 33–35 Ma and connected the northern South American coast with the Greater Antilles (known as the GAARlandia hypothesis; Iturralde-Vinent and MacPhee 1999). The phylogeographic patterns of *Ligia* in the Caribbean suggest passive overwater dispersal has been very important in the radiation of this semiterrestrial isopod in this region, which likely disperses mainly through rafting. The wide distribution of *Clade A* appears to have involved oceanic dispersal in many instances. It is unlikely that GAARlandia hypothesis explains this distribution, as the minimum and maximum COI K2P divergence within this clade are only 3.67 and 7.9%, respectively, which very unlikely represent a separation of ~32 My. The distribution of *Clade B*, albeit only found in the continent, may also indicate oceanic dispersal given the large distance separating the three localities harboring lineages of this clade. The most likely explanation for the colonization of Bermuda, a highly isolated volcanic island north of the Caribbean, by *Clade C* is recent oceanic dispersal. For the

rest of the range of *Clade C*, low sea levels during recent glacial periods may have reduced the distances among landmasses, thereby facilitating passive short distance dispersal. For example, during the last glacial maximum the Bahamas, northern Cuba and the tip of the Florida peninsula were very close (Dávalos and Russell 2012). Vicariant events, however, may have also occurred during interglacial periods in this range. Oceanic dispersal probably also occurred in *Clade D*, to explain its distribution in the Leeward Antilles and Puerto Rico. Alternatively, dispersal between the Leeward Antilles and Puerto Rico (~14–16% COI K2P divergence) could have been achieved through the terrestrial connections of GAARlandia (~32 Ma), but this scenario would imply relatively low substitution rates (i.e., ~0.5%/My or less). The GAARlandia hypothesis has been questioned (Ali 2012); although some phylogeographic studies report patterns congruent with this hypothesis (Alonso et al. 2012; Ball and Shpeley 2009; Crews and Gillespie 2010; Heinicke et al. 2007). Unfortunately, we are unable to estimate divergence times with certainty because we lack substitution rate estimates for *Ligia* in the study area, as well as reliable information on vicariant events or fossils that would allow for calibration of a molecular clock.

Phylogeographic patterns of *Ligia* in the Caribbean Sea do not appear to correspond with suggested biogeographic patterns based on population connectivity of marine organisms via larval dispersal (Cowen et al. 2006). Although *Ligia* lacks a larval stage (i.e., it is a direct developer), it is likely that long distance dispersal occurs via rafting, and thus effectively dispersing passively through surface ocean currents, such as many larvae. According to Cowen et al. (2006), the Caribbean region has four broadly defined

regions of connectivity: the eastern Caribbean; the western Caribbean; the Bahamas and the Turks and Caicos Islands; and the region at the periphery of the Colombia-Panama Gyre, with smaller areas of isolation within each region. They suggest one biogeographic break separating the eastern and western Caribbean, located from the western end of Puerto Rico south to Aruba, and another biogeographic break located around the northern edge of the Nicaraguan Rise, extending from the eastern tip of Cuba to the Honduras Caribbean coast. Separation according to these regions or biogeographic breaks is not observed, however, in the phylogeographic patterns of *Ligia*. *Clade A* is observed at both sides of the two proposed biogeographic breaks. In addition, no isolation with respect to the Colombia-Panama Gyre is observed, as *Clade A*, which was found in Panama, was also widely distributed along the Caribbean Sea, and *Clade B*, which was found in Colombia, was also found in Costa Rica and Venezuela. Furthermore, *Clade C* is found in Bahamas, but also in other localities of the northern and western Caribbean Sea. Nevertheless, it is not surprising that the phylogeographic patterns of *Ligia* in the Caribbean do not correspond with the biogeographic patterns suggested by Cowen et al. (2006), as they were proposed based on contemporary oceanographic patterns, whereas the distribution of *Ligia* was probably more affected by past oceanographic regimes.

According to Iturralde-Vinent and MacPhee (1999), oceanographic patterns in the Caribbean have been highly variable at different times in the past. These authors criticize Hedges' (1996b) proposal that dispersal patterns can be explained by the present-day predominantly unidirectional current flow in the Caribbean Sea from the

southeast to the northwest, bringing flotsam from the mouths of South American rivers to the islands of the West Indies, because colonization of the Caribbean probably occurred at times when these patterns were markedly different. In addition, they indicate that although the main present current's vector corresponds to that described by Hedges (i.e., from the southeast to the northwest), drifting experiments show that passive movements under the present-day current regime can be very unpredictable (Richardson 2005). Phylogeographic patterns of *Ligia* do not indicate colonization from the southeast to the northwest, and the somewhat scattered phylogeographic patterns of some clades may have resulted from the heterogeneity of past current patterns and stochasticity mentioned by Iturralde-Vinent and MacPhee (1999).

II.4.3 Conclusions

We have found that the *Ligia* lineages distributed in the Caribbean Sea belong to a well-supported and highly divergent clade that also has lineages in Bermuda, Florida, Bahamas and the Pacific coast of Central America and Colombia. This clade is highly genetically differentiated from other *Ligia* lineages and the external morphology of the appendix masculine enabled distinction of the members of this clade from other *Ligia* examined by us and reported in the literature, but not from specimens assigned to *L. baudiniana* by Schultz (1972) and Schultz and Johnson (1984) from Florida and Bermuda. Genetic characterization of *Ligia* specimens from the type locality of *L. baudiniana* indicates that they correspond to *L. exotica*, a cosmopolitan species highly divergent from the *Study Area Clade*. In addition, the *Study Area Clade* includes highly

divergent lineages suggesting the presence of a complex of cryptic species. Therefore, a taxonomic revision of *Ligia* in the study area is needed. The cryptic diversity observed for *Ligia* in the study area is consistent with observations in other parts of the world and the biology of this isopod. The phylogeographic patterns of *Ligia* in the Caribbean Sea suggest that passive overwater dispersal has been important, although some localized vicariant events may have occurred as well. Phylogeographic patterns, however, do not correspond with suggested biogeographic patterns based on population connectivity of marine organisms via larval dispersal, and do not indicate a colonization pattern from the southeast to the northwest hypothesized for the colonization of the Caribbean from South America by terrestrial animals. Rather, phylogenetic patterns appear to correspond with heterogeneity of past current regimes and stochasticity.

CHAPTER III

A COMPLEX EVOLUTIONARY HISTORY IN A SIMPLE ARCHIPELAGO: PHYLOGEOGRAPHY OF THE HAWAIIAN ENDEMIC *LIGIA*

III.1 Introduction

The Hawaiian Islands are well known for their rich biodiversity and endemic species (Wagner and Funk 1995). Their remoteness, representing the world's most isolated major archipelago, along with the progressive formation of these islands, are considered crucial for the striking diversification observed in several Hawaiian terrestrial organisms, which include: the Hawaiian *Drosophila* (Carson and Kaneshiro 1976; Carson 1982; O'Grady and DeSalle 2008), the silversword alliance (Baldwin and Robichaux 1995), *Succineid* land snails (Holland and Hadfield 2004; Rundell et al. 2004), honeycreeper birds (Fleischer et al. 1998; Tarr and Fleischer 1995), and others (Gillespie et al. 1994; Jordan et al. 2005; Magnacca and Danforth 2006; Rubinoff 2008). In contrast, endemism in Hawaiian marine invertebrates is strikingly lower than that in Hawaiian terrestrial organisms (Kay and Palumbi 1987), and with the exception of intertidal *Cellana* limpets (Bird et al. 2011), there are no documented marine radiations within the Hawaiian archipelago. Organisms inhabiting patchy coastal habitats of the Hawaiian archipelago have been poorly studied, but examples to date suggest that diversification is greater than in neighboring marine habitats (e.g. sandy beaches).

The shrimp *Halocaridinia rubra*, which inhabits anchialine coastal pools, shows evidence of between- and within-island divergence and is comprised of multiple divergent lineages (Craft et al. 2008; Santos 2006). An additional interesting case of diversification in a coastal patchy habitat of the archipelago is that of isopods in the genus *Ligia*, which most likely arose from a rocky supralittoral ancestor that arrived to the Hawaiian archipelago via rafting, and diversified into rocky supralittoral and inland (hereafter, terrestrial) lineages. High levels of genetic differentiation have been observed among populations of Hawaiian *Ligia* from different localities in the islands of Kaua'i and O'ahu (Taiti et al. 2003), but populations from other islands have not been studied yet. Given the rarity of reported cases of diversification for marine invertebrates in the Hawaiian archipelago, and the unusual occurrence of marine-terrestrial transitions, it is important to conduct further phylogeographic analyses of Hawaiian *Ligia*, including populations from unsampled islands, to better understand the biodiversity and evolution of this interesting group.

The genus *Ligia* has a worldwide distribution and includes 37 currently recognized species (Schmalfuss 2003); most of which are restricted to rocky supralittoral areas (Jackson 1922; Taiti et al. 2003). Seven species, however, are strictly terrestrial, inhabiting montane habitats of tropical regions (Taiti et al. 2003), and are believed to derive from supralittoral forms (Schmalfuss 1979). Supralittoral forms of *Ligia* have biological characteristics that confer them extremely low vagility (Hurtado et al. 2010), which include: direct development (lack a planktonic phase, as all peracarids); active avoidance of the open sea (they remain in the area between the splash zone and the

supralittoral); extremely low desiccation tolerance (a reason for which they stay close to the water line and are most active at night); limited motility underwater and on sandy shores (rendering them highly vulnerable to predators in these environments). Thus, supralittoral forms of *Ligia* exhibit morphological, physiological, and behavioral characteristics that are intermediate between ancestral marine and fully terrestrial isopods (Carefoot and Taylor 1995).

The biology of supralittoral *Ligia* severely constraints the movements of these isopods outside the rocky beaches they occupy, effectively isolating populations. This is reflected in the striking radiations of supralittoral *Ligia* reported in different regions of the world, with extraordinarily high levels of allopatric genetic differentiation, even between localities separated by few kilometers (Eberl et al. 2013; Hurtado et al. 2010; Jung 2008; Taiti et al. 2003; Santamaria et al. in preparation; Hurtado et al. unpublished results). These observations, as well as those by Hurtado et al. (2013), challenge earlier suggestions that littoral isopods are highly dispersive species, based on their common presence in beaches around the world (Vandel 1960). Phylogeographic patterns of *Ligia* in different regions have been shaped by past tectonic events (Hurtado et al. 2010), environmental factors, such as Sea Surface Temperature (Eberl et al. 2013), as well as oceanic dispersal events, probably through rafting (Santamaria et al. in preparation).

Two endemic species of *Ligia* are currently recognized in the Hawaiian Islands (Taiti and Howarth 1996): the rocky supralittoral *Ligia hawaiensis* (Dana 1853) and the terrestrial montane *Ligia perkinsi* (Dollfus 1900). A third species, the cosmopolitan introduced *Ligia exotica* (Roux 1828), is reported from man-made substrate (Eldredge

and Smith 2001). The supralittoral species *L. hawaiensis* occurs in rocky intertidal habitats throughout the archipelago (Jackson 1922; Taiti and Howarth 1996), whereas the terrestrial species *L. perkinsi* is found at high altitude (300–1,500 m above sea level) in wet forests on the islands of Kaua'i, O'ahu, and Hawai'i (Taiti and Howarth 1996); although the last report of *L. perkinsi* in Hawai'i was in 1896 (Taiti et al. 2003).

Taiti et al. (2003) investigated whether the terrestrial populations of *L. perkinsi* on Kaua'i and O'ahu originated from a single colonization event (i.e., *L. perkinsi* from both islands constitute a monophyletic group sister to a lineage of *L. hawaiensis*) or as two independent events, one in each island (e.g. two clades each showing reciprocal monophyly of *L. hawaiensis* and *L. perkinsi* from the same island). They conducted phylogenetic analyses with two mitochondrial genes (COI and 16S rDNA), and included individuals of *L. perkinsi* and *L. hawaiensis* from Kaua'i and O'ahu. They obtained strong support for the monophyly of the *Ligia* lineages endemic to the Hawaiian archipelago (also observed in Hurtado et al. 2010), which were divided into three main clades: one comprised of the *L. perkinsi* from Kaua'i; another clade comprised of *L. perkinsi* from O'ahu; and a third clade comprised of *L. hawaiensis* individuals. The *L. hawaiensis* clade was divided into a lineage comprised of the Kaua'i individuals and a sister lineage comprised of the O'ahu individuals. The two main *L. perkinsi* clades were paraphyletic (*L. perkinsi* from O'ahu was sister to a clade of *L. perkinsi* from Kaua'i + *L. hawaiensis*), thus, the results were inconclusive as to whether a single or two origins of the terrestrial life style occurred. In addition, they observed high divergences among populations of *L. hawaiensis*, implying long-standing isolation among them, and with

phylogeographic patterns suggesting inter-island dispersal events have been rare throughout the history of the Hawaiian endemic *Ligia* lineages (Taiti et al. 2003).

Herein, we expanded on Taiti et al.'s (2003) previous work by incorporating populations from previously unsampled main Hawaiian Islands (i.e., Maui, Moloka'i, Lana'i, and Hawai'i), increasing the number of gene markers, which include nuclear genes, and applying more current phylogenetic approaches. Sampling across all main Hawaiian Islands enables a better understanding on the diversity and evolution of endemic Hawaiian *Ligia*. Specifically, we asked: (1) whether the younger islands (i.e., Maui, Lana'i, and Moloka'i [all with an age ~ 1.3 My], and Hawai'i [0.4 My]; ages from Carson and Clague 1995) harbor highly divergent *L. hawaiensis* lineages, as observed in the older islands (i.e., Kaua'i [5.1 My] and O'ahu [3.7 My]); (2) whether evolution of *Ligia* in the Hawaiian Islands followed a pattern consistent with the progression rule (i.e., lineages from older islands are basal to those from younger islands), a back dispersal pattern (lineages from younger islands colonized the older islands), or an unresolved and/or highly stochastic pattern (indicative of a complex evolutionary history, probably with frequent inter-island dispersal); and (3) whether the new data shed light on the origin of a terrestrial life style in *L. perkinsi* (i.e., a single origin or independent origins in each island). Lastly, we incorporated geometric-morphometric analyses to determine whether different lineages of *L. hawaiensis* can be differentiated morphologically.

III.2 Materials and methods

III.2.1 Sampling

Our molecular dataset included twenty-four *L. hawaiensis* populations from across the main Hawaiian Islands and four *L. perkinsi* populations from Kaua'i and O'ahu (Figure III.1, Table III.1). We also included publicly available sequences for *L. hawaiensis* and *L. perkinsi* (Table III.1). We were unable to collect *L. hawaiensis* at Ni'ihau and Kaho'olawe because they are private property and a state reserve, respectively. Populations were sampled by hand and preserved in 70–100% Ethanol. As outgroups, we included specimens from *Ligia vitiensis*, *Ligia occidentalis*, and *Ligia exotica*, as previous research (Hurtado et al. 2010; Taiti et al. 2003) and preliminary analyses suggest they are the closest extant relatives to *Ligia* from the Hawaiian archipelago.

III.2.2 Molecular methods

We extracted total genomic DNA of *Ligia* individuals from pleopods/legs using the DNEasy Blood & Tissue kit (Qiagen Inc.), following standard protocol instructions. We PCR-amplified a 710-bp fragment of the Cytochrome Oxidase I (COI) mitochondrial (mt) gene for 1–10 individuals using the primers and conditions published by Folmer et al. (1994). A subset of these individuals (essentially one individual per locality; see Supplementary Figure S.III.1) was then amplified and sequenced for three additional mitochondrial genes using previously published primers and conditions: ~490-bp of the 16S rDNA gene (primers 16Sar/16Sbr; Palumbi 1996); ~495-bp of 12S rDNA (primers crust-12Sf/crust-12Sr; Podsiadlowski and Bartolomaeus 2005); and a 361-bp fragment

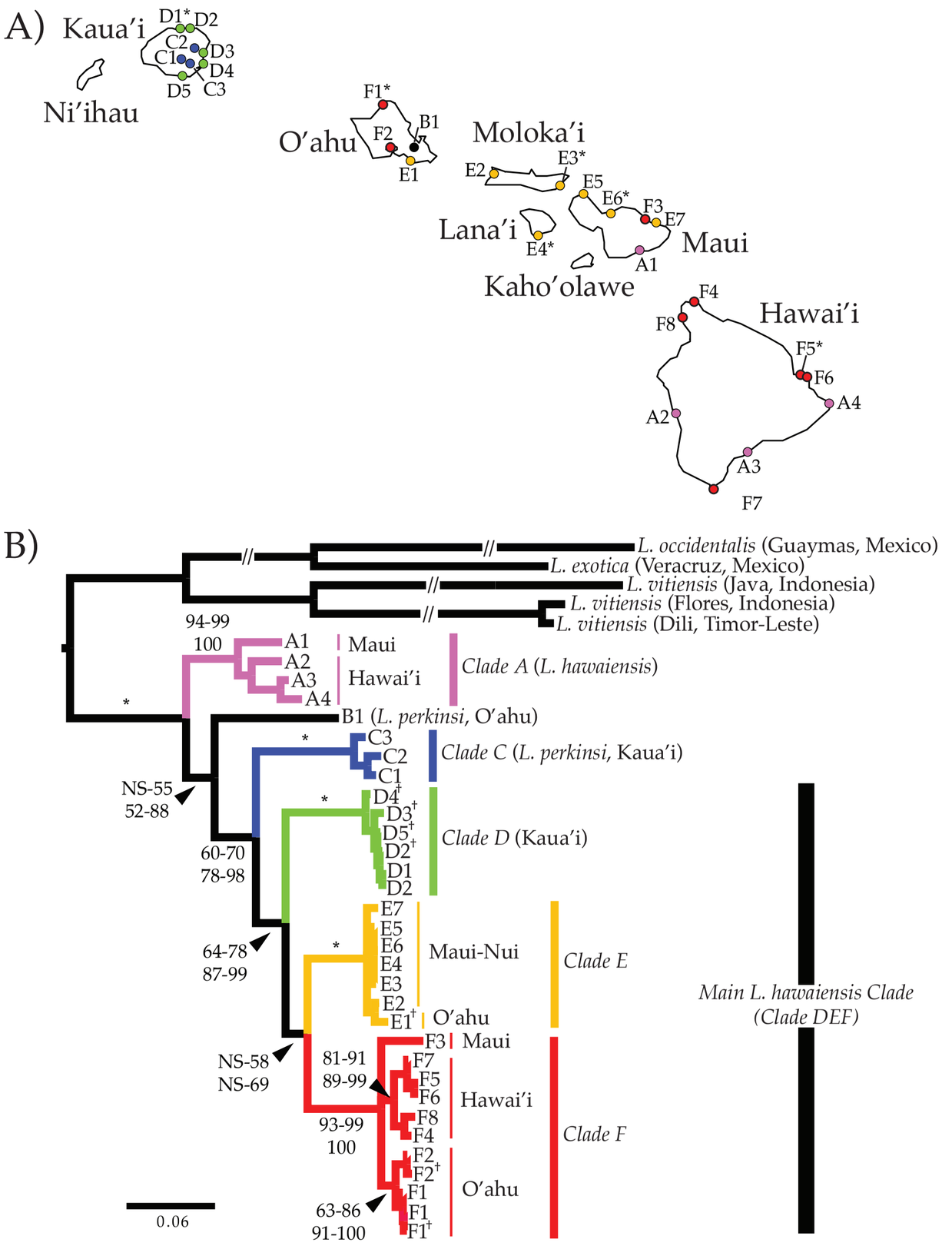

Figure III.1. Sampling localities (Panel A) and phylogenetic relationships of *Ligia* in the Hawaiian archipelago (Panel B). Colors and labels correspond those used in other figures and tables in this chapter. Detailed information for each locality is presented in Table III.1. Localities for *L. hawaiensis* are: Kaua'i: D1-Kapua'a Beach Park, D2-Kauapea Beach, D3-Kapa'a, D4-Lihu'e, D-5-Kukui'ula; O'ahu: E1-Ala Wai Canal, F1-Pupukea, F2-Pouhala Marsh; Moloka'i: E2-Papohaku Beach Park, E3-North of Puko'o; Lana'i: E4-Manele Bay; Maui: A1-Waiopai, E5-Poelua Bay, E6-Spreckelsville, E7-Keanae, F3-Honomanu Bay; Hawai'i: A2-Kealakukea Bay, A3-Pu'unalu Beach Park, A4-Isaac Hale Beach Park, F4-Keokea Beach, F5-Onekahakaha Beach Park, F6-Leleiwi Beach, F7-South Point, F8-Kapa'a State Park. Localities for *L. perkinsi* are: Kaua'i: C1-Mt Kahili, C2-Makaleha Mts, C3-Haupu Range; O'ahu: B1-Nu'uanu Pali. Localities in bold indicate those included in nuclear analyses. * indicates those localities used for geometric morphometric analyses (B) Maximum Likelihood tree of *Ligia* samples from the Hawaiian archipelago and several outgroups. The tree was obtained by analysis of the concatenated mitochondrial dataset under the GTR +Γ model in RAxML, and is rooted with all included outgroups. Clade colors and locality names correspond with those in other figures and tables in this chapter. Numbers indicate node support. Top: bootstrap support for ML methods; Bottom: Bayesian Posterior Probabilities. Nodes receiving 100% for all methods are denoted with an *

Table III.1: Localities included in this study, with corresponding GenBank accession numbers, and geographic information. ID labels correspond with those used in other figures and tables in this chapter.

