PATTERNS OF DIVERGENCE OF TERRESTRIAL *LIGIA* ISOPODS IN THE WORLD'S HIGHEST COASTAL MOUNTAIN RANGE

A Thesis

by

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Abstract

Cryptic diversity is the occurrence of two or more morphologically indistinguishable lineages within a species that are evolutionarily distinct. Tropical rainforests and marine habitats are the most species-rich habitat on Earth and might be where cryptic diversity mostly happens. Within arthropods, recognizing cryptic species is more critical. Limited knowledge of cryptic species in arthropods impacts human health, pest management, and studies of coevolution and species interactions.

Ligia species within Arthropoda phylum are halophilic, and their habitat is restricted between the supralittoral area and the waterline of rocky shores all around the world. Worldwide studies on supralittoral *Ligia* species reveal high levels of cryptic diversity within nominal species. However, for the first time, this study showed cryptic diversity in one terrestrial *Ligia* species, which lives in the Sierra Nevada de Santa Marta, Colombia.

We used two mitochondrial and one nuclear gene and by Bayesian and Maximum Likelihood analyses revealed cryptic diversity in *Ligia* SNSM. *Ligia* in the Sierra Nevada de Santa Marta was distributed according to elevation, the first one from 780 to 1,445 MASL, the second one from 1,700 to 1,817 MASL, and the third one from 1,964 to 2,198 MASL. The high cryptic diversity observed in this study for *Ligia* SNSM emphasizes the high level of biodiversity and endemism in this region. It highlights the importance of conservation studies, efforts, and strategies.

CONTRIBUTORS AND FUNDING SOURCES

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CHAPTER I Introduction And Literature Review

Cryptic diversity

Cryptic diversity or hidden diversity is defined as occurrence of two or more morphologically indistinguishable lineages within a species that are evolutionarily distinct. Two or more species are "cryptic" when they are morphologically indistinguishable but classified as a nominal species (Wattier et al., 2020). Speciation is not always accompanied by morphological changes and causes cryptic diversity. Therefore, the number of biological species is greater than the current nominal species which explains why cryptic diversity is taken into consideration in ecology and evolutionary biology (Theodoridis et al., 2019). Tropical rainforests and marine habitats are the most species rich habitat on Earth and might be the habitats that cryptic diversity mostly happens. Many Studies depicted cryptic diversity within fungi, frogs and arthropods. Recognizing cryptic species is important for conservation planning, bioprospecting, biological control and the treatment of diseases and snakebites. Within arthropods, recognizing cryptic species is more critical. Limited knowledge of cryptic species in arthropods impact human health, pest management and studies of coevolution and species interactions (Bickford et al. 2007). In this study we reveal patterns of cryptic diversity in a terrestrial lineage of *Ligia* isopods.

The Phylum Arthropoda

Arthropoda has the greatest portion of biodiversity among terrestrial species. The earliest record of arthropods on land dates to between Cambrian and Silurian (Cannicci et al., 2020; Cloudsley-Thompson, 1998).

The subphylum Crustacea

Crustacea is one of the Arthropoda groups containing lineages that succeeded in the territorialization process (Broly et al., 2012). This taxon has been categorized into five classes based on their adaptation to terrestrial life: T1 to T5, where T5 refers to fully terrestrial species that can conduct all biological activities on land (Harzsch et al., 2011). Within Crustacea, only five major lineages have achieved some degree of terrestriality: Anomura, Brachyura, Astacoidea, Amphipoda, and Isopoda. Of these, only Amphipoda and Isopoda have some species that are categorized as T5 or fully terrestrial and the other three lineages require aquatic larval stages. (Broly et al., 2012; Harzsch et al., 2011).

The Order Isopoda

The order isopoda contains 11 suborders and more than 10,300 species belonging to more than 500 genera (Dimitriou et al., 2019; Broly et al., 2012; Schmidt, 2008). There are more than 3800 terrestrial species of the order of Isopoda (Campos-Filho et al., 2019; Schmalfuss, 2003). This order has evolved many morphological characteristics and adapted to different environments, including deep sea, freshwater, and terrestrial ecosystems in all climate zones from subpolar regions to deserts. Terrestrial isopods are distributed in different elevations from sea level up to mountains (Krieger et al., 2021; Castello et al., 2020). One of the most essential factors in the distribution of isopods is soil moisture which is affected by geological conditions and precipitation (Warburg, 1993). Also, most isopods are omnivorous and feed on fungi, live or dead plants, or animals (Dean and Hodgson, 2020; Warbug, 1993).