Species	Locality Name	ID	16S rDNA Acc. No	12S rDNA Acc. No	COI Acc. No	Cyt-b Acc. No	NaK Acc. No	28S rDNA Acc. Nos	Lat.	Long.
L. hawaiensis	Waiopai, Maui	A1	KF546549	KF546573	N/A	KF546718	N/A	N/A	N/A	N/A
L. hawaiensis	Kealakukea Bay, Hawai'i	A2	N/A	KF546574	KF546627	N/A	KF546594	N/A	19°28'32.88"N	155°55'11.04"W
L. hawaiensis	Pu'unalu Beach Park, Hawai'i	A3	KF546551	KF546576	KF546628	KF546716	KF546593	KF546701	19° 8'0.60"N	155°30'18.30"W
L. hawaiensis	Isaac Hale Beach Park, Hawai'i	A4	KF546550	KF546575	N/A	KF546717	KF546586	KF546702	19°27'26.82"N	154°50'31.68"W
L. perkinsi	Nu'uanu Pali, O'ahu	B1	KF546548	KF546572	KF546661	KF546719	N/A	N/A	N/A	N/A
L. perkinsi	Mt Kahili, Kaua'i	C1	KF546546	KF546578	KF546660	N/A	N/A	N/A	N/A	N/A
L. perkinsi	Makaleha Mts, Kaua'i	C2	KF546545	KF546577	KF546659	KF546723	N/A	N/A	N/A	N/A
L. perkinsi	Haupu Range, Kaua'i	C3	KF546547	KF546579	KF546655	KF546722	KF546592	KF546683-84	N/A	N/A
L. hawaiensis	Kapua'a Beach Park, Kaua'i	D1	KF546544	KF546571	KF546598-606	KF546721	KF546585	KF546685-90	22°13'05.30"N	159°25'31.15"W
L. hawaiensis	Kauapea Beach, Kaua'i	D2	KF546543	KF546570	KF546656	KF546720	N/A	N/A	N/A	N/A
L. hawaiensis	Kauapea Beach, Kaua'i	D2	AY051343	N/A	AY051324	N/A	N/A	N/A	N/A	N/A
L. hawaiensis	Kapa'a, Kaua'i	D3	AY051344	N/A	AY051325	N/A	N/A	N/A	N/A	N/A
L. hawaiensis	Lihu'e, Kaua'i	D4	AY051346	N/A	AY051327	N/A	N/A	N/A	N/A	N/A
L. hawaiensis	Kukui'ula, Kaua'i	D5	AY051345	N/A	AY051326	N/A	N/A	N/A	N/A	N/A
L. hawaiensis	Ala Wai Canal, O'ahu	E1	AY051348	N/A	AY051329	N/A	N/A	N/A	N/A	N/A
L. hawaiensis	Papohaku Beach Park, Moloka'i	E2	KF546542	KF546569	KF546607	KF546715	N/A	N/A	21°10'46.56"N	157°15'5.88"W
L. hawaiensis	North of Puko'o, Moloka'i	E3	KF546540	KF546565	KF546608-16	KF546713	KF546587	KF546696-700	21°06'06.84"N	156°45'06.66"W
L. hawaiensis	Manele Bay, Lana'i	E4	KF546538	KF546564	KF546643-49	N/A	KF546589	KF546677-82	20°44'37.37"N	156°53'12.47"W
L. hawaiensis	Poelua Bay, Maui	E5	KF546541	KF546566	KF546657	KF546711	N/A	N/A	N/A	N/A
L. hawaiensis	Spreckelsville, Maui	E6	KF546539	KF546567	KF546650-54; 95-97	KF546712	KF546590	KF546691-95	20°54'31.38"N	156°24'40.26"W
L. hawaiensis	Keanae, Maui	E7	KF546537	KF546568	KF546658	KF546714	N/A	N/A	N/A	N/A
L. hawaiensis	Pupukea, O'ahu	F1	KF546531	KF546562	KF546617-26	KF546709	KF546591	KF546667-71	21°38'59.70"N	158°03'45.48"W
L. hawaiensis	Pupukea, O'ahu	F1	KF546533	N/A	N/A	N/A	N/A	N/A	N/A	N/A
L. hawaiensis	Pupukea, O'ahu	F1	AY051349	N/A	AY051330	N/A	N/A	N/A	N/A	N/A
L. hawaiensis	Pouhala Marsh, O'ahu	F2	KF546532	N/A	N/A	KF546710	N/A	N/A	N/A	N/A
L. hawaiensis	Pouhala Marsh, O'ahu	F2	AY051347	N/A	AY051328	N/A	N/A	N/A	N/A	N/A
L. hawaiensis	Honomanu Bay, Maui	F3	KF546530	KF546563	N/A	KF546708	N/A	N/A	N/A	N/A
L. hawaiensis	Keokea Beach, Hawai'i	F4	KF546529	KF546558	N/A	KF546703	N/A	N/A	N/A	N/A
L. hawaiensis	Onekahakaha Beach Park, Hawai'i	F5	KF546534	KF546561	KF546629-42	KF546705	KF546588	KF546672-76	19°44'16.05"N	155°02'20.15"W
L. hawaiensis	Leleiwi Beach, Hawai'i	F6	KF546535	KF546560	N/A	KF546706	N/A	N/A	N/A	N/A
L. hawaiensis	South Point, Hawai'i	F7	KF546536	KF546559	N/A	KF546707	N/A	N/A	N/A	N/A
L. hawaiensis	Kapa'a State Park, Hawai'i	F8	KF546528	KF546557	N/A	KF546704	N/A	N/A	20°12'11.52"N	155°54'6.66"W
L. exotica	Veracruz, Mexico		KF546552	KF546584	KF546664	KF546726	N/A	N/A	19°12'33.63"N	96° 7'51.39"W
L. occidentalis	Guaymas, Mexico		KF546553	KF546583	KF546666	KF546728	N/A	N/A	27°54'44.33"N	110°56'49.56"W
L. vitiensis	Parangtritis, Java, Indonesia		KF546554	KF546582	KF546665	KF546727	N/A	N/A	N/A	N/A
L. vitiensis	Dili, East Timor		KF546556	KF546581	KF546662	KF546725	N/A	N/A	N/A	N/A
L. vitiensis	Labuanbajo, Flores, Indonesia		KF546555	KF546580	KF546663	KF546724	N/A	N/A	N/A	N/A

of the Cytochrome-b (Cytb) gene (primers 144F/151F and 270R/272R; Merritt et al. 1998). We also amplified two nuclear genes for a subset of individuals (1–5 per population, see Supplementary Figure S.III.1): a ~1,000bp region of the 28S rDNA gene (primers 28SA/28SB; Whiting 2002) and a ~710bp region of the alpha-subunit of the Sodium Potassium ATPase (NaK; primers NaK forb/NaK rev 2; Tsang et al. 2008). PCR-products were cleaned with a mixture of Exonuclease I (New England Biolabs) and Shrimp Alkaline Phosphatase (USB Scientific) and cycle sequenced at the University of Arizona Genetics Core (UAGC). We assembled sequences and removed primer regions using Sequencher 4.8 (Genecodes). None of the protein-coding sequences exhibited premature stop codons or frame shifts, suggesting they are not pseudogenes.

III.2.3 Sequence alignments and mitochondrial phylogenetic analyses

We aligned the ribosomal DNA gene fragments (i.e., 16S rDNA, 12S rDNA, and 28S rDNA) with the MAFFT algorithm (Katoh et al. 2005) assuming the Q-INS-I strategy as implemented in the GUIDANCE server (Penn et al. 2010a). Because of the high divergence among lineages of *Ligia* (see Results), several regions of ambiguous alignment were observed for these genes. Therefore, we used the GUIDANCE server (Penn et al. 2010b) to estimate confidence scores for each nucleotide position (100 independent alignments based on different bootstrap guide trees were conducted), and removed all positions with a confidence score below 1.00, as well as several positions for which alignments were considered ambiguous (Table III.2). We estimated pairwise genetic distances with the Kimura-2-Parameter (K2P) correction in MEGA v5.05

Table III.2: Included and excluded characters and substitution models used for phylogenetic reconstructions of *Ligia* from Hawaii.

Gene	Taxa	Total Chars.	Exc. Chars.	Inc. Chars.	Pars. Inf.	AIC (weight)	AICc (weight)	BIC (weight)
16S rDNA	35	497	113	384	107	TIM2 +I +G (0.4412)	TIM2 +I +G (0.5449)	TIM2+I+G (0.5094)
12S rDNA	28	510	106	404	120	TIM2+I+G (0.1544)	TPM2uf+I+G (0.1767)	HKY+G (0.2746)
COI	28	615	0	615	208	012010+I+G+F (0.2669)	012010+I+G+F (0.3396)	012010+I+G+F (0.6786)
Cyt-b	26	355	0	355	146	TrN+G (0.1371)	TrN+G (0.2019)	HKY+G (0.5933)
MT Total	35	1977	219	1758	581	012313+I+G+F (0.2139)	012313+I+G+F (0.2232)	012010+I+G+F (0.5984)

(Tamura et al. 2011) for the COI and the 16S rDNA (excluding ambiguous sites) gene fragments separately.

We determined the most appropriate model of DNA substitution for each mitochondrial gene fragment and the mitochondrial concatenated dataset from among 1,624 candidate models by evaluating their corresponding likelihood scores on a fixed BioNJ-JC tree under the Akaike Information Criterion (AIC), corrected AIC (AICc), and the Bayesian Information Criterion (BIC) (Table III.2) using jModeltest v2.1.1 (Darriba et al. 2012). The chosen model was used in phylogenetic searches, with two general exceptions. First, when the selected model was not available in a particular software, we applied the next more complex model available (Table III.3). Second, as the joint estimation of Γ and I parameters can be problematic (see RAxML manual; pages 113-114 of Yang 2006), we used the simpler Γ when the chosen model included both Γ and I parameters. We also implemented several partitioning schemes: (a) all positions within a single partition; (b) partitioned by gene; and (c) the best partitioning scheme according to the BIC implemented in PartitionFinder v1.0.0 (Lanfear et al. 2012). We used the following parameters in PartitionFinder searches: branch lengths = linked; models = all; model selection = BIC; search = greedy; and *a priori* partitioning combining each gene and codon position.

We carried out maximum likelihood (ML) searches in RAxML v7.2.6 (Stamatakis 2006a; Stamatakis 2006b; Stamatakis et al. 2008) and GARLI v2.0 (Zwickl 2006). RAxML consisted of 1,000 bootstrap replicates followed by a thorough ML search under the GTR +Γ model run under the Rapid Bootstrap Algorithm, whereas GARLI analyses

Table III.3: Settings for phylogenetic analyses of the concatenated mitochondrial (MT) dataset of Hawaiian *Ligia*.

Method	Model and Priors[1]	Partitioning scheme[2]	Iterations generations/ bootstrap replicates	Sample Frequency	Runs/ chains	Burn-in	ASDSF[3]	Bayes Factors[4]/ ML Scores (-lLn)	ESS[4,5] >200	PSRF[6]
RAxML	GTR +Γ	1	1,000	n/a	n/a	n/a	n/a	-10335.851	n/a	n/a
RAxML	GTR +Γ	4 (By Gene)	1,000	n/a	n/a	n/a	n/a	-10261.31	n/a	n/a
RAxML	GTR +Γ	5 (PF)	1,000	n/a	n/a	n/a	n/a	-9701.442	n/a	n/a
Garli	TIM2 +Γ	1	1,000	n/a	n/a	n/a	n/a	-10332.685	n/a	n/a
Garli	Mixed Model	4 (By Gene)	1,000	n/a	n/a	n/a	n/a	-10247.971	n/a	n/a
Garli	Mixed Model	5 (PF)	1,000	n/a	n/a	n/a	n/a	-9795.940	n/a	n/a
MrBayes	GTR +Γ	1	200,000,000	5,000	4	25%	0.001663	-10392.418	Yes	1
MrBayes	GTR +Γ	4 (By Gene)	200,000,000	5,000	4	25%	0.001071	-10358.250	Yes	1
MrBayes	GTR +Γ	5 (PF)	200,000,000	5,000	4	25%	0.001462	-10060.935	Yes	1
Phycas	GTR +Γ	1	1,000,000	50	1/1	25%	n/a	-10398.907	n/a	n/a
Phycas	GTR +Γ	4 (By Gene)	1,000,000	50	1/1	25%	n/a	-10347.169	n/a	n/a
Phycas	GTR +Γ	5 (PF)	1,000,000	50	1/1	25%	n/a	-9759.840	n/a	n/a

[1] All others default; [2] different partitions separated by comma; [3] Average standard deviation of split frequencies; [4] estimated in Tracer v.1.5; [5] Effective Sample Size; [6] Potential Scale Reduction Factor for all parameters; PF = PartitionFinder v.1.0: (A) 12S+16S+Cytb2 (GTR +G), (B) COI1 (TrNef+I); (C) COI2+Cytb1 (F81 +I), (D) COI3 (HKY +G); (E) Cytb3 (TrN +G).

consisted of 1,000 bootstrap replicates under the appropriate model of evolution identified by jModeltest. All other settings were as default. We calculated majority-rule consensus trees for each analysis with the SumTrees command of DendroPy v3.10.1 (Sukumaran and Holder 2010).

We carried out Bayesian phylogenetic reconstructions in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) and Phycas v1.2.0 (Lewis et al. 2008). We implemented polytomy priors (Lewis et al. 2005) in Phycas to alleviate the potential overestimation of posterior probabilities (i.e., "star-tree paradox") known to affect Bayesian approaches (Suzuki et al. 2002). We present the number of independent MCMC runs, chains, and generations in Table III.3, with all other parameters as default. We determined if Bayesian analyses had reached stationarity by: (a) stable posterior probability values; (b) high correlation between the split frequencies of independent runs as implemented in AWTY (Nylander et al. 2008); (c) small and stable average standard deviation of the split frequencies of independent runs; (d) Potential Scale Reduction Factor close to 1; and (e) an Effective Sample Size (ESS) > 200 for the posterior probabilities, as evaluated in Tracer v1.5 (Rambaut and Drummond 2009). Samples prior to stationarity were discarded as "burnin" (Table III.3). To estimate the posterior probability for each node, we built majority-rule consensus trees of the stationary stage of each run using the SumTrees command (Sukumaran and Holder 2010).

III.2.4 Nuclear gene analyses

Given the low variation levels observed in both nuclear genes amplified (see Results), we visualized relationships between nuclear alleles on networks constructed using the cladogram estimation algorithm of Templeton et al. (1992) as implemented by TCS v1.21 (Clement et al. 2000). We calculated the 95% most parsimoniously plausible branch connections between alleles, with all other settings as default.

III.2.5 Geometric-morphometric methods

We captured digital images of the dorsal side of *L. hawaiensis* specimens using QCapture v3.1.2 and an Olympus QColor3 digital camera attached to an Olympus SZ61 stereomicroscope. We removed all pereopods (i.e., legs) to ensure specimens laid flat. Dissected pereopods were not used for morphometric comparisons. During dissections, we determined and noted the sex of each specimen by visually inspecting the endopod of the 2nd pleopod. Sex was noted as either: male (M); gravid female (F); or juvenile/non-gravid female (J). We characterized body shape by digitizing 27 landmarks (LMs), using TpsDig v2.16 (Rohlf 2004), on the periphery of *Ligia* bodies (Figure III.2). We included landmarks that capture taxonomically informative regions and can be measured unambiguously. For example, we placed landmarks on the medial and the lateral boundaries of the eyes at the body periphery. These landmarks capture the relative size of the eyes and the inter-ocular distance, characters used to distinguish *Ligia* species (Taiti et al. 2003). We characterized the relative width of body segments and overall body shape, also important in *Ligia* taxonomy (Jackson 1922; Khalaji-Pirbalouty and

Wägele 2010; Lee 1994; Schultz and Johnson 1984; Taiti et al. 2003), by placing landmarks on the lateral posterior tergite tips. Lastly, we captured the shape of the pleotelson, another trait used in *Ligia* taxonomy (Khalaji-Pirbalouty and Wägele 2010; Schultz 1974; Taiti et al. 2003), by placing landmarks at its posterior tip and the lateral posterior points.

As the body plan of *Ligia* is bilaterally symmetric, all but the pleotelson tip LMs are anatomically homologous and may not be treated as independent in statistical analyses. As suggested by Zelditch et al. (2004), we reflected and averaged homologous landmarks across the body midline as defined by the pleotelson tip and the midpoint between the medial eye LMs. Corrected landmarks were centered, scaled and rotated, to best align with the consensus, using the method of generalized least squares, and projected to a flat shape space using tpsRelw v1.49 (Rohlf 2006). We calculated principal components of aligned coordinates to yield orthogonal shape variables, retaining for analysis 11 variables, representing ~95 % of overall variation. These principle components were taken as shape variables to test for differences in shape between *L. hawaiensis* lineages, sexes, and size classes. We used the centroid size (the summed square distances of landmarks from the centroid; Bookstein 1991) as an estimate of body size.

III.2.6 Statistical analyses

We carried out full factorial MANCOVA analyses of shape variables as a function of lineage, sex, size, and all interactions, to discern the meaningful correlates of body

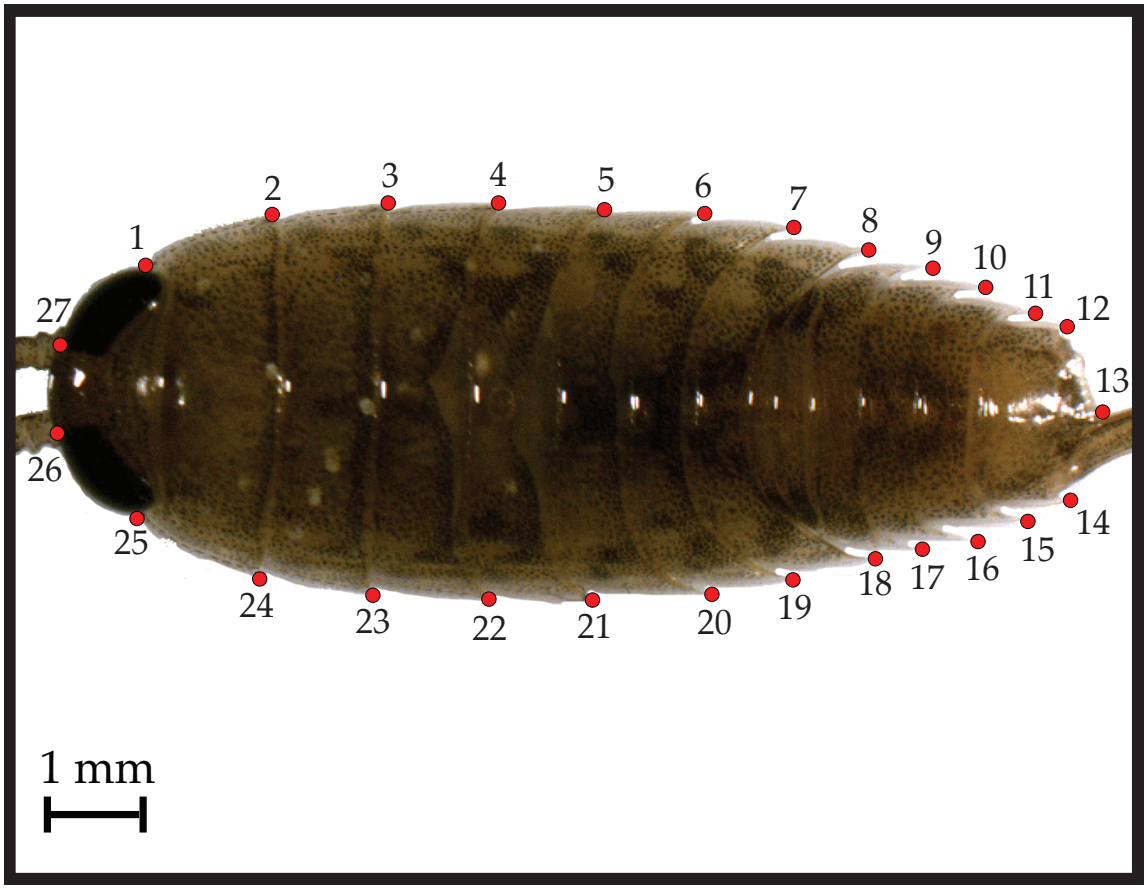

Figure III.2: Placement of landmarks used in geometric-morphometric analysis of *L. hawaiensis*. LMs 1 and 25 represent the posterior margin of eye; LMs 2-11 and 15-24 are the posterior most point of each segment; LMs 12 and 14 are the lateral posterior points of the pleotelson while LM 13 is the posterior most point of the pleotelson; LMs 26 and 27 correspond to the inner most margin of the eyes.

shape. When interaction terms were not significant, we removed them from the model, in a hierarchically manner, and repeated analyses. We estimated effect strengths by calculating partial eta squared values (η_p^2), which is the multivariate analog of R^2 in simple regression models (Tabachnick and Fidell 2001). We further explored differences between *L. hawaiensis* lineages with quadratic Discriminant Function Analyses (DFAs). To focus on these between-group differences, we first accounted for other predictors by conducting a preliminary MANCOVA and saving residual variation (Langerhans and DeWitt 2004). We used successful DFAs classification rates as an intuitive metric of the power of morphological divergence to correctly assign an individual to its genetic lineage based solely on its morphology. All DFA results were validated using leave-one out cross validation (LOOCV). All statistical tests were carried out in JMP v9.0.1. Lastly, we visualized shape differences between all main effects by producing thin-plate-spline transformations of LM positions in tpsRegr v1.37 (Rohlf 2005).

III.3 Results

All new sequences produced in this study have been deposited in GenBank under accession numbers KF546528–KF546728 (Table III.1).

III.3.1 Mitochondrial phylogenetic results

The final concatenated mitochondrial dataset (mt) included thirty-two individuals from throughout the Hawaiian archipelago (Figure III.1, Table III.1), and five individuals

from three *Ligia* species (*L. exotica*, *L. occidentalis*, and *L. vitiensis*) as outgroups. We excluded 219 poorly aligned positions (16S rDNA: 113-bp; 12S rDNA: 106-bp), resulting in a final alignment of 1758 characters, 581 of which were parsimony informative (Table III.2). Selection criteria in jModeltest did not agree on a single model for the dataset. The Bayesian Information Criterion (BIC) selected a model with three substitution rates (rate matrix: 012010, see jModeltest manual), variable nucleotide frequencies (+F), and both +I and +Γ parameters. Akaike Information Criteria strategies (AIC, AICc) selected a slightly more complex model consisting of four substitution rates (rate matrix: 012313, see jModeltest manual) and +F, +I, and +Γ parameters. Given the low weights observed for these models under the AIC and AICc (Table III.2), and that the 95% confidence interval included the BIC selected model, we applied the latter in GARLI analyses. For all other software (e.g. RAxML, MrBayes), we applied the GTR +Γ model instead, as the chosen models cannot be implemented.

Phylogenetic relationships inferred from the mitochondrial dataset are shown in Figure III.1. The endemic Hawaiian archipelago *Ligia* clade (i.e., ingroup) was highly supported: 100 Bootstrap Support (BS) and 100 Posterior Probability (PP). Within this clade, we observed three basal lineages with divergences among them between 11.85 and 16.74% COI K2P (Table III.4): (1) *Clade A* (lavender in Figure III.1), which is a well-supported clade (94–99 BS; 100 PP) that included some *L. hawaiensis* populations from Maui and Hawai'i, and represents a new lineage that was not previously identified by Taiti et al. (2003); (2) *Lineage B* (black in Figure III.1), which includes the *L. perkinsi* population from O'ahu (B1), and was previously reported by Taiti et al. (2003);

Table III.4: Estimates of evolutionary divergence, as measured by Kimura-2-parameter distances, among the main *Ligia* lineages in the Hawaii archipelago and outgroups. Above matrix: COI gene distances; below matrix: 16S rDNA gene distances. Diagonal (in bold) indicate within-clade distances (upper values: COI; below: 16S rDNA).

	Clade A	*Clade B*	*Clade C*	*Clade D*	*Clade E*	*Clade F*	*L. exotica*	*L. occidentalis*	*L. vitiensis*
Clade A	**5.88** **2.15-4.39**	14.52-14.81	11.85-13.79	13.57-16.74	13.96-14.63	14.36-15.96	23.77-25.04	21.17-21.18	19.76-24.54
Clade B	6.34-7.36	**N/A** **N/A**	14.22-14.87	15.14-15.81	15.75-16.65	13.04-14.39	24.11-24.11	22.96-22.96	19.08-24.54
Clade C	6.69-8.72	7.67-8.01	**0.88-2.51** **0.30-1.22**	13.57-15.37	12.69-14.24	12.03-14.44	24.62-25.13	20.93-21.90	19.26-25.23
Clade D	7.02-7.72	5.65-6.31	4.36-5.33	**0.00-1.60** **0.00-0.91**	10.53-12.93	12.20-13.99	23.66-24.42	23.33-23.83	20.52-26.53
Clade E	7.04-9.49	6.32-6.67	5.66-6.68	3.72-5.36	**0.00-2.51** **0.00-1.84**	10.51-12.91	23.93-24.73	23.08-23.58	22.16-26.45
Clade F	7.74-10.22	8.39-10.17	5.66-6.68	5.36-7.72	4.07-6.40	**0.18-5.30** **0.00-1.85**	22.17-23.02	22.12-23.60	20.47-24.57
L. exotica	14.89-16.04	16.41-16.41	12.88-13.25	14.04-14.81	14.83-15.25	15.32-16.12	**N/A** **N/A**	22.65-22.65	22.82-24.34
L. occidentalis	19.02-20.66	19.88-19.88	18.58-19.85	18.26-19.05	20.69-21.12	19.09-20.35	13.00-13.00	**N/A** **N/A**	22.65-23.32
L. vitiensis	19.05-21.90	18.37-20.20	17.77-20.59	16.01-17.77	15.19-18.55	17.92-19.16	21.20-23.90	24.02-25.24	**0.30-17.89** **3.06-23.02**

and (3) *Clade CDEF* (blue, green, orange, and red, respectively, in Figure III.1; 60–70 BS and 78–98 PP), which contained all *L. perkinsi* samples from Kaua'i (*Clade C*) and the rest of the *L. hawaiensis* samples from the archipelago (*Clade DEF*).

Within *Clade A*, we detected three divergent lineages (maximum COI K2P divergence = 5.88%; Table III.4): (1) one found in a single Maui population (A1); (2) another in a single population from western Hawai'i (A2); and (3) the last in eastern Hawai'i (A3, A4). The analyses suggest the Maui lineage may represent the most basal split within *Clade A* and that lineages from Hawai'i form a monophyletic group; support for this relationship, however, was variable (66–85 BS; < 65 PP).

Within *Clade CDEF* we observed a basal split between *Clade C* (blue in Figure III.1; 100 BS; 100 PP), which contained all *L. perkinsi* localities from Kaua'i (C1–C3), and the *L. hawaiensis* Clade *DEF* (64–78 BS; 87–99 PP). Maximum COI K2P divergence within *Clade C* was 2.51% (Table III.4); and this lineage was previously identified by Taiti et al. (2003). Within *Clade DEF*, relationships among clades *D*, *E*, and *F* were unresolved. *Clade D* (light green in Figure III.1; 100 BS and 100 PP) includes four *L. hawaiensis* localities from Kaua'i (D2–D5) previously sampled by Taiti et al. (2003), and one newly sampled in this study (D1). This clade corresponds with the *L. hawaiensis* lineage from Kaua'i reported by Taiti et al. (2003). Maximum within-clade COI K2P divergence in *Clade D* was 1.60%. A member of *Clade E* (orange in Figure III.1; 100 BS and 100 PP) was previously sampled in Taiti et al. (2003) from O'ahu (E1); we discovered that this clade distribution extends to Moloka'i (E2, E3), Lana'i (E4), and Maui (E5–E7). Maximum within-clade COI K2P divergence in *Clade E* was

2.50%. Lastly, *Clade F* (red in Figure III.1; 93–99 BS and 100 PP) contained previously sampled populations from O'ahu (F1–2), and new localities from Maui (F3) and Hawai'i (F4–F8). We recovered three lineages within *Clade F*: (1) an O'ahu lineage (F1–2; 63–86 BS and 91–100 PP) that corresponds to the *L. hawaiensis* O'ahu clade reported by Taiti et al. (2003); and two new lineages: (2) a Hawai'i lineage (F4–F8; 81–91 BS and 89–99 PP); and (3) a lineage formed by a single population from Maui (F3). Maximum COI K2P divergence among *Clade F* lineages was 5.30%.

III.3.2 Nuclear gene patterns

We sequenced two nuclear genes for all *Study Area* lineages with the exception of the *L. perkinsi* lineage from O'ahu (*B*). Multiple attempts to amplify nuclear genes from this population proved unsuccessful. The patterns inferred from the nuclear genes were in general, consistent with those inferred from the mitochondrial genes. For the NaK gene, we only observed six alleles, separated by 1–6 steps. For *Clade A* members, we detected two alleles separated by a single step. These alleles were separated from the other alleles by 2–6 steps, which is concordant with the high divergence observed in mitochondrial genes between *Clade A* and all other lineages. The allele observed for the individual of *Clade C* (*L. perkinsi* from Kaua'i) was divergent from the other alleles by 5–6 steps, also consistent with the mitochondrial results. The NaK results show a closer relationship among members of clades *D*, *E*, and *F*, also congruent with the mitochondrial results. Three alleles were observed for members of these clades, which were separated by only 1–2 steps, with *Clade E* members from Lana'i, Moloka'i and

Maui sharing an allele with a *Clade F* member from Hawai'i (F5); whereas another member of the *Clade F* from O'ahu (F1) harbored a unique allele. The *Clade D* individual from Kaua'i harbored a unique allele.

For the 28S rDNA gene, we excluded 60 poorly aligned positions, resulting in a final alignment of 967 characters, 31 of which were parsimony informative. For this gene, we detected thirteen alleles. Seven of these alleles were recovered from *Clade E* individuals (Figure III.3; colors for clades correspond with those in Figure III.1), with the other lineages harboring one or two alleles. Concordant with mitochondrial phylogenetic findings, alleles from different major lineages appeared highly differentiated, with most lineages separated by 10–21 steps. We note, however, that the single allele found in *Clade D* (Kaua'i; D1) was separated by only two steps from one of the two alleles observed in *Clade F* (from the O'ahu F1 locality). The F1 and D1 alleles were in turn separated by 11 steps from the other *Clade F* allele (from the Hawai'i F5 locality). This pattern could be the result of incomplete lineage sorting or a past hybridization event. Examination of additional informative nuclear markers in individuals from multiple populations per clade is needed to resolve this question. Indeed, multispecies coalescent analyses of numerous unlinked markers are likely needed to resolve relationships with more certainty (Degnan and Rosenberg 2009). Nevertheless, as shown by Mateos et al. (2012), datasets with three loci, including one with strong phylogenetic signal (i.e., the mitochondrial dataset) and one or two with low phylogenetic signal (e.g. NaK) are not well suited for multispecies coalescent approaches, and different methods lead to

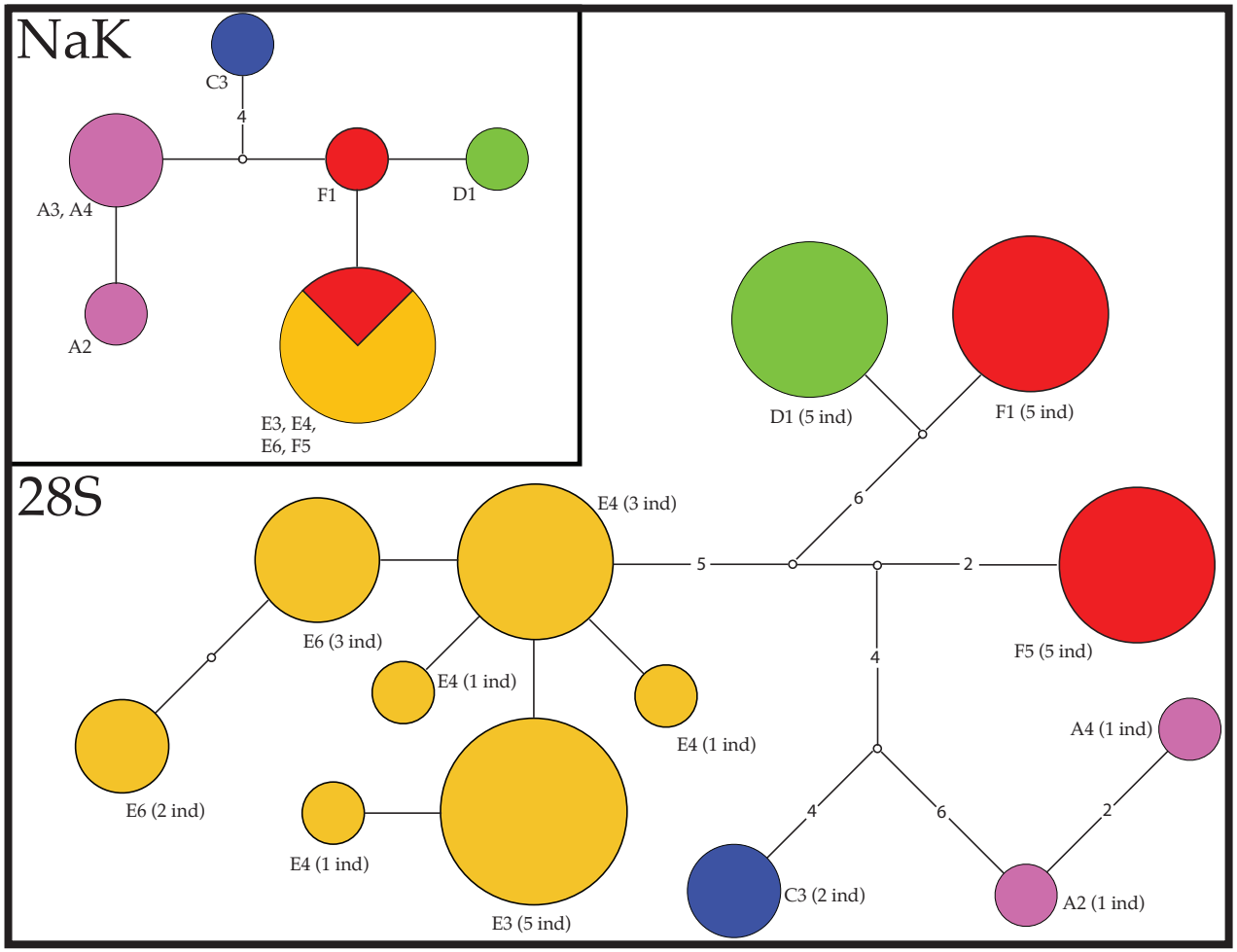

Figure III.3: Haplotype networks for the 28SrDNA and NaK gene fragments for *Ligia* from the Hawaiian archipelago. Colors and labels correspond with those in Figures III.1 and III.3. Geographic origin of alleles is indicated by the labels next to the allele, and corresponds with those in other figures and tables in this chapter. White circles represent unsampled (i.e., missing) alleles. The size of circles is proportional to the frequency at which an allele was recovered.

different results. Therefore, we consider that at the present stage, the mitochondrial phylogeny represents the most plausible hypothesis.

III.3.3 Geometric morphometrics

We analyzed a total of 84 *L. hawaiensis* individuals from six localities (D1, E3, E4, E6, F1, and F5), representing three of the major lineages detected for *L. hawaiensis* and all of the Hawaiian Islands sampled. We did not include individuals from *Clade A* in geometric morphometric analyses due to limited sampling (i.e., < 3 individuals per population). Principal component analysis generated 24 non-zero eigenvectors and the first eleven accounted for 96.20% of the variance, and thus were included in posterior analyses.

The full factorial MANCOVA yielded no significance for the three-way interaction term, thus we removed it and repeated the analysis. This simpler MANCOVA model (Table III.5) yielded significant results for the effects of Lineage ($\lambda_{wilks} = 0.358$, $d.f._{num} = 22$, $d.f._{den} = 120$, $P < 0.0001$), Sex ($\lambda_{wilks} = 0.257$, $d.f._{num} = 22$, $d.f._{den} = 120$, $P < 0.0001$), Size (as measured by Log Centroid) ($F = 5.178$, $d.f._{num} = 11$, $d.f._{error} = 60$, $P < 0.0001$), and for the Lineage*Sex interaction term ($\lambda_{wilks} = 0.372$, $d.f._{num} = 44$, $d.f._{den} = 231.5$, $P = 0.0209$). All significant effects had partial eta squared values indicative of large effect sizes ($\eta_p^2 > 0.2$), with the main effects of Lineage, Sex, and Size having values > 0.4.

Quadratic DFAs of MANCOVA residuals indicated significant differences between lineages ($\lambda_{wilks} = 0.580$, $d.f._{error} = 140$, $P < 0.0089$). We achieved an initial correct assignment of individuals to their lineage in 91.67% of cases. After LOOCV, the

Table III.5: Results of multivariate analyses of covariance examining overall body shape in *L. hawaiensis*. Significant effects with a ηp2 value >0.2 are indicated in bold.

	λ_{wilks}	F	$d.f._{num}$	$d.f._{den}$	*p*	η_p^2
Lineage	**0.358**	**3.660**	**22**	**120**	**<.0001**	**0.4016**
Sex	**0.257**	**5.308**	**22**	**120**	**<.0001**	**0.4932**
Size	**0.949**	**5.178**	**11**	**60**	**<.0001**	**0.4870**
Lineage*Sex	**0.372**	**1.553**	**44**	**231.5**	**0.0209**	**0.2279**
Sex*Size	0.729	0.936	22	120	0.5498	0.1464
Lineage*Size	0.783	0.710	22	120	0.8225	0.1151

Table III.6: Classification rates for *L. hawaiensis* lineage DFAs. Rows indicate actual clade of origin, while columns indicate predicted clade membership. We present the percentage of individuals correctly assigned to their clade of origin for the original model first, followed by LOOCV rates.

	Clade D	*Clade E*	*Clade F*
Clade D	**100**	0.00	0.00
	26.67	46.67	26.66
Clade E	2.63	**89.47**	7.89
	8.57	**62.86**	28.57
Clade F	0.00	8.11	**91.89**
	0.00	29.41	**70.59**

successful classification rate dropped to 59.52%, with per-lineage validated correct classification rates as follows: 26.67% for *Clade D*, 70.59% for *Clade E*, and 62.86% for *Clade F*. Individuals for *Clade D* were more likely to be identified as *Clade F* (7/15) than to their original lineage (4/15). Most misclassified individuals after LOOCV were assigned to *F* (17 of 34) and *E* (14 of 34) (Table III.6).

III.3.4 Shape variation in L. hawaiensis

We present thin-plate-spline transformations for all major lineages (Figure III.4) and for sex categories (Figure III.5). Visualizations are presented at either 3*X or 10*X the normal range for ease of comparison. Visualizations for the individuals with the highest canonical score for each lineage are shown in Supplementary Figure S.III.2.

L. hawaiensis lineages differ most prominently in two areas: the width of the body, relative to the total body length, and the distance between the eyes, as measured by the distance between their medial boundaries. We also detect minor differences in the distal-most point of the pleotelson. Individuals from *Clade D* have an oblong-ovate body with a mid-body narrower than individuals from clades *E* and *F*. The distance between the eyes appears to be ~2/3 the total eye length. *Clade E* exhibits an ovoid shape and eyes that appear to be separated by a distance greater than the total eye length. Furthermore, the distal-most point of the pleotelson protrudes much more prominently than in either *Clade D* or *F*. Individuals from *Clade F* exhibit an overall body shape similar to those from *Clade D*; however, the pleotelson is much more similar to that seen in *Clade D*. Also, distance between the eyes appears to be equal to the length of the eyes.