Body Structure

The habitat of the isopods significantly impacts their morphological and behavioral characteristics (Schmalfuss 1984). Isopods have long, arched or flat bodies and have seven pairs of legs (Dean and Hodgson, 2020). All the isopods have cuticle integument, which contains

spherical pigment granules. The isopods' integuments change during their development (Warburg, 1993). In terrestrial isopods, there are two phases of molting. In the first phase, the molting occurs in the posterior half of the body, and after a few days, the second phase happens in the anterior half (Verhoeff, 1940). Some isopods such as *Ligia baudiniana* and *Ligia exotica* have the ability to change their body color when exposed to different backgrounds (Warburg, 1993). The respiratory organs of the marine isopods are gills. However, terrestrial isopods are evolved to develop pleopodal lungs (Wright and Ting, 2006; Unwin, 1931).

The genus *Ligia*

In 1798, Fabricius described the genus *Ligia* as a member of Ligiidae and suborder Oniscidea, which is one of the suborders of Isopoda (Fabricius, 1798). Since 1995, the monophyly of Oniscidae and Ligiidae has been controversial (Zhang et al., 2021). In 2000, based on mtDNA markers, Michel-Salzat and Bouchon stated that *Ligia* is closely related to the suborder of Sphaeromatidea (Dimitriou et al., 2019; Michel-Salzat and Bouchon, 2000). In 2017, based on Bayesian analysis on both protein and non-protein coding genes, it was suggested that *Ligia* is not a well-supported group in the Oniscidea suborder (Lins et al., 2017). In 2019, Dimitriou et al. conducted a study based on two nuclear genes and two ribosomal RNA genes to show relationships among major Oniscidea clades. In their study, genus *Ligia* is paraphyletic and appeared to be more closely related to marine suborder clades. while the phylogenetic pattern of other species of Oniscidea implied a complex history of the transition from the marine environment to land (Dimitriou et al., 2019). The most recent study based on complete mitogenomes suggests that Ligiidae is much more primitive than other terrestrial isopods and should be considered a suborder, parallel to Oniscidea. Furthermore, the genus of *Ligia* is closer to marine suborders, including Valvifera, Cymothoida, and Sphaeromatidae (Zhang et al., 2021).

Currently, 42 species in the genus of *Ligia* are recognized (Santamaria et al., 2013). Most *Ligia* species are halophilic, and their habitat is restricted between the supralittoral area and the waterline of rocky shore worldwide (Hurtado et al., 2010). The supralittoral areas or sea-land interface areas are influenced by both sea and land (Winder et al., 2017). Seal-land interface areas are exposed to harsh conditions such as extreme temperatures, fresh water from the rain and seawater from wave splash and different predators from land and sea birds. These conditions make sea-land interface areas a harsh place to live. However, most *Ligia* species are adapted to live in the supralittoral regions. The supralittoral isopod species (Table 1) are direct developers and they do not have planktonic larval stage. These isopods have limited swimming abilities and avoid entering the water (Hurtado et al., 2014). The supralittoral isopod species exhibit intermediate physiological, morphological, biochemical, and behavioral characteristics between totally marine and terrestrial species (Carefoot & Taylor 1995). Worldwide studies on supralittoral Ligia species reveal high levels of cryptic diversity within nominal species. In 2010, Hurtado et al. conducted a study on supralittoral *Ligia* rocky intertidal shores from central California to central Mexico. Maximum Likelihood and Bayesian phylogenetic analyses on two mitochondrial genes (Cytochrome Oxidase I and 16S ribosomal DNA) represented high levels of allopatric genetic divergence among many localities in *Ligia occidentalis* in this region (Hurtado et al., 2010). In addition, a study conducted in 2013 by Eberl et al. showed high levels of allopatric differentiation among *Ligia Occidentalis* at Point conception related to temperature (Ebrel et al., 2013). Santamarta et al. represented high levels of allopatric divergences among Caribbean supralittoral *Ligia*. *Ligia baudiniana* is the only valid native supralittoral species of *Ligia* in the Caribbean coastal region. This study revealed 17.04−23.97% of genetic divergences between different lineages of *L. baudiniana* in this region (Santamarta et al., 2014).