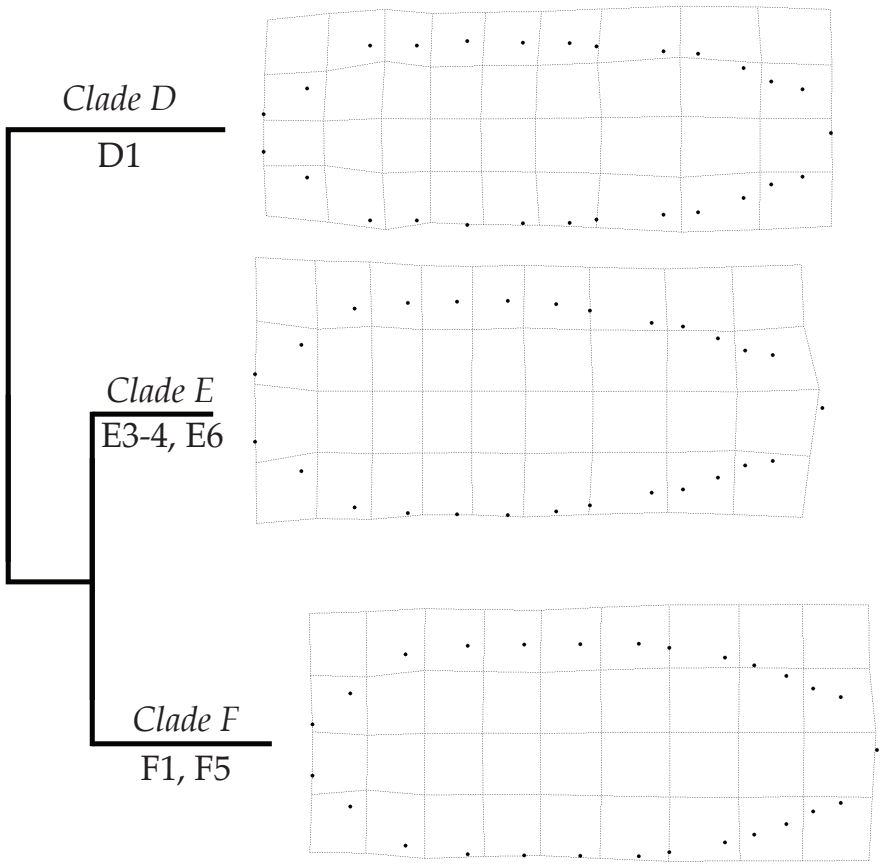

Figure III.4: Thin-plate-spline transformations of LM positions for *L. hawaiensis* lineages. Transformations are shown at 10*X natural range to aid visualization.

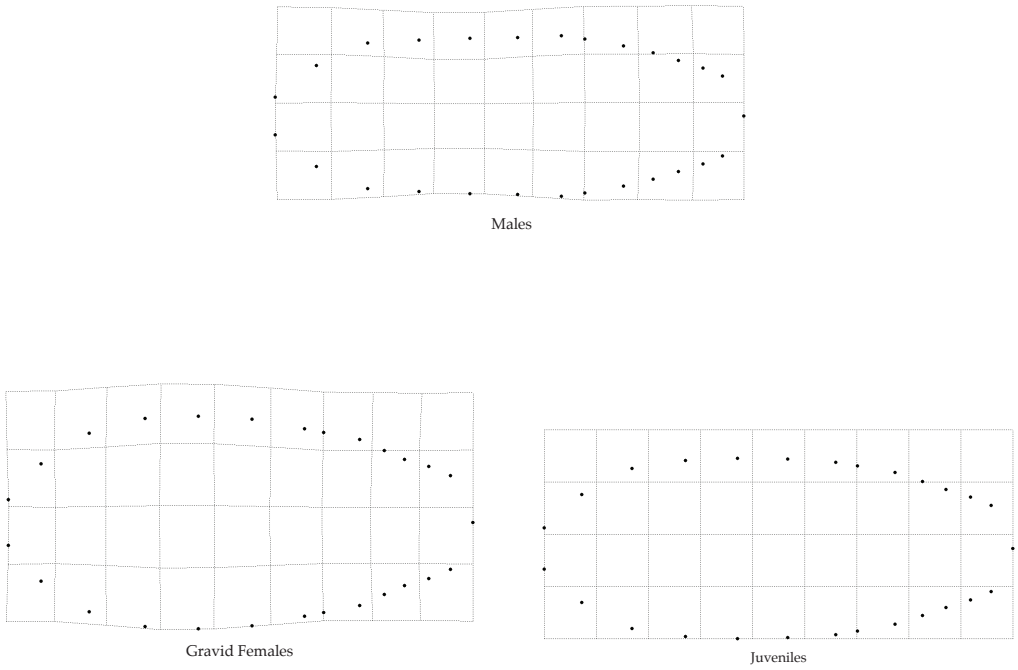

Figure III.5: Thin-plate-spline transformations of LM positions for *L. hawaiensis* sexes.
Transformations are shown at 10*X natural range to aid visualization.

Differences between the sexes appear to be mostly localized in the anterior end of the body, with females having wider segments than both males and juveniles (Figure III.5). Juveniles and males appear to have very similar body shapes, but the latter appear to have a slight invagination of the body around the midline (Figure III.5). We do not present shape visualizations for each sex and lineage combination separately due to limited sample sizes (i.e., limited number of juveniles and females). Differences between sizes were also evident, with larger individuals having a somewhat broader body than smaller individuals (not shown), a pattern reported for other *Ligia* species (Carefoot 1973; Santamaria et al. in preparation).

III.4 Discussion

In this study, we conducted phylogeographic analyses of the *Ligia* lineages endemic to the Hawaiian archipelago including a comprehensive sampling of these isopods across the main Hawaiian Islands, which greatly enhances our understanding on the diversity and evolution of this group. Previous genetic work by Taiti et al. (2003) on *Ligia* from the Hawaiian islands of Kaua'i and O'ahu reported highly divergent lineages of the terrestrial *L. perkinsi* and the coastal *L. hawaiensis* in these islands. By including populations from previously unsampled main Hawaiian Islands, we have detected additional lineages and populations of *L. hawaiensis*. We report the discovery of a new lineage of *L. hawaiensis* (i.e., *Clade A*), distributed in Maui and Hawai'i, which is highly divergent (> 10% COI K2P) from previously reported *Ligia* lineages in the Hawaiian archipelago (Taiti et al. 2003). We also discovered that the distribution of a lineage

(*Clade E*) previously sampled in a locality in O'ahu (E1) extends to Moloka'i, Lana'i, and Maui. Similarly, we discovered that *Clade F*, which was previously identified in Taiti et al. (2003) from O'ahu, contains two additional lineages; one distributed in Maui (F3) and one in Hawai'i (F4–F8). Divergence among the three *Clade F* lineages is > 3% COI K2P. Considering that intra-specific divergences in marine invertebrates are typically < 3% for the same COI fragment used in this study [73], both *L. hawaiensis* and *L. perkinsi* likely represent cryptic species complexes.

High levels of genetic differentiation among populations of the Hawaiian *Ligia* are congruent with studies of *Ligia* in other parts of the world (Eberl et al. 2013; Hurtado et al. 2010; Jung 2008; Santamaria et al. in preparation; Hurtado et al. unpublished results). Biological characteristics of these isopods severely restrict the dispersal potential of this isopod, contributing effectively to the isolation of populations, and, in the long-term, to allopatric genetic differentiation (Hurtado et al. 2010). Nonetheless, phylogeographic patterns indicate that past dispersal events have been important in shaping the evolutionary history of these isopods in the Hawaiian archipelago.

The monophyly of the endemic Hawaiian *Ligia* lineages is well supported, suggesting that evolution of this group likely occurred within the archipelago. Lineages identified as the closest relatives of the endemic Hawaiian *Ligia* are highly divergent and found in other Pacific localities (Hurtado et al. unpublished results). Large divergences observed among Hawaiian *Ligia* lineages also suggest a long evolutionary history for this group. In addition, the phylogeographic patterns observed, although not fully resolved, do not support simpler patterns of evolution, such as colonization from older to

younger islands (i.e., progressive rule), or vice versa (i.e., from younger to older islands), but rather a complex evolutionary history. Illustrating the complexity of the evolutionary history of the Hawaiian *Ligia*, the three most basal lineages include one found only in the younger islands (*Clade A*), a terrestrial lineage restricted to O'ahu (*Clade B*), and one that is the most diverse. This latter clade (*CDEF*) includes supralittoral and terrestrial lineages, and divergent lineages restricted either to the older island of Kaua'i or to the other islands. Dispersal and local extinctions likely contributed to shape this complex evolutionary history.

Despite the complex evolutionary history of Hawaiian *Ligia*, some phylogeographic patterns emerge, which appear congruent with the geological history of the main Hawaiian Islands. First, Kaua'i, the oldest of the main Hawaiian Islands, harbors only one endemic *L. hawaiensis* lineage (*Clade D*), which is highly divergent from the other lineages. This is consistent with the older geological history of this island and its high degree of isolation, as no overland connections are thought to have existed between Kaua'i and other Hawaiian islands (Carson and Clague 1995). Second, in contrast to the pattern observed in Kaua'i, sharing of highly divergent lineages is observed among the other main Hawaiian Islands, suggesting inter-island dispersal among these islands. The geological history of these islands may have provided opportunities for the exchange of colonizers from divergent lineages. Moloka'i, Maui, Lana'i, and Kaho'olawe, are thought to have existed as a single landmass (i.e., Maui Nui Complex) throughout most of their geological history, first splitting up some ~0.6 million years ago (Ma) and retaining land connections during glacial low sea stands (Price and Elliott-Fisk 2004).

The Maui Nui complex is thought to have been connected via a land bridge to O'ahu between 2.2–1.9 Ma forming the short lived O'ahu Nui complex (Carson and Clague 1995; Price and Elliott-Fisk 2004). These past connections may have facilitated the dispersal of the *Clade E L. hawaiensis*, restricted to the Maui Nui islands and O'ahu. The low genetic divergences observed among *Clade E* populations (< 2.5% COI K2P) may indicate a recent history of isolation among populations of this clade. Dispersal of *Clade F* across these islands may also have been facilitated by these connections. Third, the oceanic channel separating Maui and Hawai'i does not appear to constitute a very effective barrier for inter-island dispersal of coastal organisms that disperse through rafting, as two divergent lineages are shared between Hawai'i and Maui (i.e., clades *A* and *F*). Remarkably, however, members from *Clade E* were not found in Hawai'i, despite their wide distribution in the Maui Nui islands. Finally, evolution of the terrestrial life style appears to have occurred very early during the diversification of the Hawaiian *Ligia*, as clades *B* and *C* are highly divergent and occupy a very basal position in the phylogeny of Hawaiian *Ligia*.

With regard to the evolution of the terrestrial life style in Hawaiian *Ligia*, our results confirm the paraphyly of *L. perkinsi*, previously observed by Taiti et al. (2003). Therefore, from the phylogeographic patterns it is uncertain (1) whether evolution of the terrestrial lifestyle occurred independently in each island (i.e., Kaua'i and O'ahu) or (2) whether this life style evolved once. Both of the above hypotheses are equally parsimonious assuming a coastal ancestor for the endemic Hawaiian *Ligia* clade, which we consider is most likely as the closest lineages are coastal and because of the

predominantly coastal nature of the genus. Under the observed phylogenetic patterns, an independent origin of the terrestrial lifestyle would have required two steps: the evolution of the terrestrial lifestyle along the branches of clades *B* and *C*. Conversely, a single origin of the terrestrial lifestyle may only be explained by invoking the adaptation to terrestrial habitats followed by a reversal to the marine habitat (i.e., 2 steps). Taiti et al. (2003) considers the independent adaptation to terrestrial habitats in Hawaiian *Ligia* as the most plausible explanation for the origin of *L. perkinsi* populations in O'ahu and Kaua'i. They consider unlikely a shift from terrestrial to seashore conditions, given that most of the species in the genus occupy supralittoral habitats, and that terrestrial forms appear to have derived from supralittoral forms (Schmalfuss 1979). Similar conditions could have acted in O'ahu and Kaua'i that facilitated the independent colonization of freshwater habitats. Nonetheless, not enough is known about the biology of *L. perkinsi* to discard the possibility of a reversal. This species is often found in the rocky shores of streams (Taiti and Howarth 1996), with populations in O'ahu occurring less than 3 Km from shore and at low altitude (Lichtwardt 1986). Furthermore, *L. perkinsi* exhibit the highest osmoregulatory capabilities observed in the genus (Carefoot et al. 2000). Post-hoc analyses from Approximately Unbiased (AU) tests implemented in CONSEL (Shimodaira and Hasegawa 2001) rejected the monophyly of *L. perkinsi* ($P = 1 \times 10^{-6}$), which would have supported a single origin of the terrestrial lifestyle These tests also rejected the monophyly of *L. hawaiensis* ($P = 2 \times 10^{-5}$), as well as the monophyly of *L. perkinsi* from Kaua'i + *L. hawaiensis* from Kaua'i.

Morphological differences have been previously observed between *L. perkinsi* lineages (*B*, *C*), but not between *L. hawaiensis* lineages (*D*, *F*) (Taiti et al. 2003). Previous comparisons, however, relied on classic taxonomic characters. Geometric-morphometric approaches have proven useful in identifying differences between otherwise cryptic species in other invertebrates (Carvajal-Rodríguez et al. 2006; Francuski et al. 2009; Milankov et al. 2009; Mitrovski-Bogdanovic et al. 2013), including crustaceans (Bertocchi et al. 2008; Zuykova et al. 2012). By applying these powerful tools, we have detected statistically significant differences between three highly genetically divergent lineages of *L. hawaiensis*. Thin-plate-spline visualizations indicate that *L. hawaiensis* lineages differ in traits widely used in the taxonomy of *Ligia* (Jackson 1922; Khalaji-Pirbalouty and Wägele 2010; Lee 1994; Schultz and Johnson 1984; Taiti et al. 2003), including: their relative body width; the distance between the eyes; and the protrusion of the distal-most point of the pleotelson. As such, the observed differences may be of use for taxonomic revisions of *L. hawaiensis*. For example, individuals from *Clade D* exhibit traits (i.e., a narrow oblong-ovoid body shape and distances between the eyes that are shorter than for other *L. hawaiensis* lineages) that clearly distinguish them from all other *L. hawaiensis*, and that were previously used to describe *Ligia kauaiensis* (Edmondson 1931). This species, now considered a synonym of *L. hawaiensis* (Schmalfuss 2003), was first described from individuals from the shores of Kalihiwai Bay, Kaua'i (Edmondson 1931), the same location (D1) included in our molecular and morphometric analyses. Therefore, the deep genetic divergence of

Clade D (at least 13.57% COI K2P), its discrete geographic distribution, and apparently distinct morphology suggest that *L. kauaiensis* may be a valid species.

We also detected significant differences in overall body shape between sexes, with females exhibiting wider anterior segments than males and juveniles. These differences may be caused by the development of the ventral marsupium (i.e., brood pouch) in females. This structure forms from thoracic sterna and overlapping oostegites prior to copulation in mature females, and was present in all samples identified as females. As we did not observe obvious differences between males and juveniles and females were classified based on the presence of the marsupium, a temporary structure (Hornung 2011), differences between the sexes in *L. hawaiensis* may be temporary, and thus, not relevant to the taxonomy of *Ligia* in the Hawaiian archipelago. Lastly, we detected an effect of body size on body shape for *L. hawaiensis*, with larger individuals exhibiting a broader, less oval shape than smaller individuals; a pattern that has been reported for other *Ligia* species (Carefoot 1973; Santamaria et al. in preparation).

CHAPTER IV

CONSTRAINED DIFFERENTIATION IN BODY SHAPE DESPITE HIGH GENETIC DIVERGENCES AMONG LINEAGES OF *LIGIA OCCIDENTALIS SENSU LATO*

IV.1 Introduction

Morphologically cryptic species (i.e., two or more distinct species classified as a single species) are widespread (Pfenninger and Schwenk 2007) and, although recognized for almost 300 years (Winker 2005), their ubiquity in nature was not realized until the proposal of the biological species concept (Mayr 1942). More recently, the advent of DNA sequencing has lead to a surge in documentation of cryptic species in a wide array of taxa (Bickford et al. 2007), including the discovery of deeply divergent cryptic species complexes (Colborn et al. 2001; Lefebure et al. 2006; Rocha-Olivares et al. 2001). Whether or not such lineages are truly cryptic is important for practical purposes (i.e., taxonomy and conservation), as well as for addressing questions of ecological and evolutionary relevance. This task is daunting, however, as traditional taxonomy appears to be of limited use in such cases. Geometric-morphometric methods offer an alternative powerful tool to examine morphological differences, which has been successfully used in an array of taxa including vertebrates and invertebrates, for which traditional morphological characters were not diagnostic (Carvajal-Rodríguez et al. 2006; Francuski et al. 2009; Milankov et al. 2009; Mitrovski-Bogdanovic et al. 2013). Herein, we

employed landmark-based geometric-morphometric methodology to examine whether previously identified highly divergent allopatric lineages of the supralittoral isopod *Ligia occidentalis* Dana 1853, one of the most remarkable cases of cryptic diversification in the coastal realm, exhibit morphological differences.

Based on morphology, *Ligia occidentalis* is currently recognized as a single species throughout its entire distribution range (Pacific coast of North America between southern Oregon and Central Mexico, including the Gulf of California). No junior synonyms have been described (Espinosa-Pérez and Hendrickx 2001; Schmalfuss 2003). Phylogeographic analyses, however, suggest that *L. occidentalis* represents a cryptic species complex (hereafter *L. occidentalis sensu lato*) comprised of several highly divergent lineages (Eberl et al. 2013; Hurtado et al. 2010). Divergences among lineages are as high as 29.89% (Kimura-2-parameter; K2P) for the Cytochrome Oxidase I gene (COI), with > 60% of the pairwise comparisons between localities exhibiting COI K2P divergences > 10% (Hurtado et al. 2010). Deep divergences among lineages suggest a long evolutionary history of *L. occidentalis s. l.* in the region, possibly since the Miocene (Hurtado et al. 2010). High levels of genetic differentiation between populations indicate gene flow is severely restricted, even over small geographical distances, suggesting long-standing isolation of populations (Hurtado et al. 2010). In addition, crossbreeding experiments suggest post-mating reproductive barriers may exist between some lineages (Eberl et al. 2013; McGill 1978), and surveys of mitochondrial and nuclear gene markers do not show evidence of hybridization among divergent lineages (Eberl et al. 2013; Hurtado et al. unpublished data).

The high levels of allopatric genetic differentiation observed within *L. occidentalis s. l.* are consistent with its biological characteristics (Carefoot and Taylor 1995; Hurtado et al. 2010), which restrict this and the other species of coastal *Ligia*, to a very narrow vertical portion of rocky intertidal shores, between the splash and supralittoral zones. Members of the genus *Ligia* actively avoid entering the open sea; although they are capable of performing underwater gas exchange and swimming short distances (i.e., few meters). Likely as a result of their extremely low desiccation resistance, they tend to remain hidden under rocks and in crevices during the day and become more active at night. They remain close to the water line, where they can acquire water from wet substrate, droplets, puddles, spray, and air humidity. Their locomotion underwater and on sandy shores is very limited, rendering them highly vulnerable to predators outside the rocky habitat. Furthermore, they are direct developers (i.e., lack a planktonic phase; a characteristic of all peracarids), which further restricts dispersal of these isopods outside their rocky beaches. The above characteristics have likely contributed to the high isolation observed among populations of *Ligia* in several other regions, where the existence of cryptic species complexes is also suggested [i.e., the Caribbean (Santamaria et al., in preparation), the Hawaiian Archipelago (Taiti et al. 2003), the Korean Peninsula (Jung 2008)]. Nevertheless, the number and divergence of lineages within *L. occidentalis s. l*. represents the most striking example.

Members of *Ligia* exemplify some of the few animals that have adapted to live exclusively in the supralittoral, an environment characterized by harsh conditions (Hurtado et al. 2013). These include regular exposure to extreme temperatures, to air, to

fresh water from rain, to seawater from wave splash and storm surge, and to predation by land and marine animals and seabirds (Brown 2001; Ellis et al. 2007; Menge 1976). On the one hand, such harsh environmental conditions might impose strong stabilizing selection on morphology, limiting the morphological divergence that can accompany speciation (Bickford et al. 2007). On the other hand, different lineages of *L. occidentalis* are exposed to markedly different environmental conditions, which might be expected to promote morphological divergence.

Ligia occidentalis s. l. is distributed along a ~3,000 Km latitudinal gradient that encompasses several proposed marine biogeographic provinces (reviewed in Robertson and Cramer 2009). In general, the main lineages have non-overlapping geographic distributions, which largely match marine ecoregions (Spalding et al. 2007), with distributional limits usually corresponding with sharp environmental changes. The geographical limit between the two most divergent clades of *L. occidentalis* (20–25% divergence for COI) occurs at the Point Conception biogeographical boundary (Eberl et al. 2013). This boundary separates the Oregonian zoogeographical province, ranging from northern California and Oregon south to Point Conception, and the Californian province, which ranges from Point Conception to approximately Magdalena Bay, Pacific coast of Baja California. This area is defined by a transition between cold northern and warm southern water masses, as well as marked changes in coastal hydrography, dissolved oxygen, salinity and topography, and convergent ocean currents (Briggs 1974; Browne 1994; Seapy and Littler 1980). The geographical limit between these two main clades largely reflects the changes in sea surface temperature that define the Point

Conception biogeographical boundary along the shores of both, the mainland and the Californian Channel Islands (Eberl et al. 2013). Within the Gulf of California, sharp environmental differences are also observed in the distribution of the two most divergent lineages of *Ligia* (15–26% K2P COI) occupying this basin (Hurtado et al. 2010). One lineage occurs in the Northern Gulf of California (from the mouth of the Colorado River to the Midriff islands), whereas the other is distributed in the Southern Gulf of California. The Northern Gulf of California is characterized by strong seasonal variation in water temperatures (> 30 ºC in the summer; 8–12 ºC in the winter), large tidal regimes (up to 10m), and high summertime salinities (35–40 ppt) (Brusca 2006; Santamaría-del-Angel et al. 1994); whereas the Southern Gulf of California is characterized by somewhat lower salinities, smaller tidal regimes, and moderate seasonal variation in water temperatures (30–32 ºC in the summer; 18–20ºC in the winter) (Brusca 2006 and references therein).

In this study, we used landmark-based geometric-morphometric analyses to examine whether morphological differences in body shape are detectable among highly divergent lineages of *L. occidentalis*. We characterized body shape, including landmarks that captured taxonomically informative regions that have been used to distinguish *Ligia* species. Our results show significant differences in body shape among lineages and among localities within lineages. Large overlap, however, is observed among clades, which limits the use of body shape as a taxonomical diagnostic character. We discuss the potential factors that may be constraining body shape divergence among genetically differentiated clades of this isopod.

IV.2 Materials and methods

IV.2.1 Samples

We analyzed a total of 492 *Ligia* individuals from 53 Pacific localities distributed between central California and central Mexico, including the Gulf of California (Figure IV.1; Table IV.1). Samples were collected by hand during 2007–2010 and stored in 100% ethanol. Once in the laboratory, they were stored in ethanol at −80°C until dissection. These specimens were part of the original collections obtained for the *Ligia* phylogeographic study of this region by Hurtado et al. (2010); and represent the eight main highly divergent lineages identified in that study, which, for the most part, have non-overlapping distributions (Eberl et al. 2013). For consistency among studies, we use the same names for these lineages as in Hurtado et al. (2010). (1) A Central California clade (*Clade A*; grey in Figures IV.1 and IV.2) distributed from southern Oregon to north of Point Conception, CA, and some localities of the Northern Channel Islands, mainly in the western part (Eberl et al. 2013). (2) A Southern California clade (*Clade B*; light green in Figures IV.1 and IV.2) found on the mainland from south of Point Conception to San Diego, and in Santa Catalina Island. (3) A California clade (*Clade C*; orange in Figures IV.1 and IV.2) distributed from San Diego, CA to Ensenada, Mexico, and in some localities in the eastern part of the Northern Channel Islands. (4) A Baja Pacific North clade (*Clade D*; magenta in Figures IV.1 and IV.2) distributed from Ensenada, Mexico, to north of the Guerrero Negro Lagoon. (5) A Baja Pacific South clade (*Clade E*; light blue in Figures IV.1 and IV.2) found from south of this lagoon to Puerto San Carlos, Mexico. (6) A Careyes Clade (*Clade F*; brown in Figures IV.1 and

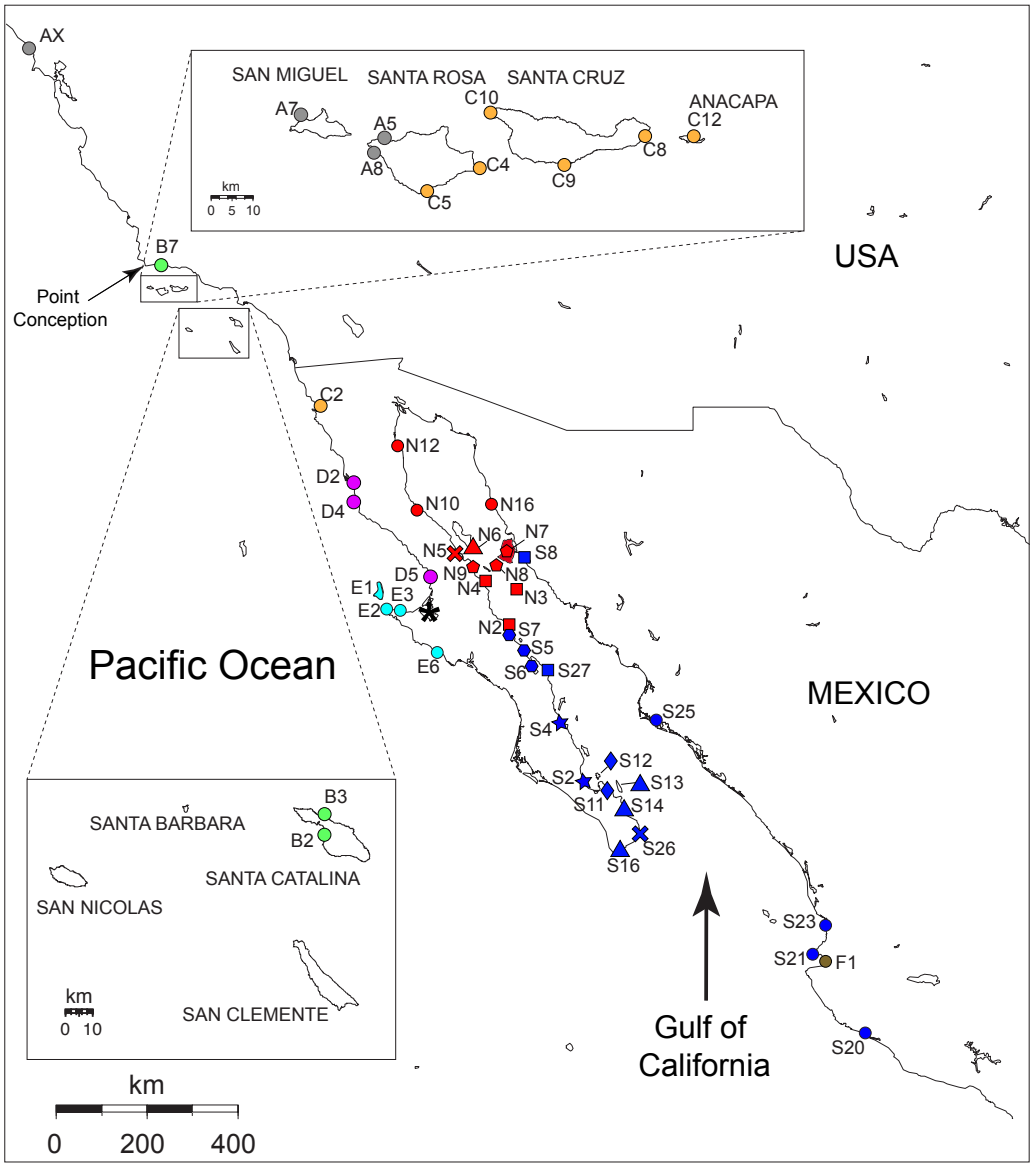

Figure IV.1: Map of sampled localities. Color and shape correspond to those in Hurtado et al. (2010). AX-Bodega Marine Laboratory; A5-N.W. Talcott; A7-Otter Harbor; A8-Fossil Reef; B2-Little Harbor; B3-Ithsmus Cove; B7-Refugio; C2-Corona Ensenada; C4-East Point; C5-Johnsons Lee; C8-Smugglers Cove; C9-Willows; C10-Fraser Cove; C12-Frenchys; D2-San Quintin; D4-Arroyo Ancho; D5-Tomatal; E1-Cedros Is.; E2-Punta Eugenia; E3- El Queen; E6-San Hipolito; F1-Vallarta; S2-Cajete; S4-San Cosme; S5-Mulege; S6- Bahía Armenta; S7-San Lucas; S8-Bahía Kino; S11- La Paz; S12-Isla Espiritu Santo; S13-Isla Cerralvo; S14-Barriles; S16-Cabo San Lucas; S20- Boquita; S21-Punta Mita; S23-Aticama; S25-Topolobampo; S26-Cabo Pulmo; S27-San Nicolas; N2-Santa Rosalia; N3-Isla San Pedro Martir; N4-San Francisquito; N5-Bahía de los Angeles; N6-Isla Angel de la Guarda (2 localities); N7-Isla Tiburon (2 localities); N8-Isla San Esteban; N9-San Rafael; N10-San Luis Gonzaga (2 localities); N12-San Felipe; N16-Puerto Libertad. * denotes Guerrero Negro Lagoon.

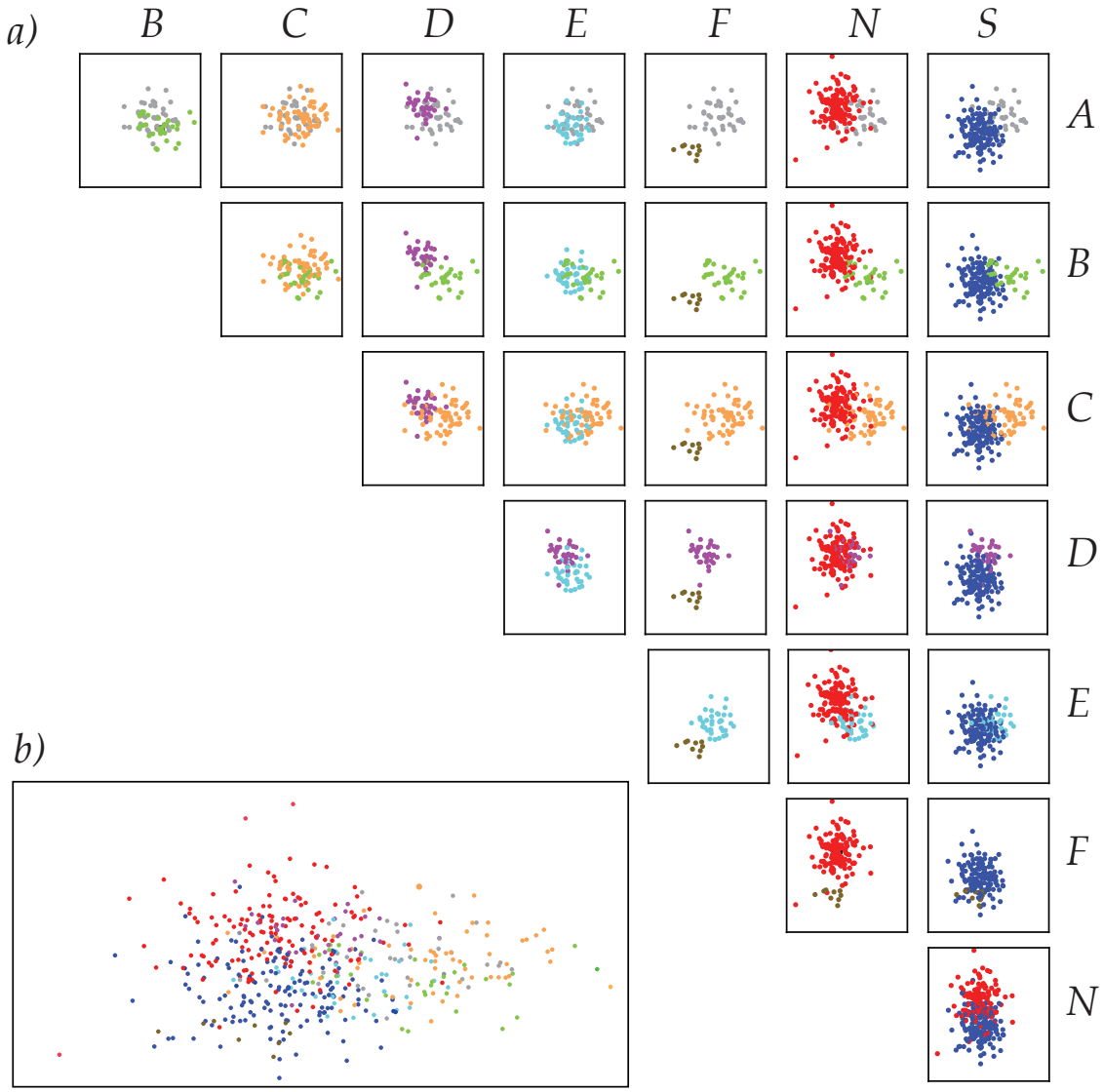

Figure IV.2: Results of Discriminant Function Analyses (DFA) for *Ligia* lineages from Central California to Central Mexico. Lineages are identified by color, which correspond to those in other figures and in Hurtado et al. (2010). Canonical plots are shown for each pairwise comparison between *Ligia* lineages (Panel a) and for the overall dataset (Panel b). The first and second canonical axes explained 43.13% and 32.20% of the variance, respectively

Table IV.1: Sampled localities by lineage with corresponding sample size. Label IDs correspond with those in Figure IV.1.

Label ID	N	Population	
AX	9	Bodega Marine Laboratory	Clade A
A8	10	Fossil Reef	N=38
A5	9	N.W. Talcott	
A7	10	Otter Harbor	
B3	9	Ithsmus Cove	Clade B
B2	10	Little Harbor	N=31
B7	12	Refugio Beach	
C4	10	East Pt. Beach	Clade C
C2	10	Ensenada (Corona Beach)	N=61
C10	6	Fraser Cove	
C12	9	Frenchy's	
C5	8	Johnson's Lee Beach	
C8	10	Smugglers Cove	
C9	8	Willows' Anchorage	
D4	10	Arroyo Ancho	Clade D
D2	14	South of Quintin	N=33
D5	9	El Tomatal	
E1	9	I. Cedros	Clade E
E2	8	Punta Eugenia	N=37
E3	10	Campo Queen	
E6	10	San Hipolito	
F1	9	Puerto Vallarta	Clade F
			N=9
N6	10	Angel de la Guarda	Clade GN
N5	10	Bahia de los Angeles	N=137
N16	10	Puerto Libertad	
N7	10	Ratolandia	
N8	17	San Esteban	
N12	10	San Felipe	
N4	9	San Francisquito	
N9	10	San Rafael	
N10	10	San Luis Gonzaga	
N10	4	San Luis Gonzaga (Mudflat)	
N3	12	San Pedro Martir	
N2	9	Santa Rosalia	
N7	9	El Tordillo	
N6	7	Viborita	
S23	10	Aticama	Clade GS
S6	18	Bahia Armenta	N=147
S14	6	Los Barriles	
S20	7	Boquita	
S26	9	Cabo Pulmo	
S2	8	El Cajete	
S13	8	I. Cerralvo	
S12	8	Espiritu Santo (Cathedral)	
S16	6	Cabo San Lucas	
S8	8	Kino	
S5	10	Mulege	
S11	10	La Paz (Malecon)	
S21	8	Punta Mita	
S4	9	San Cosme	
S7	5	San Lucas	
S27	9	San Nicolas	
S25	7	Topolobampo	

IV.2) reported only from populations in Puerto Vallarta and Careyes, Mexico. (7) A Gulf North clade (*Clade N*; red in Figures IV.1 and IV.2), which includes populations in the northern Gulf of California, including localities in the midriff islands. (8) A Gulf South clade (*Clade S*, blue in Figures IV.1 and IV.2) distributed in the southern Gulf, and mainland south of the Gulf to the State of Guerrero coast.

IV.2.2 Geometric-morphometric methods

We captured digital images of the dorsal side of each *Ligia* specimen using QCapture v. 3.1.2 software and an Olympus QColor3 digital camera attached to an Olympus SZ61 stereomicroscope. All pereopods (i.e., legs) were removed prior to image capture to ensure the cephalon and pereon laid flat. Dissected pereopods were not used in the morphometric study. We defined the sex of each individual as: gravid female (denoted as F), mature male (denoted as M), or others, which could be immature males or non-gravid females (denoted as J). Gravid females harbor a ventral thoracic pouch with tens of yellow eggs or embryos. Mature males can be recognized by visually inspecting the presence of gonopodia in the endopod of the 2nd pleopod.

We characterized body shape by digitizing 27 landmarks, using TpsDig v. 2.16 (Rohlf 2004), on the periphery of *Ligia* bodies (Figure IV.3). Care was taken to include landmarks that captured taxonomically informative regions and that can be measured unambiguously. Landmarks were placed on medial and lateral boundaries of the eyes at the body periphery. They capture the relative size of the eyes and the distance between them, both characters used to distinguish *Ligia* species (Taiti et al. 2003). Landmarks were also placed on lateral posterior tergite tips to aid in characterizing relative width of

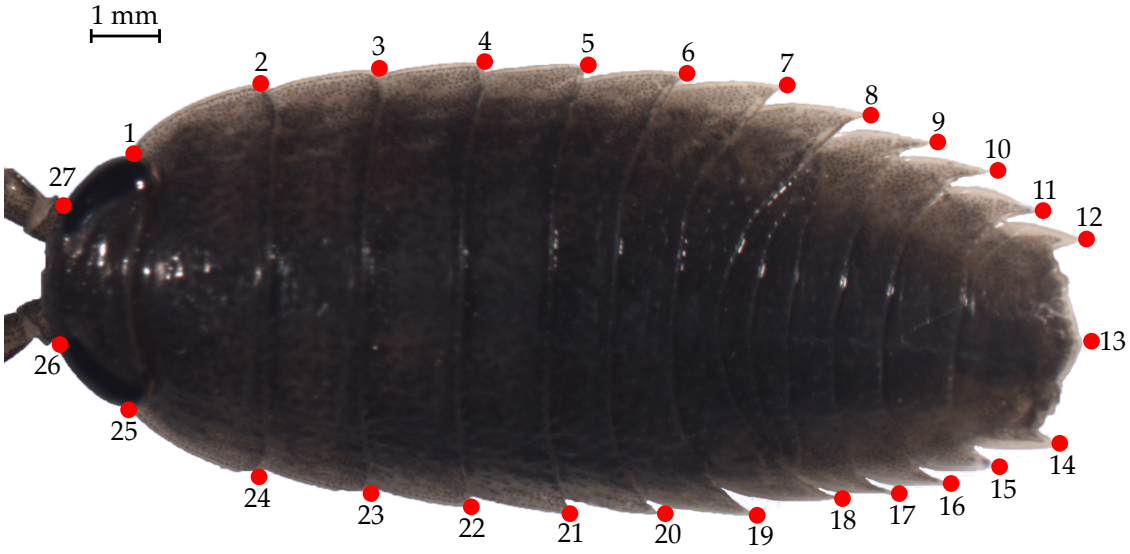

Figure IV.3: Placement in *Ligia occidentalis s. l.* of landmarks (LMs) used in geometric-morphometric analyses. LMs 1 and 25 represent the posterior margin of eye; LMs 2-11 and 15-24 are the lateral posterior tergite tips of each segment; LMs 12 and 14 are the lateral points of the pleotelson while LM 13 is the distal most point of the pleotelson; LMs 26 and 27 correspond to the inner most margin of the eyes.

each body segment and overall body shape, also important in *Ligia* taxonomy (Jackson 1922; Khalaji-Pirbalouty and Wägele 2010; Lee 1994; Schultz and Johnson 1984; Taiti et al. 2003). Finally, landmarks were placed at the posterior tip and the lateral posterior points of the pleotelson. Relationships between these landmarks capture the shape of the pleotelson, another trait used in *Ligia* taxonomy (Khalaji-Pirbalouty and Wägele 2010; Schultz 1974; Taiti et al. 2003). We used the centroid size (summed squared distances of landmarks from the centroid; Bookstein 1991) as a measure of body size.

As the body plan of *Ligia* is bilaterally symmetric, all but the pleotelson tip landmarks are anatomically homologous and should not be treated as independent in statistical analyses. We therefore reflected and averaged homologous landmarks across the midline (Zelditch et al. 2004), which was defined as a line connecting the pleotelson tip and the midpoint between the medial eye landmarks. Corrected landmarks were centered, scaled and rotated, to best align with the consensus, using the method of generalized least square, and projected to a flat shape space using tpsRelw v. 1.49 (Rohlf 2006). We calculated principal components of aligned coordinates to yield orthogonal shape variables (i.e., Relative Warps).

IV.2.3 Statistical analyses

We conducted full factorial MANCOVA analyses of shape variables as a function of Lineage (i.e., *Clade*), Population nested within Lineage, Sex, Size, and all interactions, to discern the meaningful correlates of body shape. When interaction terms were not significant, we removed them from the model, hierarchically by order (i.e., from the more complex to the simpler), and repeated the analyses (Engqvist 2005). We estimated

effect strengths by calculating partial eta squared values (η_p^2), which is the multivariate analog of R^2 in simple regression models (Tabachnick and Fidell 2001).