In this study we revealed patterns of cryptic diversity in a terrestrial lineage of *Ligia* isopods for the first time. Eight strictly terrestrial species of *Ligia* have been recorded (Table 2). They presumably evolved strict terrestriality independently from supralittoral ancestors. The strictly terrestrial species are not dependent on standing water for feeding, movement, and reproduction (Cloudsley-Thompson, 1998; Taiti et al., 2003; Santamaria et al., 2013).

Table 1. List of all recognized coastal terrestrial Ligia species

(Schmalfuss, 2003)

Species Name	Reported by	Distribution
Ligia australiensis	Dana, 1853	Coast of Australia, including Tasmania and Lord Howe Island
Ligia baudiniana	Milne Edwards, 1840	Atlantic and Pacific shores of America from Florida to Brazil and from California to Ecuador, including Galapagos Islands
Ligia cinerascens	Budde-Lund	Japan; Kuril Islands
Ligia curvata	Vandel, 1948	Angola: Lobito
Ligia dentipes	Budde-Lund, 1885	Andaman and Nicobar Islands
Ligia dilatata	Brandt, 1833	Namibia: Lüderitzbucht; South Africa: Cape Peninsula
Ligia dioscorides	Taiti & Ferrara, 2004	Yemen: Socotra Archipelago
Ligia exotica	Roux, 1828	Circumtropical
Ligia ferrarai	Kersmaekers & Verstraeten, 1990	Madagascar: "Songoritelo Barn-Hill"
Ligia filicornis	Budde-Lund, 1893	NW-Venezuela: Puerto Cabello
Ligia glabrata	Brandt, 1833	Namibia: Lüderitzbucht; South Africa: Cape Peninsula
Ligia gracilipes	Budde-Lund, 1885	West coast of Africa from Senegal to northern Angola
Ligia hachijoensis	Nunomura, 1999	Japan: Izu Islands
Ligia hawaiensis	Dana, 1853	Hawaii Islands and Fiji Islands
Ligia italica	Fabricius, 1798	Coasts of Black Sea, Mediterranean Sea, Atlantic in northern Africa down to Cape Verde, Macaronesian Islands

Table 2. List of all recognized coastal terrestrial Ligia species

(Schmalfuss, 2003)

Species Name	Reported by	Distribution
Ligia litigiosa	Wahrberg, 1922	Coasts of Chile and Peru; Juan Fernández Islands
Ligia malleata	Pfeffer, 1889	East Africa: "Bagamoyo"
Ligia miyakensis	Nunomura, 1999	Japan: Izu Islands
Ligia natalensis	Collinge, 1920	South Africa: southeastern coast from Knysna to Natal
Ligia novizealandiae	Dana, 1853	New Zealand and Kermadec Islands
Ligia occidentalis	Dana, 1853	Coasts of California (USA and Mexico)
Ligia oceanica	Linnaeus, 1767	Atlantic coasts of Europe, coasts of western Baltic Sea, and some places at the Atlantic coast of North America (introduced?)
Ligia pallasii	Brandt, 1833	Pacific coast of North America from the Aleutian Islands and Alaska to Santa Cruz in California
Ligia pallida	Jackson, 1938	Polynesia: Christmas Island
Ligia pigmentata	Jackson, 1922	Red Sea, Persian Gulf and coast of Somalia
Ligia rugosa	Jackson, 1938	Southeastern Polynesia
Ligia ryukyuensis	Nunomura, 1983	Japan
Ligia saipanensis	Nunomura, 2001	Micronesia: Saipan Island
Ligia vitiensis	Dana, 1853	Sulawesi; Singapore; New Guinea; Melanesia; Polynesia; Somalia (introduced ?)
Ligia yamanishii	Nunomura, 1990	Japan: Tokyo Prefecture