We explored differences among lineages, and among populations within lineages with Discriminant function analyses (DFAs). To focus exclusively on these between group differences, we first accounted for other predictors by conducting a preliminary MANCOVA and saving residual variation. Residuals were used in the DFAs to remove the effects of size and sex (Langerhans and DeWitt 2004). We tested whether all groups in our data shared a covariance matrix with the Box's M test. Although quadratic DFAs do not assume a homogeneous covariance matrix, singularities in the data matrix may prevent their correct use. In cases where neither linear nor quadratic DFAs could be correctly applied, we used regularized DFAs (Friedman 1989), as a compromise approach. We determined the best combination of Lambda (i.e., the degree of shrinkage of the individual class covariance matrix estimates towards the pooled estimate) and Gamma (i.e., the degree of shrinkage toward a multiple of the identity matrix) values by evaluating the risk of misclassification under several combinations of these parameters as suggested by Friedman (1989). Using this procedure, we attempted to assign individuals to 1) their clade of origin and 2) their population of origin within their corresponding clade. All results were validated using leave-one out cross validation (LOOCV).

We tested for associations between phylogeny and morphological variation by estimating Pagel's λ (Pagel 1999) and Blomberg's *K* (Blomberg et al. 2003) for all shape variables (i.e., relative warps), using Ives et al.'s (2007) method to account for multiple

observations per terminal branch. The use of relative warps in these analyses is justified, as relative warps are aligned to the main axis of variation and maintain inter-object distances (Perez et al. 2011). Both statistics provide an univariate measure of the strength of phylogenetic signal in the data, with values close to zero indicating no phylogenetic signal, and values close to one indicating the character has evolved under a Brownian motion (BM; i.e., phylogenetic signal explains the observed patterns). We tested whether observed λ values were statistically different from those expected under a null model (i.e., BM = 0) and a fully Brownian model (i.e., BM = 1) using a likelihood ratio test. In addition, we tested whether observed *K* values departed from the null hypothesis of no phylogenetic signal by using 10,000 permutations (Blomberg et al. 2003). All computations were carried out using the Picante (Kembel et al. 2010) and GEIGER (Harmon et al. 2008) packages in R.

We also tested whether genetic divergence is related to multivariate morphological divergence. We estimated genetic distances from Cytochrome Oxidase I (COI) sequences published by Hurtado et al. (2010) using the Kimura-2-Parameter (K2P) model in PAUP* (Swofford 2003). We calculated pairwise Euclidean distances for all localities on the residual variation (see above) using PopTools v. 3.2 (Hood 2010) in Microsoft® Excel. We tested for correlations between COI K2P distances and Euclidean Morphological Distances using a Mantel Test. P-values were calculated by permutation in addition to the parametric approach of the Mantel test. All statistical tests were carried out in JMP v. 9.0.1.

Finally, to visualize shape differences between lineages, we produced thin-plate-spline transformations of LM positions in tpsRegr v. 1.37 (Rohlf 2005). We also produced transformations for smallest and largest individuals for each clade and in the dataset overall to visualize the effect of body size on shape. We used these transformations to describe the general shapes of individuals within lineages and make comparisons among lineages.

IV.3 Results

IV.3.1 MANCOVA

Principle components analysis generated 24 non-zero eigenvectors. The first eleven warps accounted for 95.39% of the variance, and were included in subsequent analyses, whereas the other thirteen were discarded. The full factorial MANCOVA yielded no significant three- or four-way interaction terms, which were removed prior to repeating the analysis. This simpler MANCOVA model (Table IV.2) yielded significant results for the effects of Population nested within Lineage ($\lambda_{wilks} = 0.263$, $d.f._{num} = 242$, $d.f._{den} = 3347.6$, $P < 0.0001$), Lineage ($\lambda_{wilks} = 0.196$, $d.f._{num} = 77$, $d.f._{den} = 2020.9$, $P < 0.0001$), Size ($F = 41.776$, $d.f._{num} = 11$, $d.f._{den} = 336$, $P < 0.0001$), and for two interaction terms: Population x Sex [Lineage] ($\lambda_{wilks} = 0.154$, $d.f._{num} = 495$, $d.f._{den} = 3631.4$, $P < 0.0001$), and Population x Size [Lineage] ($\lambda_{wilks} = 0.074$, $d.f._{num} = 495$, $d.f._{error} = 3631.4$, $P < 0.0001$). Of these, the only effects with a partial eta square (η_p^2) value above 0.2 were

Table IV.2: Results of multivariate analyses of overall body shape in *Ligia* isopods. Significant effects with a η_p^2 value > 0.2 are indicated in bold.

	F	$d.f._{num}$	$d.f._{den}$	*P*	η_p^2
Population [Lineage]	1.9977	242	3347.6	<0.0001	0.126
Lineage	**8.1999**	**77**	**2020.9**	**<0.0001**	**0.238**
Sex	1.2442	11	336	0.2563	0.039
Size	**41.776**	**11**	**336**	**<0.0001**	**0.578**
Population*Sex [Lineage]	1.4006	495	3631.4	<0.0001	0.160
Population*Size [Lineage]	**2.0240**	**495**	**3631.4**	**<0.0001**	**0.216**

Lineage, Size, and the interaction term Population x Size [Lineage]. The main effect of Sex was not significant ($F = 1.244$, $d.f._{num} = 11$, $d.f._{den} = 336$, $P = 0.2563$).

IV.3.2 DFAs

Results of the Box's M test indicate that covariance matrices are heterogeneous across lineages (Box's M: 1123.2, $d.f._{error} = 72611.9$, $P < 0.0001$), suggesting linear DFAs were inappropriate for our dataset. We could not implement quadratic DFAs, however, due to singularities (i.e., possible correlations in Relative Warps 8 to 10) in our data matrix. Therefore, we implemented regularized DFAs. We used Lambda and Gamma values of 0.1 in final analyses, as low values for Lambda and Gamma are recommended when covariances are different, data are abundant, and when variables may be correlated (Inc 2007). Also, these values produced the lowest misclassification rates in preliminary analyses under a variety of Lambda and Gamma combinations. Regularized DFAs of residuals indicated significant differences between Lineages ($\lambda_{wilks} = 0.165$, $d.f._{error} = 2847.8$, $P < 0.0001$). No distinct clusters, however, were seen in canonical plots, which may be explained by the extensive overlap between most lineages in pairwise comparisons (Figure IV.2).

Initially, a correct assignment of individuals to their lineage of origin was achieved in 72.8 % of cases, but dropped to 57.9 % after leave-one out cross validation (LOOCV), which may be explained by the overlap between lineages (Figure IV.2). Per-lineage validated correct classification rates were: 57.89% for *Clade A*; 38.71% for *Clade B*; 44.26% for *Clade C*; 69.70% for *Clade D*; 59.46% for *Clade E*; 44.44% for *Clade F*;

59.12% for *Clade N*; and 64.38% for *Clade S*. Most misclassified individuals were assigned to geographically nearby lineages. A full breakdown of classification results is shown in Table IV.3. We observed similar patterns under different combinations of Lambda and Gamma between 0.1–0.5 (data not shown).

We conducted regularized DFAs (Lambda = 0.1, Gamma = 0.1) by Lineage attempting to assign individuals to the localities of origin. All lineages and localities were used except *Clade F*, as it only consisted of one locality (Puerto Vallarta). All DFAs proved significant (*Clade A*: $\lambda_{wilks} = 0.130$, $d.f._{error} = 71.412$, $P < 0.003$; *Clade B*: $\lambda_{wilks} = 0.20$, $d.f._{error} = 36$, $P < 0.031$; *Clade C*: $\lambda_{wilks} = 0.028$, $d.f._{error} = 240.9$, $P < 0.0001$; *Clade D*: $\lambda_{wilks} = 0.21$, $d.f._{error} = 40$, $P < 0.017$; *Clade E*: $\lambda_{wilks} = 0.067$, $d.f._{error} = 33$, 68.5, $P < 0.0001$; *Clade N*: $\lambda_{wilks} = 0.017$, $d.f._{error} = 975.5$, $P < 0.0001$; *Clade S*: $\lambda_{wilks} = 0.012$, $d.f._{error} = 1128.3$, $P < 0.0001$). The percentage of individuals (initial/LOOCV; respectively) correctly assigned to their locality within each clade are: 100/44.74 for *Clade A*; 100/61.29 for *Clade B*; 98.36/42.62 for *Clade C*; 96.97/54.55 for *Clade D*; 100/24.32 for *Clade E*; 97.08/25.55 for *Clade N*; and 97.26/36.99 for *Clade S*.

IV.3.3 Tests of phylogenetic signal

We did not detect any evidence of phylogenetic structure in all shape variables using both λ and *K* statistics, with the exception of the fifth relative warp (Table IV.4). This last result, however, may represent a false positive, as no obvious differences were observed between lineages upon inspection. Although the statistical power of these tests is maximized when $N > 20$ (Blomberg et al. 2003; Pagel 1999), Pagel's λ values are

Table IV.3: Classification rates for Discriminant Function Analyses of *Ligia* isopods to lineage of origin. Clades of origin are indicated on the first column, while predicted membership rates are indicated in remaining columns. We present the percentage of individuals correctly assigned to their clade of origin for the original model (upper) and LOOCV rates (lower).

	A	*B*	*C*	*D*	*E*	*F*	*N*	*S*
	73.68	2.63	18.42	0.00	2.63	0.00	0.00	2.63
A	**57.89**	5.26	26.32	0.00	5.26	0.00	0.00	5.26
	3.23	**83.87**	3.23	6.45	3.23	0.00	0.00	0.00
B	16.13	**38.71**	29.03	6.45	3.23	0.00	3.23	3.23
	14.75	4.92	**63.93**	1.64	8.20	0.00	6.56	0.00
C	19.67	11.48	**44.26**	1.64	11.48	0.00	8.20	3.28
	0.00	0.00	0.00	**100.00**	0.00	0.00	0.00	0.00
D	0.00	0.00	9.09	**69.70**	6.06	0.00	9.09	6.06
	0.00	2.70	5.41	2.70	**86.49**	0.00	0.00	2.70
E	0.00	5.41	13.51	2.70	**59.46**	0.00	8.11	10.81
	0.00	0.00	0.00	0.00	0.00	**100.00**	0.00	0.00
F	0.00	0.00	0.00	0.00	11.11	**44.44**	0.00	44.44
	2.92	2.19	0.00	4.38	9.49	0.00	**64.96**	16.06
N	2.92	2.19	2.92	4.38	9.49	0.00	**59.12**	18.98
	2.05	5.48	1.37	4.79	8.90	2.05	5.48	**69.86**
S	2.74	6.16	1.37	5.48	9.59	2.05	8.22	**64.38**

Table IV.4: Results of analyses of phylogenetic signal for shape variables (i.e. relative warps) included in multivariate analyses of shape.

	Blomberg's *K*	P^A	Pagel's λ	M.L. (lnl)^B	$P = 0^C$	$P = 1^D$
RW1	0.335	0.116	1.0E-07	21.7	1.000	0.069
RW2	0.067	0.491	2.0E-05	24.8	1.000	0.000
RW3	0.316	0.093	1.0E-07	24.8	1.000	0.064
RW4	0.016	0.948	4.5E-05	25.2	1.000	0.000
RW5	0.825	0.062	1.0E+00	29.6	0.080	1.000
RW6	0.034	0.663	1.1E-05	33.1	1.000	0.000
RW7	0.022	0.799	1.7E-06	34.4	1.000	0.000
RW8	0.012	0.975	8.0E-02	35.5	0.862	0.000
RW9	0.009	0.982	7.0E-06	35.2	1.000	0.000
RW10	0.010	0.938	7.9E-06	32.8	1.000	0.000
RW11	0.007	0.985	4.8E-06	36.0	1.000	0.000

[A]: p-value for observed Blomberg's *K* value based on 10,000 randomizations
[B]: Likelihood of observed Pagel's λ value
[C, D]: probability observed λ diverges from a null (BM = 0) and fully-brownian model (BM = 1)

robust to the number of taxa included, whereas Blomberg's *K* values decrease as additional taxa are included (Münkemüller et al. 2012). Exploratory analyses incorporating guide-trees with major clades subdivided into component lineages produced results consistent with these expectations and concordant with the results presented herein. We do not present these results, as the statistical significance of *K* values cannot be inferred using unresolved guide trees (Kembel et al. 2010). K2P genetic distances ranged from 0.00–28.5% (mean = 20.2%, median = 21.6%). Euclidean distances in the morphological dataset ranged from 0.008–0.09 ($\mu = 0.034$, Median = 0.033). Regression of pairwise morphological distances against pairwise K2P genetic distances (Figure IV.4) was significant ($F = 79.0491$, $d.f._{error} = 1375$, $P < 0.0001$); however, the R^2 value suggested a poor fit between the data and the model ($R^2 = 0.054$). Thus, the significant correlation appears to be a false positive. Mantel tests are known to be afflicted by high type-I error rates (Lapointe and Legendre 1995; Nunn et al. 2006; Oberrath and Bohning-Gaese 2001), and their use in phylogenetic comparative analyses has been discouraged (Harmon and Glor 2010). Nonetheless, we incorporated Mantel tests to determine whether the combination of all shape variables produced patterns different than those seen by evaluating shape variables independently.

IV.3.4 Visualization of shape variation in Ligia

We present thin-plate-spline transformations for all major clades at 10*X of the normal range for ease of comparison (Figure IV.5). Because such magnifications have as much to do with statistical power as well as with the magnitude of effects, we selected

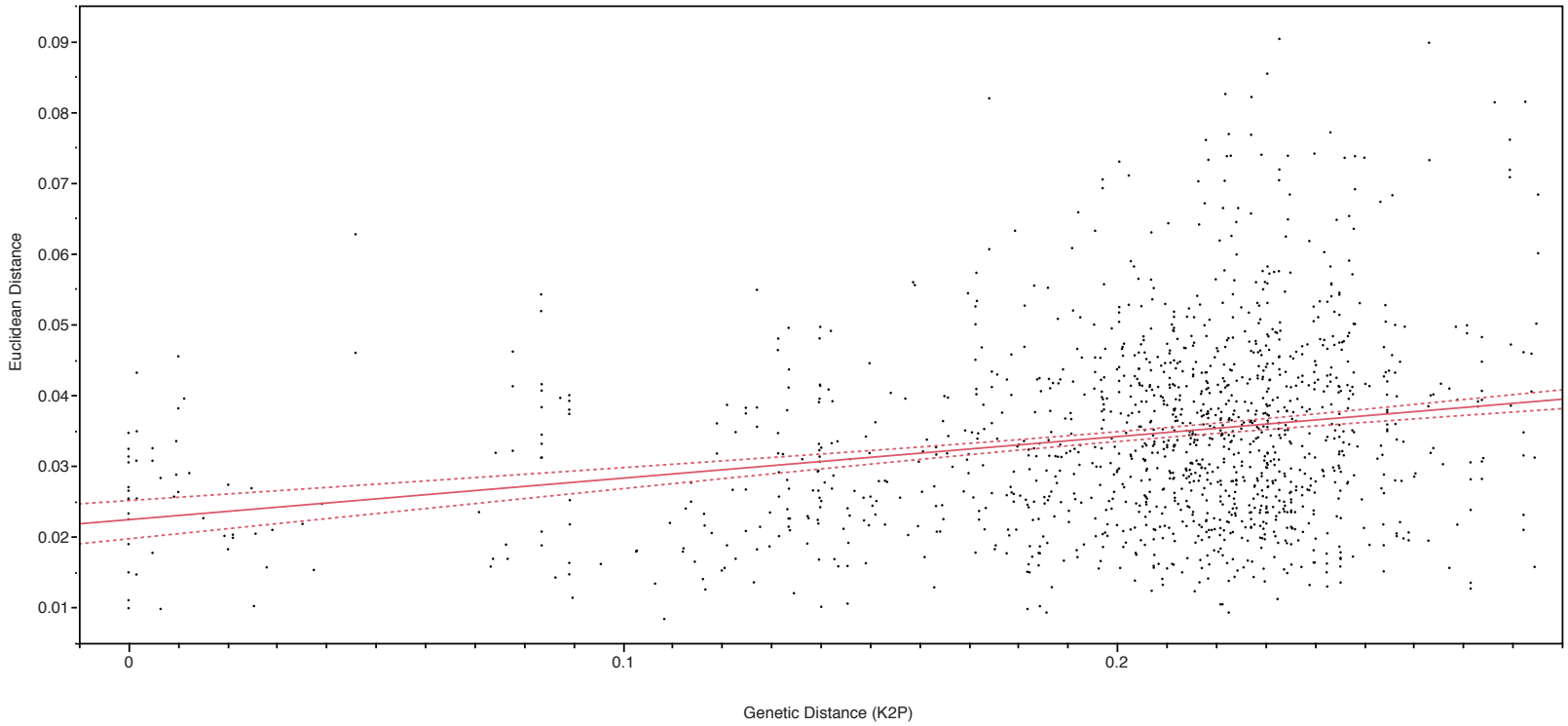

Figure IV.4: Correlation between pairwise genetic and Euclidean distances. The solid line indicates best-fit line and dotted line indicates confidence limits at 95%.

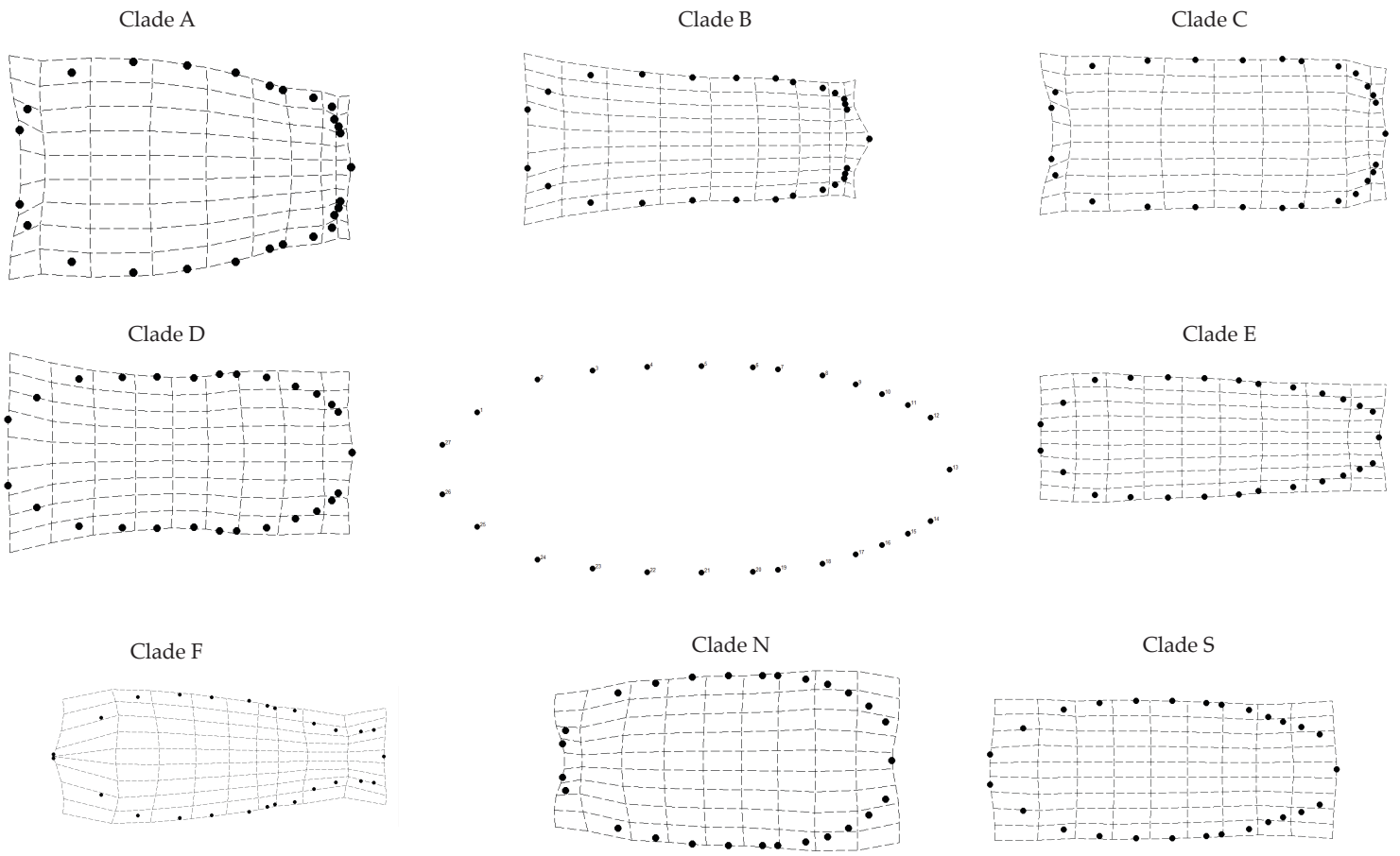

Figure IV.5: Thin-plate-spline transformations of LM positions for each *Ligia* lineage. Transformations are shown at 10*X natural range to aid visualization.

individuals from each clade with the highest canonical score, and provide those images as supplemental material (Supplementary Figure S.IV.1). Between-clade differences appear most pronounced in the cephalon, pleotelson, and midbody regions. *Ligia* individuals in *Clade A* exhibit an enlarged cephalon with small eyes. Their pleotelson is compressed, with the lateral posterior and the distal most point almost parallel. *Clade C* has a pleotelson similar to that of *Clade A*; however, a somewhat rectangular body shape and small eyes with no enlarged cephalon may distinguish individuals from *Clade C*. Individuals in *Clade B* are characterized by an oval body-shape with a normal sized cephalon and medium-sized eyes. As in *Clade A*, the pleotelson is compressed; however, the distal-most point protrudes well beyond the lateral posterior points. *Clade D* exhibits a slight invagination in the midbody region and medium-sized eyes on a regular cephalon. Their pleotelson appears less compressed than other clades, with the exception of *Clade E*. Although exhibiting a similar pleotelson, *Clade E* has no midbody invagination. Also, individuals from this clade exhibit medium sized eyes with a slightly larger cephalon than the rest of the body (e.g. the body tapers posteriorly). *Clade S* exhibits a body shape similar to those in *Clade E*; however, the body does not appear to taper, and the distal point of the pleotelson appears to protrude more extensively than in *Clade E*. *Clade F* specimens have very large eyes, with a drastic invagination in the segments prior to the pleotelson. *Clade N* has small eyes, with an oval body shape and a non-compressed pleotelson. It also exhibits a large 1st segment.