Table 3. List of all recognized fully terrestrial Ligia species

(Nunomura et al., 2011; Schmalfuss, 1978)

Species Name	Reported by	Distribution
Ligia perkinsi	Dollfus, 1900	Hawaiian Islands
Ligia simoni	Dollfus, 1893	Northern Venezuela and northern Colombia
Ligia platycephala	Van Name, 1925	Venezuela; Guyana; Trinidad
Ligia philoscoides	Jackson, 1938	Southeastern Polynesia
Ligia latissima	Verhoeff, 1926	New Caledonia
Ligia boninensis	Nunomura, 1979	Japan: Bonin Islands
Ligia taiwanensis	Lee, 1994	Taiwan
Ligia torrenticola	Nunomura, 2011	Chichijima and Anijima Islands

Terrestrialization

After the origin of the planet Earth and the multicellularity development, terrestrialization is considered to be the third most significant event in the history of life on Earth. Terrestrialization is explained as the invasion of land habitat by plants and animals (Vecoli et al., 2010; Selden 2001). The terrestrialization processes require some physical and morphological adaptations. These adaptations reduce water dependency and support physicochemical environmental changes. Organisms that colonized the land from the sea habitat experienced remarkable changes in their dispersal and locomotion as well as digestive, reproductive, and respiratory systems. Previous studies suggest that terrestrial organisms modify their water management and osmoregulation (Broly et al., 2012; Little, 2009; Selden, 2001). Evolving new respiratory organs such as lungs were one of the most significant developments in intertidal and terrestrial organisms. Before the evolution of respiratory organs, gills were responsible for respiratory activities, ionic, osmotic, and pH regulation (Cannicci et al., 2020).

The high level of diversity among the terrestrial species shows that terrestrialization happened several times in various phyla of plants and animals during the Paleozoic, i.e., between 540 to 251 million years ago (Cannicci et al., 2020; Broly et al., 2012). However, only a few animal phyla such as the Chordata, the Mollusca, and the Arthropoda were able colonize the land, overcome their dependency on moist habitat to complete their life cycles outside the water, and become fully terrestrial (Lozano-Fernandez et al., 2016; Rafferty, 2010).

The Sierra Nevada de Santa Marta (SNSM)

Colombia is one the richest countries as far as biological aspects are concerned. The Sierra Nevada de Santa Marta (SNSM) is a unique biosphere reserve in Colombia and a well-known habitat for many endemic and migratory species (Duran-Izquierdo and Olivero-Verbel, 2021). This region has extraordinary biodiversity and endemism and is considered one of the most irreplaceable places on Earth for threatened species (Le Saout et al., 2013; IUCN.org, 2013). There are 1800 species of flora, 631 species of birds, 50 species of amphibians, 189 species of mammals, and 92 species of reptiles in SNSM. One hundred thirty-three of these species (about 5%) are endemic or restricted in this region (Duran-Izquierdo and Olivero-Verbel, 2021). SNSM is an isolated triangle-shaped mountain range with a 13,700 sq km area (Tschanz et al., 1974). SNSM is the world's most elevated coastal range, reaching 5,775 m within only 48km from the shore (Le Saout et al., 2013; Reenen and Gradstein, 1983). Wide alluvial plains separate SNSM from the main mountain ranges in Colombia. This region is surrounded by the Caribbean Sea and the plains of the lower Guajira Peninsula from the north, Magdalena River plain from the west, and the valleys of the Rios Cesar and Ranchería from the southeast side (Tschanz et al., 1974). In SNSM, vegetation is varied and dependent on climate and location in different seasons. The north and northwest of SNSM is covered with evergreen forest and has the shortest dry season (January to

March). The western SNSM receives a significant amount of rainfall and is covered with semi-deciduous forest (Adams and Bernard, 1977).

The *Ligia* isopod in SNSM

In 1913, Pearse collected *Ligia* samples between Minca (~800 MASL) to Cuchilla San Lorenzo (>2000 MASL) in SNSM. He described them as *Ligia richardsonae.* Later, in 1952, Vandel synomized *Ligia richardsonae* with *Ligia simoni* i.e., the species first identified in the mountains of Venezuela at 1,200 meters above sea level (Schmalfuss, 2003; Schmalfuss, 1978). Schmalfuss also categorized *Ligia* in SNSM as *Ligia simoni*. *Ligia* SNSM is recorded between 780 m and 2,198 m above sea level (Schmalfuss, 1978). Schmalfuss's hypothesis states that the presence of *Ligia* at high altitudes is attributed to supperalitoral *Ligia* species escaping from a rocky intertidal crab, *Grapsus grapsus,* and inhabited in the mountainous mist forests.

Justification and Hypothesis

The previous studies on coadstal *Ligia* show high levels of allopatric differentiation. In this study we are interested to know if high levels of allopatric differentiation is also observed in this terrestrial lineage given its broad altitudinal distribution and what factors may be the determining differentiation. In addition, we conducted molecular phylogenetic analyses of *Ligia* SNSM with other *Ligia* species, including those in the Caribbean study (Santamaria et al. 2014) to determine whether the SNSM lineage originated within the present-day Caribbean clade or it has an earlier split. Herein, we conducted maximum likelihood and Bayesian phylogenetic analyses to infer the evolutionary history of the *Ligia* lineages in the world's highest coastal mountain range in tropic regions. To understand the evolution pattern of *Ligia* in SNSM we provided information on the number of lineages as well as phylogenetics relationship to find their affinities to other *Ligia* species around the world.

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Sampling

Specimens of terrestrial *Ligia* individuals were collected from Pozo Azul, Minca (780 MASL) to the El Dorado Nature Reserve (2198 MASL) in SNSM, Colombia (Figure 1). Samples were preserved in 95% ethanol and stored at -20°C.

Figure 1. The elevation map of the study area (SNSM, Colombia)

The numbers on the map refer to the samples ID which pin to the point that they were collected. The red arrows indicate the lowest point (Pozo Azul, Minca - 780 MASL) where the sampling was started (Google Earth Pro,v7.3.4, April 03, 2019).
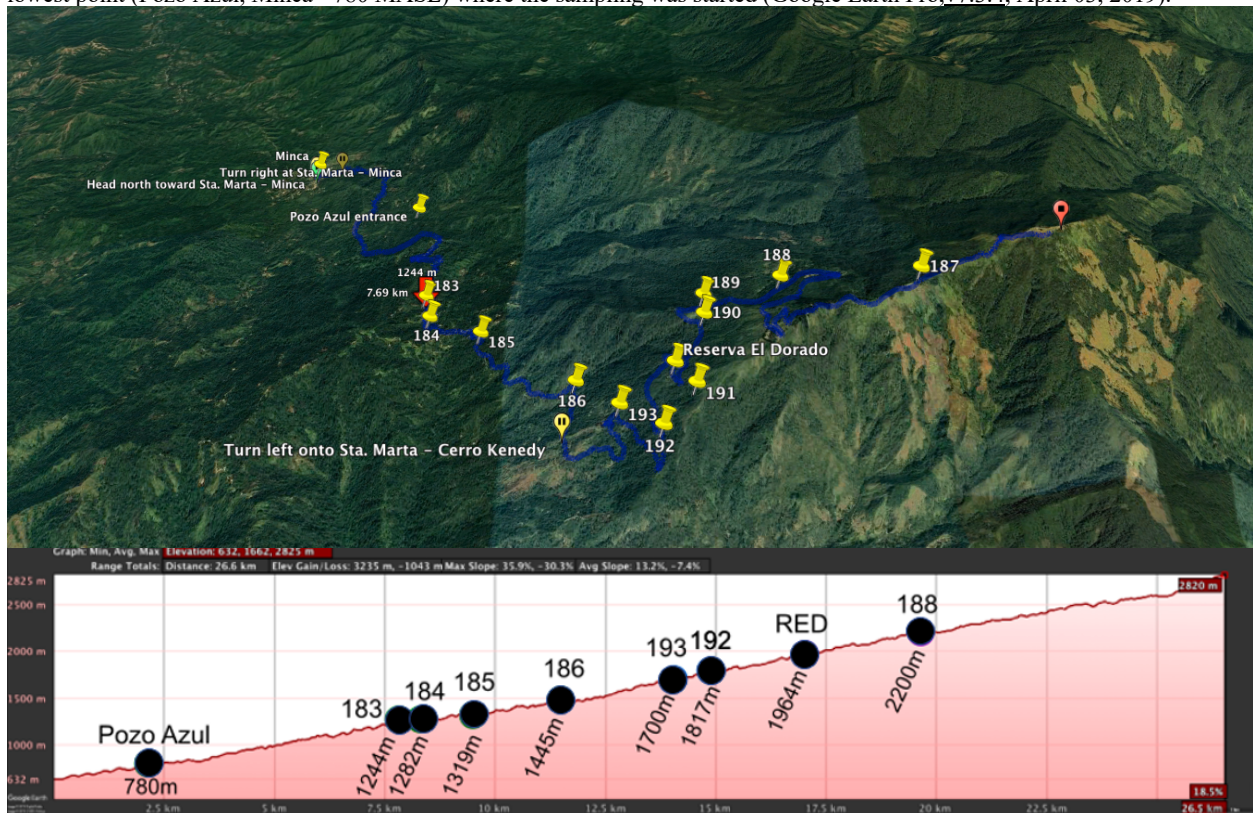
Molecular methods