We also present thin-plate-spline transformations at 3*X of the normal range for the largest and smallest individuals for both the overall dataset (i.e., Size effect), and for

each lineage (i.e., Lineage*Size) (Figure IV.6). In general, larger individuals exhibit a broader body and smaller eyes (relative to the total body size), with a distal point of the pleotelson that is slightly more protruding. All lineages appear to exhibit similar patterns, differing mostly in the magnitude of the effect. Individuals in clades *A*, *C*, *D*, and *E* exhibit the most obvious allometric effects (Figure IV.6). Much subtler differences are observed between the large and small individuals in clades *B*, *N*, and *S* (Figure IV.6).

IV.4 Discussion

IV.4.1. Body shape variation among lineages

Landmark-based geometric-morphometrics have been employed successfully to detect morphological differences between otherwise cryptic species in several invertebrate taxa (Carvajal-Rodríguez et al. 2006; Francuski et al. 2009; Milankov et al. 2009; Mitrovski-Bogdanovic et al. 2013), including crustaceans (Bertocchi et al. 2008; Zuykova et al. 2012). We used these methods to investigate whether differences in body shape occur among highly divergent lineages of *L. occidentalis s. l.*, as well as among populations within these lineages. MANCOVA detected a statistically significant effect of lineage, size, and the interaction population x size [lineage] on body shape with high values of partial eta square (η_p^2). DFAs also found significant differences among lineages and among populations within lineages. Large overlap in overall body shape, however, occurs among lineages, as evidenced by canonical plots (Figure IV.2) and the low classification rates of cross-validated DFAs. Differences among lineages in general

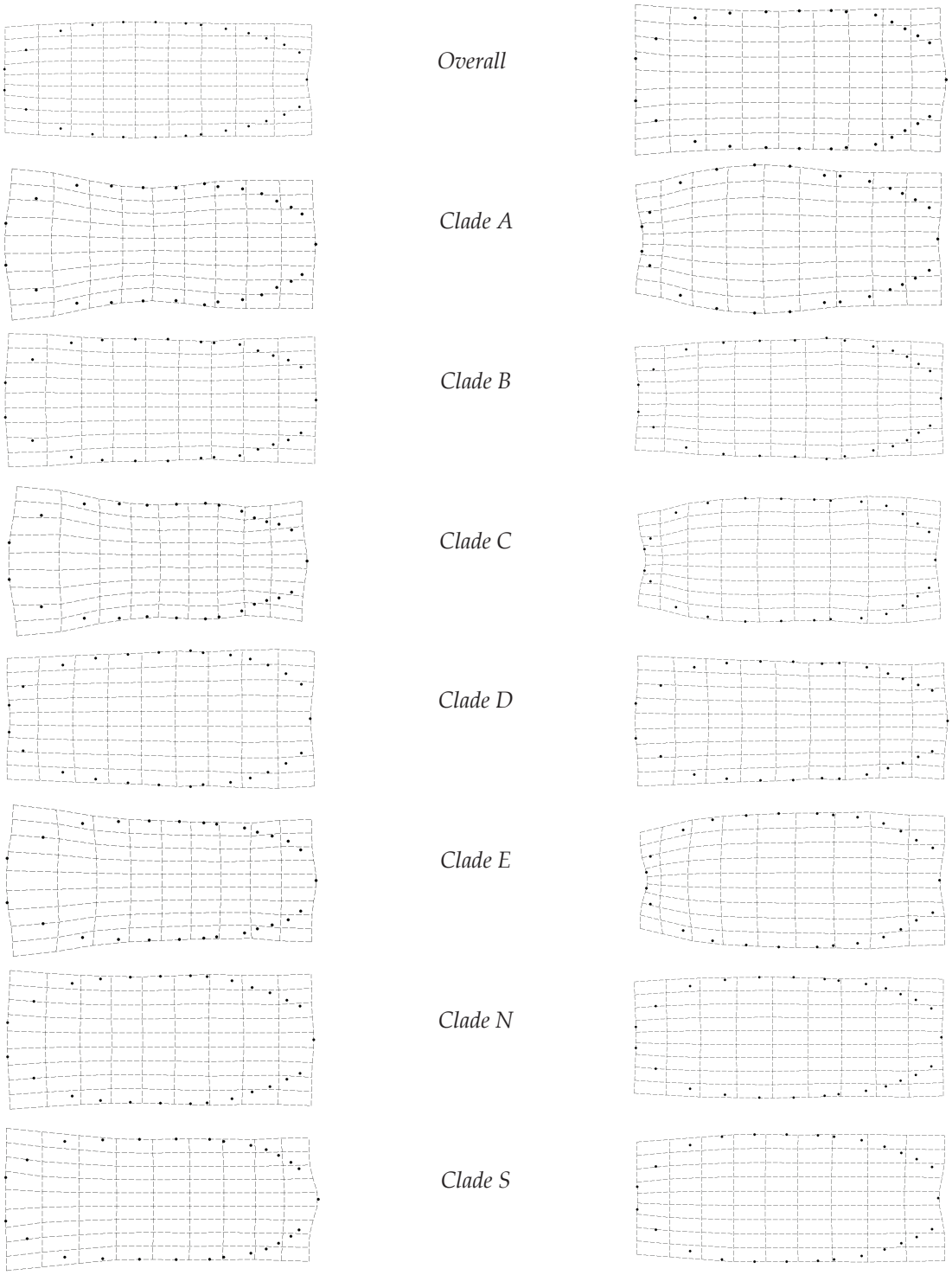

Figure IV.6: Thin-plate-spline transformations of LM positions for *Ligia* size minima (left) and maxima (right) for the overall dataset, and for each lineage. *Clade F* is not presented due to small sample sizes. Transformations are shown at 3*X the natural range to aid visualization.

body shape were observed in thin-plate-spline transformations at great magnification (i.e., 10*X). Allometric effects were observed in general, but are more pronounced in some clades. Larger individuals tended to exhibit a broader body and smaller eyes relative to the total body size, with a distal point of the pleotelson that is slightly more protruding. Finally, genetic and morphological distances are poorly correlated and tests of phylogenetic signal failed to detect an association between phylogenetic relatedness and body shape.

Despite finding significant differences in body shape among highly divergent lineages of *L. occidentalis s.l.*, and even among populations within lineages, body shape cannot be used as a diagnostic character for taxonomic purposes. Correct classification rates of cross-validated DFAs were low (overall = 57.9%), with correct assignments for most clades below 60% and as low as 39% for *Clade B*. Correct classification rates before cross-validation were much higher (overall = 72.8%), being 100% for two clades (*D* and *F*), indicating the model is very unstable and sensitive to the exclusion of just one individual. The probable explanation for the low rates of correct classification in the validated DFAs is the large overlap observed in body shape among clades, as evidenced in the canonical plots (Figure IV.2).

Similarities in body shape among lineages may be related to phylogenetic relatedness, geographic proximity (which may imply exposure to more similar environmental/ecological conditions), and/or stochasticity. We note, however, that phylogenetic relatedness and geographic proximity are highly confounded. According to Hurtado et al. (2010) and unpublished results from Hurtado et al., *Clade A* is sister to

Clade BCDE, with the relationships within this last group being (*B* (*E* (*C D*))); indicating adjacent distributions between phylogenetically closer lineages. Similarly, *Clade N* is sister to *Clade S*, and both occupy adjacent distributions in the Gulf of California; the former in the northern Gulf, whereas the latter in the southern Gulf. *Clade F* is a highly divergent lineage, whose distribution overlaps with that of *Clade S*. The relationships amongst the highly divergent *ABCDE*, *NS*, and *F* clades are uncertain.

Even though phylogenetic relatedness and geographic proximity are confounded, data on the clades to which misclassified individuals were assigned (Table IV.3) suggest that geographic proximity influences the degree of body shape similarity among clades. Individuals from geographically adjacent clades appear to have more similar body shapes. For example, the majority of the misclassified individuals from *Clade A* were placed in *Clade C*. Similarly, the majority of the misclassified individuals from *Clade C* were placed in *Clade A*. Most of the localities sampled from clades *A* and *C* were in the Northern Channel Islands. Therefore, geographic proximity appears to be more relevant for the misclassification of individuals from clades *A* and *C*. Similar situations are observed for misclassified individuals of the other clades with the exception of *Clade E*. A noteworthy case is that of the misclassified individuals of *Clade F*, which were classified as members of *Clade S* in equal proportion to the correctly assigned individuals (i.e., 44.4%). Clades *F* and *S* are phylogenetically distant, but the localities of *Clade F* examined are nested within those of *Clade S*. The pair-wise canonical plots show a remarkable separation between *Clade F* and all other clades, except *Clade S*, with which it exhibits complete overlap (Figure IV.2). Another example is that of *Clade*

S, for which most misclassified individuals were assigned to geographically nearby clades *N* and *E*. Whereas clades *N* and *S* are sister lineages, *Clade E* is distantly related. Misclassification of individuals in *Clade E* appears to be more stochastic, with few individuals being incorrectly assigned to the geographically adjacent *Clade D*. The lack of signal from phylogeny and genetic distance on body shape variation is consistent with the apparent effect of geographical proximity described above.

The somewhat constrained evolution of body shape in *L. occidentalis s. l.*, despite its apparently long evolutionary history, may be explained by one or more of the following mechanisms: (a) strong stabilizing selection (Charlesworth et al. 1982; Simpson 1953; Wiens and Graham 2005); (b) strong functional constraints (i.e., a range of phenotypes having roughly equivalent fitness, but any phenotypes outside the bounds having zero fitness) (Schlichting and Pigliucci 1998); and (c) intrinsic genetic and developmental constraints, which can result from correlations among traits (Erwin 2007; Schlichting and Pigliucci 1998). Strong stabilizing selection and strong functional constraints on body shape could be exerted by the harsh supralittoral environment and/or by the fact that *L. occidentalis s. l.*, and all coastal *Ligia* spp., are restricted to rocky habitats. Due to its effect on locomotor function, substrate type is a critical determinant of morphology (Goodman 2008; Losos et al. 1997; Vervust et al. 2007). Nevertheless, restriction to rocky substrate has not prevented greater body shape differentiation of more genetically divergent lineages of coastal *Ligia*. For example, canonical plots show greater separation between *L. hawaiensis* and *L. occidentalis s. l.* (Supplementary Figure S.IV.2) than among lineages of *L. occidentalis s. l.* Similarly, although not examined with

geometric morphometrics, *L. occidentalis s. l.* is clearly morphologically distinct on the basis of distance between the eyes and the shape of the caudal peduncle of the uropod from its highly genetically divergent relative *L. pallasi*, with which it overlaps in northern California and southern Oregon (Eberl 2012). The lack of strong morphological divergence within *L. occidentalis s. l.* also suggests that directional selection related to sexual selection via visual mating signals does not operate for body shape in this isopod, which might be expected given its predominantly nocturnal activity (Hurtado, personal observation).

The apparent influence of geographic proximity on shape similarity suggests however, that the environment might impose at least some weak directional selection on shape variation. For example, the similarity between clades *A* and *C*, most of which were sampled on the Northern California Channel Islands, despite the marked differences in SST among insular localities occupied the two clades (Eberl et al. 2013), suggests that insular ecological factors may be relevant to body shape (e.g. different or fewer terrestrial predators may be present in the islands). Determination of the influence of extrinsic and intrinsic factors on body shape variation in *L. occidentalis s. l.* will require studies of ecological parameters, as well as of the genetic architecture of body shape, including an assessment of phenotypic plasticity (Schlichting and Pigliucci 1998; Wake 1991).

IV.4.2 Allometric effects

We also detected allometric effects on the overall body shape of *Ligia* from the study

area, with η_p^2 values (Table IV.2) suggesting body size to be the strongest determinant on overall body shape. In general, larger *Ligia* individuals exhibit a disproportionately wider body than smaller individuals, a pattern reported for *L. pallasii* (Carefoot 1973) and *L. hawaiensis* (Santamaria et al., in preparation). Thin-plate-spline visualizations, however, suggest the widening of the body is not uniform across lineages. Either developmental (Stern and Emlen 1999) or ecological differences (Pfennig 1992) may be responsible for the differences in the magnitude of this effect observed in *Ligia* from the study area. Three of the lineages (*C*, *D*, *E*) exhibiting the deepest widening of the body form a well supported monophyletic clade (Hurtado et al. 2010), with those exhibiting no obvious allometric effects (*N* and *S*) also forming a monophyletic group (Hurtado et al. 2010). These patterns may be indicative of the shared evolutionary history of these lineages, and may be due to shared developmental constrains. On the other hand, environmental factors may be at play. Growth rates of isopods are known to be affected by environmental factors such as temperature (Donker et al. 1998; Holdich and Tolba 1981; Strong and Daborn 1980), food availability (Reichle 1968), and exposure to pollutants (Donker et al. 1993). In general, lineages in the colder Pacific Ocean (A, C, D, E) exhibited obvious allometric effects, whereas those in the warmer Gulf of California (*N*, *S*) did not. Furthermore, we observed some obvious allometric effects in some *Clade N* localities (N7, N9, N12), suggesting that differences in allometric effects on body shape may also correspond to ecological factors and not phylogenetic trajectory. As the distribution of *Ligia* lineages closely matches changes in sea surface temperatures (Eberl et al. 2013), additional work is needed to establish the contributions of ecologic

differences and phylogenetic relatedness on the observed differences in the magnitude of allometric changes.

CHAPTER V

SUMMARY

In this Dissertation, we applied phylogenetic and morphological approaches to study different evolutionary aspects of *Ligia* isopods across three highly dynamic geological regions. Biological characteristics of these isopods severely restrict their dispersal potential, which leads to isolation of populations and high levels of allopatric genetic differentiation. Consistently, phylogeographic studies of *Ligia* in different parts of the world have revealed high levels of allopatric genetic differentiation. Phylogeographic patterns have also shed light on factors that have been important in shaping regional evolutionary histories. In this study, we examined phylogeographic patterns of *Ligia* in the region comprised by the Caribbean and the Pacific coast of Central America and Colombia (Chapter II). We also studied phylogeographic patterns of *Ligia* in the Hawaiian Archipelago (Chapter III) and conducted geometric-morphometric analyses in some supralittoral lineages to test for body shape differences among them. Finally, we also applied these geometric-morphometric analyses to test for within and among body shape differences in highly genetically divergent lineages found in the region between central California and central Mexico, including the Gulf of California (Chapter IV). Our aim is to obtain a better understanding on the evolutionary history of *Ligia* isopods in these three geologically dynamic regions.

In Chapter II, we found that *Ligia* populations from the Caribbean Sea, Bermuda, Florida, Bahamas, and the Pacific coast of Central America and Colombia, belong to a

well-supported and highly differentiated clade within *Ligia*. Highly divergent lineages are observed within this clade, suggesting it corresponds to a cryptic species complex. Genetic characterization and examination of the external morphology of the appendix masculina of *Ligia* specimens from the type locality of *Ligia baudiniana*, the only currently valid *Ligia* species endemic to the Caribbean region, indicates that these individuals correspond to *L. exotica*. This is a species that has been introduced to artificial littoral habitats around the world and that is highly genetically divergent from the *Ligia* clade found in the study area. The specimens from the study area have a very distinct external morphology of the appendix masculina, which allows distinction from other members of *Ligia*, but not from specimens that have been assigned to *L. baudiniana* from Florida and Bermuda. The phylogeographic patterns of *Ligia* in the Caribbean Sea indicate that passive overwater dispersal has played an important role in the evolution of these isopods in this region. Some localized vicariant events, however, may have occurred as well. Observed phylogeographic patterns do not correspond with suggested biogeographic patterns based on contemporary population connectivity of marine organisms via larval dispersal. They also do not indicate a colonization pattern from the southeast to the northwest that has been hypothesized for the colonization of the Caribbean from South America by terrestrial animals. Phylogeographic patterns of *Ligia* in the Caribbean appear to reflect heterogeneity of past current regimes and stochasticity.

In Chapter III, we expanded previous phylogeographic work on *Ligia* that focused on Kaua‘i and O‘ahu, by incorporating populations from other main Hawaiian Islands,

increasing the number of gene markers, which include nuclear genes, and applying more current phylogenetic approaches. Our results revealed new lineages and expanded our knowledge on the geographic range of previously reported lineages. The phylogeographic patterns of *Ligia* in the Hawaiian Archipelago suggest a complex evolutionary history for these isopods in this region, and were inconsistent with simple evolutionary models proposed for the Hawaiian Islands, such as the progression rule. Some phylogeographic patterns, however, appear congruent with the geological history of the main Hawaiian Islands. Kaua‘i, the oldest of the main Hawaiian Islands, harbors only one endemic supralittoral lineage, which is highly divergent from the other lineages. This is consistent with the older geological history of this island and its high degree of isolation. In contrast, sharing of highly divergent lineages is observed among the other main Hawaiian Islands, suggesting inter-island dispersal among these islands. This may have been facilitated by previous land connections between the Maui Nui Islands, and between them and O‘ahu. Dispersal of *Ligia* has also occurred through the oceanic channel separating Maui and Hawai‘i. Geometric morphometric approaches detected significant differences in overall body-shape among highly genetically divergent supralittoral lineages. Evolution of a terrestrial lifestyle in *L. perkinsi* from Kaua‘i and O‘ahu appears to have occurred early during the diversification of the Hawaiian *Ligia*, but it is uncertain whether it occurred independently in each island or evolved once.

In Chapter IV, we used geometric morphometric approaches to test for body shape differences within and among highly divergent genetic lineages of *Ligia* from Central

California and Central Mexico, including the Gulf of California. Despite finding significant differences among these lineages, body shape appears to be a poor diagnostic character for taxonomic purposes. Low correct classification rates were obtained in cross-validated DFAs and large overlap in body shape is observed among clades. Similarities in body shape among lineages may be related to phylogenetic relatedness, geographic proximity (which may imply exposure to more similar environmental and/or ecological conditions), and/or stochasticity. Although phylogenetic relatedness and geographic proximity are highly confounded, some patterns suggest geographic proximity, and not phylogenetic relatedness, influences the degree of body shape similarity among some lineages. Our results also suggest that the evolution of body shape in *Ligia* isopods of this region is somewhat constrained. Given the deep divergences among lineages, such constrained evolution does not appear to be the result of limited time since divergence. Instead, it may be explained by one or more of the following mechanisms: (a) strong stabilizing selection; (b) strong functional constraints (i.e., a range of phenotypes having roughly equivalent fitness, but any phenotypes outside the bounds having zero fitness); and (c) intrinsic genetic and developmental constraints, which can result from correlations among traits.