The total genomic DNA was extracted from pleopods and/or legs of *Ligia* specimens by DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. A 310bp fragment of the 16s rDNA gene was amplified with published primers 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3')

(Palumbi, 1996). Each PCR reaction contained 1 ul DNA template, 0.4 uL of each primer 10 mM, 0.1 uL OneTaq DNA polymerase, 0.4 uL dNTPs 10 mM, 0.64 uL MgCl2 25 mM and 4 uL 5x PCR buffer. The PCR protocol used was: 2 min at 94°C followed by 30 cycles of 30s at 94°C; 40s at 52°C, 1 min at 68°C; and a final extension at 68°C for 10 min.

A 490bp fragment of the 12s rDNA gene was amplified with published primers crust-12sf (5'-CAGCAKYCGCGGTTAKAC-3') and crust-12sr (5'-ACACCTACTWTGTTACGACTTATCTC-3') with the following PCR conditions: 4 min at 94°C, 34 cycles of 1min at 94°C, 30 s at 56°C, 1.5 min at 72°C and final extension at 72°C for 4 min. Each PCR reaction included 6.25 uL of RedTaq polymerase , 0.5 uL of each primer 10 mM and 2.75 uL DNA template.

A 680bp fragment of the NaK gene was amplified with primers NaK forb (5'-ATGACAGTTGCTCATATGTGGTT-3') and NaK rev2 (5'-ATAGGGTGATCTCCAGTRACCAT-3') with the following PCR temperature profile : 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 54°C, 1.5 min at 72°C and final extension at 72°C for 10 min. Each PCR reaction included 6.25 uL of RedTaq polymerase, 0.5 uL of each primer 10 mM and 2.75 uL DNA template. PCR products were cleaned by Applied Biosystems Exosap-itTM (Thermo Fisher Scientific, Waltham, MA, USA). We used Sequencher 4.8 (Genecodes, Ann Arbor, MI, USA) and CodonCode Aligner software package 9.0.1 (CodonCode Corporation, Dedham, MA, USA) to assemble and edit the sequences, and trim the primers. There were no stop codons in NaK sequences. All the other localities' sequences were downloaded from GenBank. The species name as well as their locations, IDs and sources are gathered in Table 3. Sequences were aligned with the online MAFFT version 7 (Katoh et al., 2019), assuming Q-INS-I strategy with default parameters. The NEXUS and FASTA files of all datasets were downloaded for further

analysis. The NEXUS files were uploaded on CIPRES portal for Bayesian analysis and FASTA files were uploaded on IQTree web server for Maximum Likelihood analysis.

Table 4.List of Ligia species genes used in this study.

Included localities and corresponding GenBank Accession Numbers, Latitude and Longitude and Sequence Labels.

pecies	Location	ID	16s rDNA Accession no.	12s rDNA Accession no.	NaK Accession no.	Lat.	Long.	Source
L. hawaiensis	Pu'unalu Beach Park, Hawai'i	1	KF546551	KF546576	KF546593	19° 8'0.60"N	155°30'18.30"W	Santamaria, CA., et al. 2013
L. perkinsi	Haupu Range, Kaua'i	2	KF546547	KF546579	KF546592	N/A	N/A	Santamaria, CA., et al. 2013
L. hawaiensis	Kapua'a Beach Park, Kaua'i	3	KF546544	KF546571	KF546585	22°13'05.30"N	159°25'31.15"W	Santamaria, CA., et al. 2013
L. hawaiensis	Manele Bay, Lana'i	4	KF546538	KF546564	KF546589	20°44'37.37"N	156°53'12.47"W	Santamaria, CA., et al. 2013
L. hawaiensis	Pupukea, O'ahu	5	KF546531	KF546562	KF546591	21°38'59.70"N	158°03'45.48"W	Santamaria, CA., et al. 2013
L. exotica	Kanagawa, Japan	6	KX447727	MG676405	MG676443	35°9'25"N	139°36'43"E	Hurtado L., et al., 2018
L. exotica	Qingdao, Shandong, China	7	KX447748	MG676407	MG676431	36°3'58"N	120°22'10"E	Hurtado L., et al., 2018
L. exotica	Okinawa, Japan	8	KX447742	MG676412	MG676429	26°28'46"N	127°55'40"E	Hurtado L., et al., 2018
L. exotica	Zhujiajian Island, Zhejiang, China	9	KX447739	MG676418	MG676440	29°54"N	122°53'E	Hurtado L., et al., 2018
L. exotica	Toyohashi, Japan	10	KX447741	MG676417	MG676435	N/A	N/A	Hurtado L., et al., 2018
L. exotica	Municipal Harbor, Port Aransas, TX, USA	11	KX447720	MG676421	MG676436	27°50'24"N	97°3'50"W	Hurtado L., et al., 2018
L.cinerascens	Cheju, South Korea	12	KX447756	MG676404	MG676425	33°22'N	126°32'E	Hurtado L., et al., 2018
L. baudiniana	Fort Sherman, Panama	13	KF555764	KF555811	KF555884	09∘21_51.36__N	79∘56_55.56__W	Santamaria et al., 2014

L. baudiniana	Santa Marta, Colombia	14	KF555776	KF555819	KF555885	11∘20_07.74__N	73∘ 58_31.26__ W	Santamaria et al., 2014
L. baudiniana	Long Bird Bridge, Bermuda	15	KF555782	KF555829	KF555873	32∘21_05.34__N	64∘ 42_35.16__ W	Santamaria et al., 2014
L. baudiniana	Fajardo, Puerto Rico	16	KF555793	KF555807	KF555874	18∘21_38.84__N	65∘ 37_28.51__ W	Santamaria et al., 2014
L. baudiniana	Veracruz, Panama	17	KF555795	KF555837	KF555878	08∘53_28.30__N	79∘ 35_35.19__ W	Santamaria et al., 2014
L. baudiniana	El Morro, Venezuela	18	KF555789	KF555833	KF555875	N/A	N/A	Santamaria et al., 2014
L. baudiniana	Buenaventura, I. Palma, Colombia	19	KF555797	KF555834	KF555877	N/A	N/A	Santamaria et al., 2014
L. italica	Italy	20	KF555800	KF555840	N/A	N/A	N/A	Santamaria, C.A. et al., 2013
L. italica	Italy	21	DQ183054	DQ182954	N/A	N/A	N/A	Klossa-Kilia, E., et al, 2006
L. oceanica	Germany	22	KF555802	KF555839	N/A	N/A	N/A	Santamaria et al., 2014
L. oceanica	Concarneau, France	23	JQ814400	N/A	N/A	47° 52' N	3° 55' W	Raupach,M. J., et al, 2014
L.cinerascens	Tianjin, China	25	KX447752	N/A	N/A	39°08'N	117°11'E	Hurtado, L.A., et al,2018
L. perkinsi	Nu'uanu Pali, O'ahu	26	KF546548	KF546572	N/A	N/A	N/A	Santamaria, CA., et al. 2013
L. exotica	Veracruz, Mexico	27	KF546552	KF546584	N/A	19°12'33.63" N	96° 7'51.39"W	Santamaria, CA., et al. 2013
L. occidentalis	Guaymas, Mexico	28	KF546553	KF546583	N/A	27°54'44.33" N	110°56'49.56"W	Santamaria, CA., et al. 2013
L. vitiensis	Parangtritis, Java, Indonesia	29	KF546554	KF546582	N/A	N/A	N/A	Santamaria, CA., et al. 2013
L. vitiensis	Basin Cabri, Aldabra Atoll, Seychelles	30	MF828579	MF828590	MF805565	N/A	N/A	Santamaria, CA., et al. 2017
L. vitiensis	Dili, Timor-Leste	31	KF546556	KF546581	N/A	N/A	N/A	Santamaria, CA., et al. 2017