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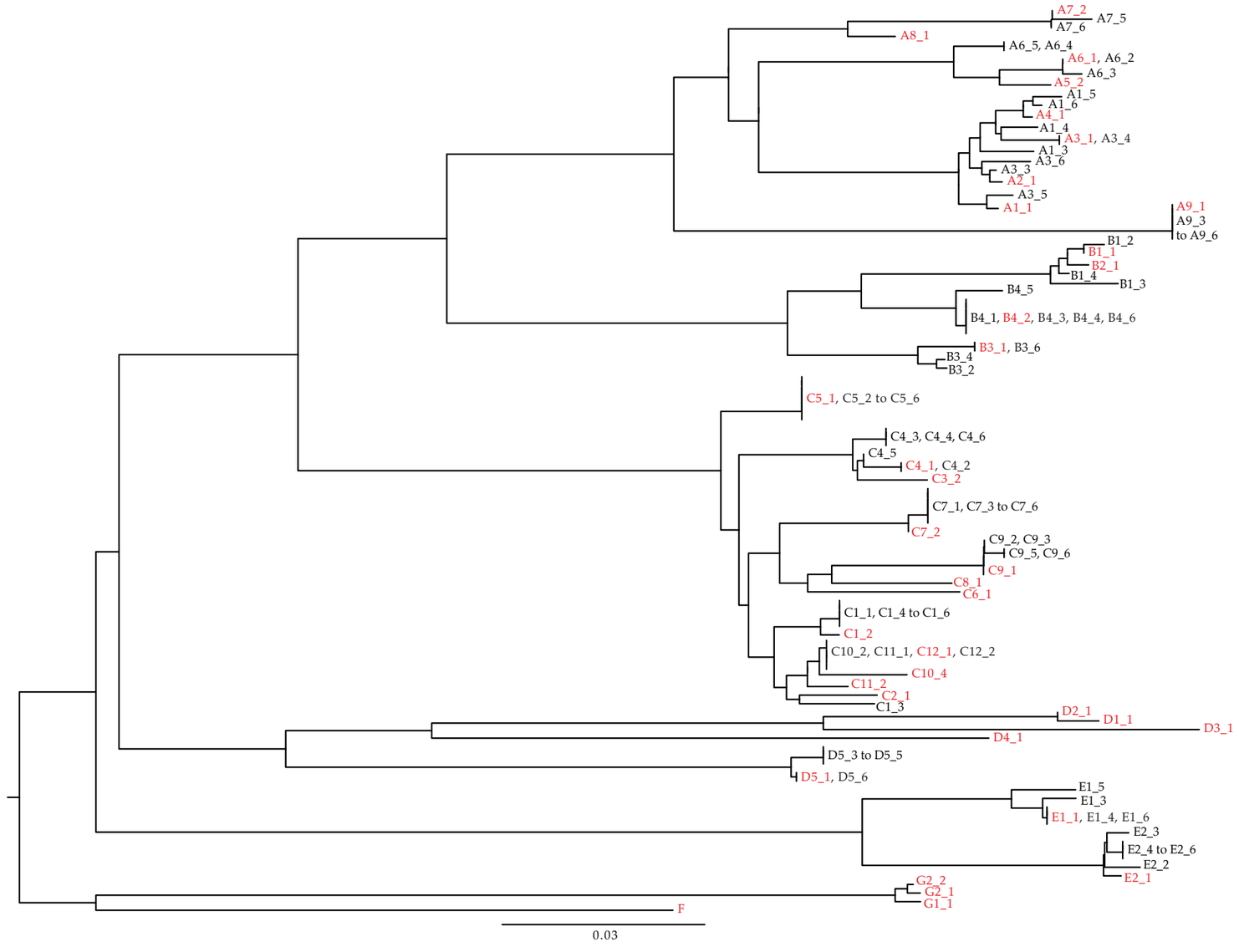
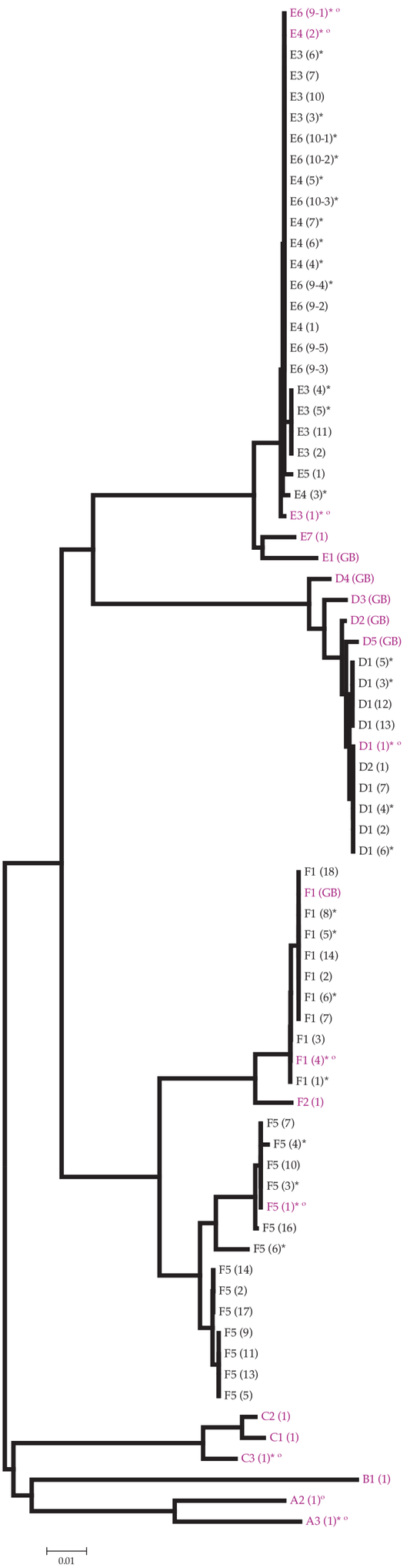
APPENDIX A

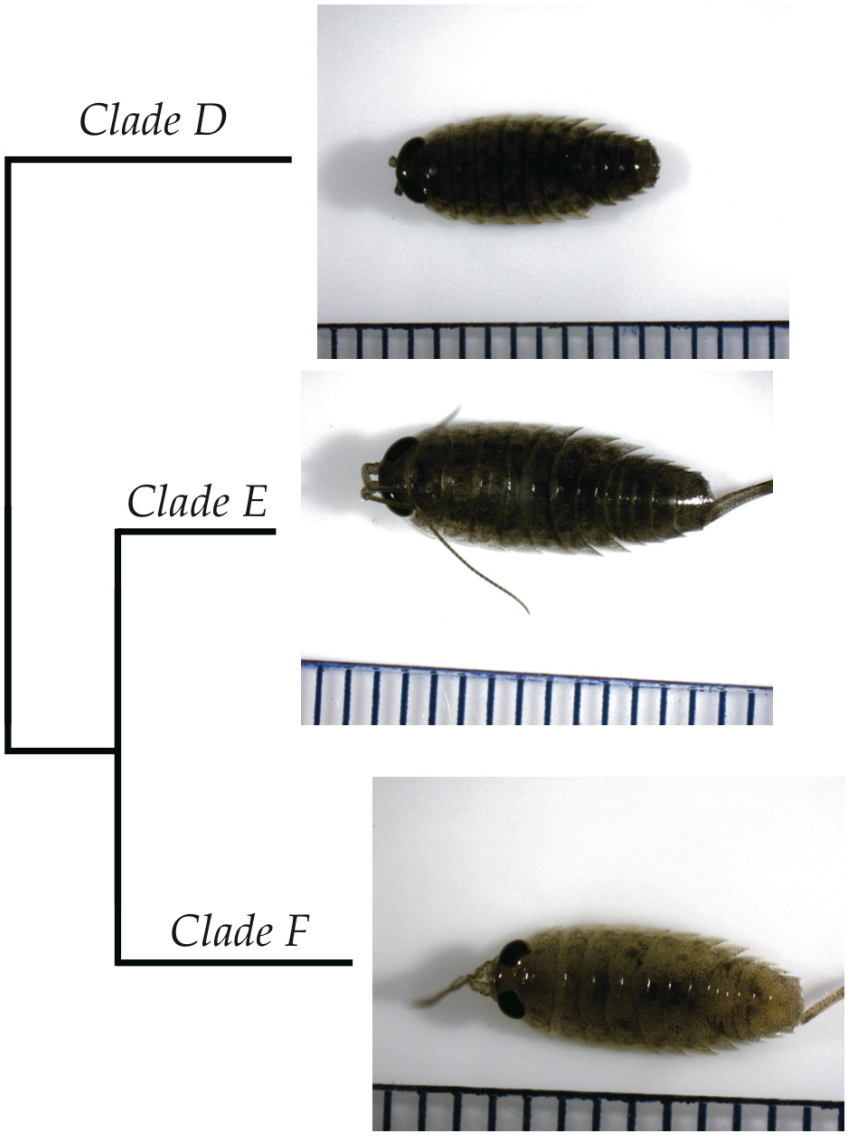
SUPPLEMENTARY MATERIAL



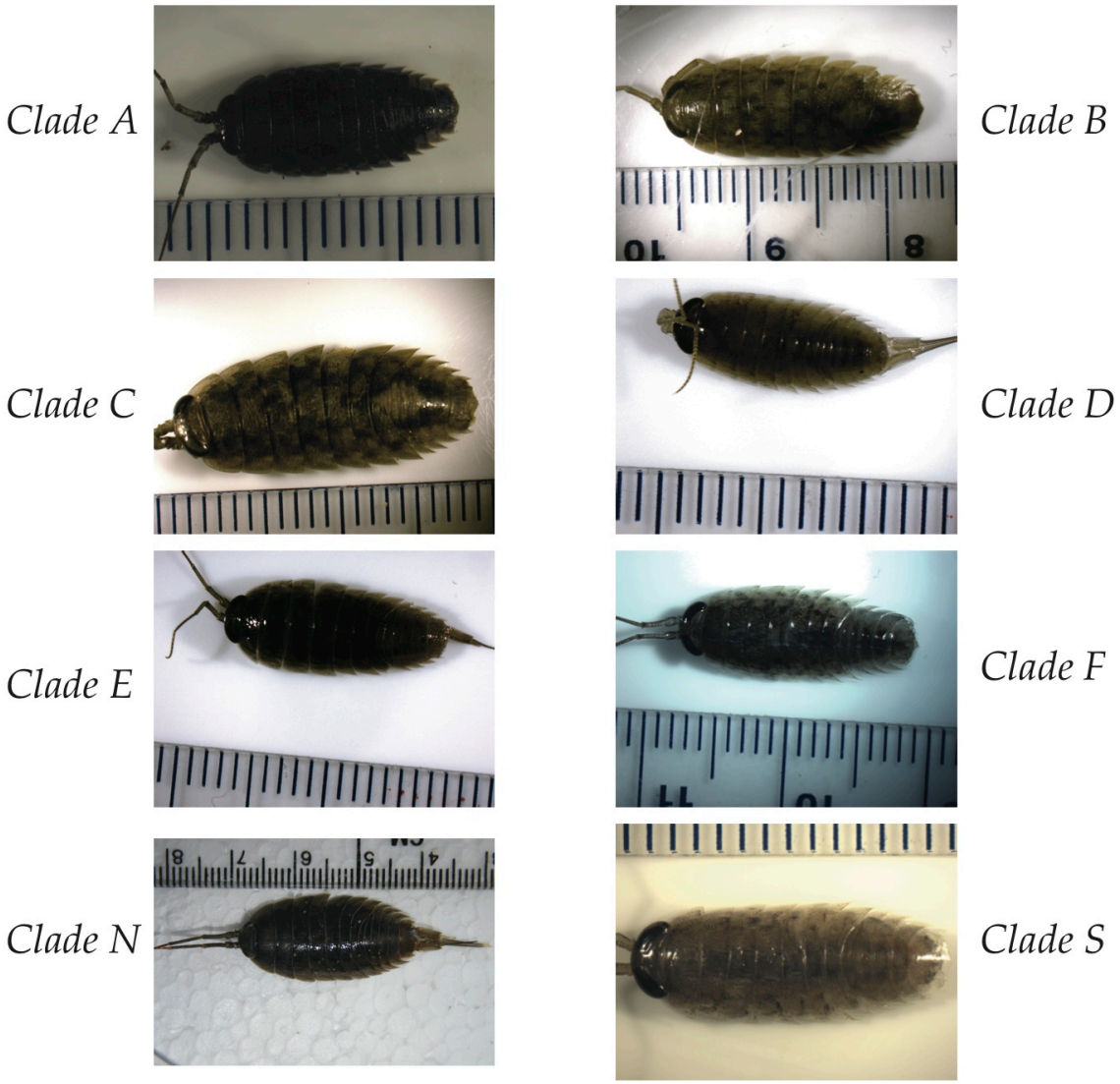
Supplementary Figure S.II.I: Neighbor joining tree of Cyt-b all individuals sequenced from the Caribbean study area. Individuals in red indicate those used in mitochondrial phylogenetic reconstructions.



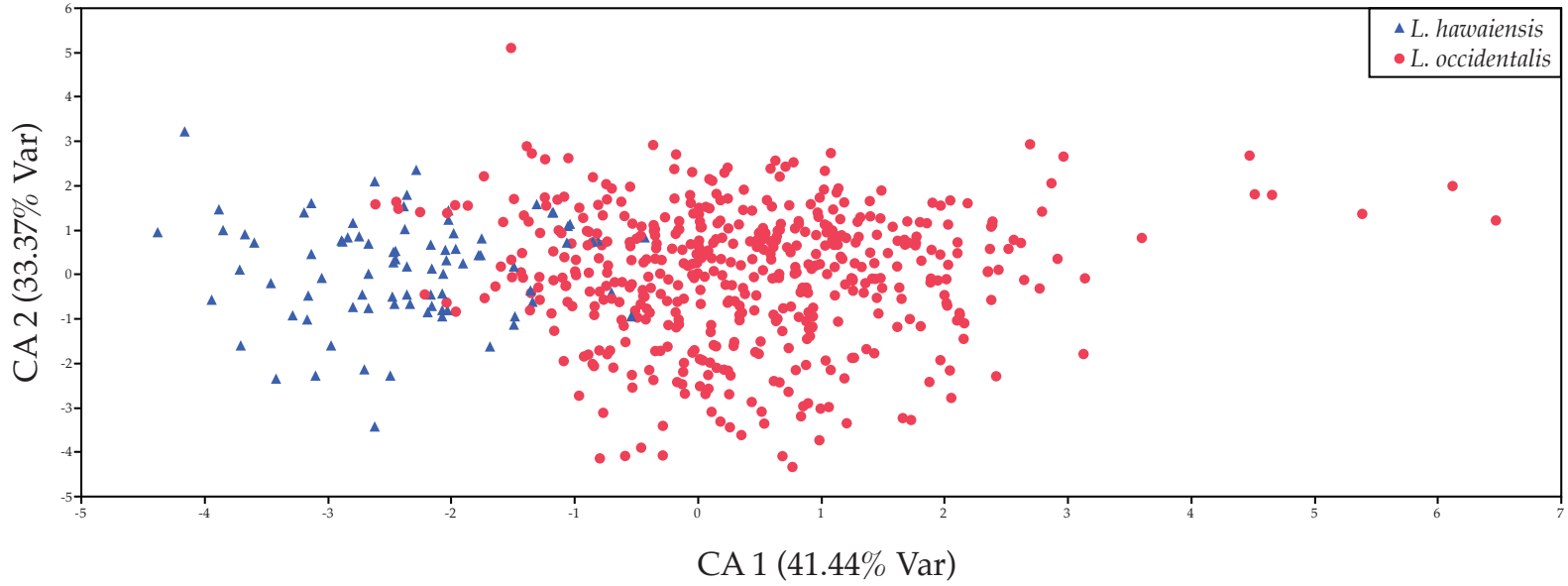
Supplementary Figure S.II.1: Neighbor joining tree of COI all individuals sequenced from the Hawaiian study area. Individuals in color indicate those used in mitochondrial phylogenetic analyses. *: Individuals for which the 28S rDNA nuclear gene was sequenced. °: Indicates those individuals for which the NaK nuclear gene was sequenced.



Supplementary Figure S.III.2: Individuals with the highest canonical score for each *L. hawaiensis* lineage.



Supplementary Figure S.IV.I: Individuals with the highest canonical score for each *L. occidentalis s. l.* lineage.



Supplementary Figure S.IV.2: Canonical plots of morphological comparisons of *L. hawaiensis* (blue triangles) and *L. occidentalis s. l.* (red circles) lineages.

Supplementary Table S.II.1: Estimates of evolutionary divergence, as measured by Kimura 2-parameter distances, for populations within *Clade A*. Minima and maxima are given for all pairwise comparisons. Above matrix: COI gene distances. Lower matrix: 16S rDNA gene distances. Diagonals: within-clade divergence (upper values: COI; lower values: 16S rDNA).

	Portobelo, Panama (A)	Portobelo, Panama (B)	Portobelo, Panama (C)	Fort Sherman, Panama	Yaguanabo, Cuba	Playa Ancon, Cuba	Condado Beach, P.R.	Marigot, St. Martin	Boca Chica, Dom. Rep.
Portobelo, Panama (A)	-----	0.22	0.67	0.45	6.14	7.16	N/A	N/A	3.69
Portobelo, Panama (B)	0.7	-----	0.9	0.22	6.39	7.41	N/A	N/A	3.93
Portobelo, Panama (C)	0.35	0.35	-----	1.12	6.88	7.91	N/A	N/A	4.16
Fort Sherman, Panama	0.35	0.35	0	-----	6.14	7.16	N/A	N/A	3.69
Yaguanabo, Cuba	1.05	1.05	0.7	0.7	-----	1.81	N/A	N/A	5.63
Playa Ancon, Cuba	0.7	1.4	1.05	1.05	0.35	-----	N/A	N/A	7.14
Condado Beach, P.R.	1.4	1.4	1.05	1.05	1.05	1.4	-----	N/A	N/A
Marigot, St. Martin	1.4	1.4	1.05	1.05	1.05	1.4	0	-----	N/A
Boca Chica, Dom. Rep.	1.05	1.05	0.7	0.7	0.7	1.05	1.05	1.05	-----

Supplementary Table S.II.2: Estimates of evolutionary divergence, as measured by Kimura 2-parameter distances, for populations within *Clade B*. Minima and maxima are given for all pairwise comparisons. Above matrix: COI gene distances. Lower matrix: 16S rDNA gene distances. Diagonals: within-clade divergence (upper values: COI; lower values: 16S rDNA).

	Playa Bonita, Costa Rica	Piuta, Costa Rica	Santa Marta, Colombia	Maracas Bay, Trinidad and Tobago
Playa Bonita, Costa Rica	-----	0.22	4.88	N/A
Piuta, Costa Rica	0.35	-----	5.12	N/A
Santa Marta, Colombia	1.76	1.4	-----	N/A
Maracas Bay, Trinidad and Tobago	1.05	0.7	1.05	-----

Supplementary Table S.II. 3: Estimates of evolutionary divergence, as measured by Kimura 2-parameter distances, for populations within *Clade C*. Minima and maxima are given for all pairwise comparisons. Above matrix: COI gene distances. Lower matrix: 16S rDNA gene distances. Diagonals: within-clade divergence (upper values: COI; lower values: 16S rDNA).

	Nassau, The Bahamas	Jaws Beach, The Bahamas	Summerland Key, FL.	Indian Key, FL.	Duck Key, FL.	Cozumel, Mexico	Habana, Cuba	Carrie Bow Cay, Belize	Tela, Honduras
Nassau, The Bahamas	-----	4.16	2.5	2.27	N/A	1.35	5.35	N/A	N/A
Jaws Beach, The Bahamas	1.4	-----	3.46	3.22	N/A	3.69	6.62	N/A	N/A
Summerland Key, FL.	1.05	0.35	-----	0.67	N/A	2.04	4.88	N/A	N/A
Indian Key, FL.	1.05	0.35	0	-----	N/A	1.81	5.12	N/A	N/A
Duck Key, FL.	1.05	1.76	1.4	1.4	-----	N/A	N/A	N/A	N/A
Cozumel, Mexico	0.35	1.05	0.7	0.7	0.7	-----	5.11	N/A	N/A
Habana, Cuba	1.76	1.05	0.7	0.7	1.4	1.4	-----	N/A	N/A
Carrie Bow Cay, Belize	1.05	0.35	0	0	1.4	0.7	0.7	-----	N/A
Tela, Honduras	2.11	2.11	1.76	1.76	2.47	1.76	2.47	1.76	-----

Supplementary Table S.II.4: Estimates of evolutionary divergence, as measured by Kimura 2-parameter distances, for populations within *Clade D*. Minima and maxima are given for all pairwise comparisons. Above matrix: COI gene distances. Lower matrix: 16S rDNA gene distances. Diagonals: within-clade divergence (upper values: COI; lower values: 16S rDNA).

	Fajardo, Puerto Rico	East Coast, Aruba	Piscaderabaai, Curaçao	Spaans Lagoen, Aruba	Donkey Beach, Bonaire
Fajardo, Puerto Rico	-----	13.95	15.82	15.52	16.12
East Coast Aruba	3.22	-----	14.55	14.55	15.13
Piscaderabaai Curaçao	3.2	2.11	-----	0.9	9.15
Spaans Lagoen Aruba	3.2	2.11	0	-----	8.89
Donkey Beach, Bonaire	2.48	2.12	1.4	1.4	-----