L. vitiensis	Stone Town, Zanzibar, Tanzania	3 2	MF8285 78	MF8285 89	N/A	6°09′33.8″S	39°11′26.4″ E	Santamaria, CA., et al. 2017
L. dentipes	Galle, Sri Lanka	3 3	MF8285 76	MF8285 84	N/A	6°01′49.9″N	80°13′03.3″ E	Santamaria, CA., et al. 2017
L. dentipes	Silhouette Island, Seychelles	3 4	MF8285 73	MF8285 86	MF8055 64	4°29′08.4″S	55°15′12.4″ E	Santamaria, CA., et al. 2017
L. dentipes	Dutch Bay, Trincomale e, Sri Lanka	3 5	MF8285 77	MF8285 83	N/A	8°33′52.6″N	81°14′27.8″ E	Santamaria, CA., et al. 2017
L. occidental is	Guaymas, Mexico	3 6	KF5465 53	KF5465 83	N/A	27°54′44.3″ N	110°56′49.6 ″W	Santamaria, CA., et al. 2017
L.occident alis	Harris Point	3 7	HM5698 86	N/A	KC9531 03	N/A	N/A	Eberl R., et al., 2013
L.occident alis	Isthmus Cove	3 8	HM5699 12	N/A	KC9531 13	N/A	N/A	Eberl R., et al., 2013
L.occident alis	Corona Ensenada	3 9	HM5698 97	N/A	KC9531 21	N/A	N/A	Eberl R., et al., 2013
L.occident alis	Tomatal	4 0	HM5698 96	N/A	KC9531 35	N/A	N/A	Eberl R., et al., 2013
L.occident alis	Bahia Asuncion	4 1	HM5699 03	N/A	KC9531 42	N/A	N/A	Eberl R., et al., 2013
L. glabrat a	Luderitz, Namibia	4 2	N/A	N/A	MH1731 52	26°39′47″S	15°04′55″E	Greenan et al., 2018
L. dilatata	Koelbaai, South Africa	4 3	N/A	N/A	MH1731 53	34°14′51″S	18°51′15″E	Greenan et al., 2018
L. natalen sis	Summerstr and, Port Elizabeth, South Africa	4 4	N/A	N/A	MH1731 54	33°59′01″S	25°40′16″E	Greenan et al., 2018
L. natalen sis	Kidd's Beach, South Africa	4 5	N/A	N/A	MH1731 55	33°08′50″S	27°42′10″E	Greenan et al., 2018
L. SNSM (83, 84, 85, 86, pa)	Sierra Nevada de Santa Marta, Colombia	L						This Study
L. SNSM (92, 93)	Sierra Nevada de Santa Marta, Colombia	M						This Study
L. SNSM (88)	Sierra Nevada de Santa	H						This Study

Species	Location	No.						Reference
	Marta, Colombia							
Sphaeroma serratum (outgroup)	Italy	46	N/A	N/A	MN2342 62	N/A	N/A	Dimitriou et al., 2019
Sphaeroma serratum (outgroup)	Italy	47	N/A	N/A	MN234 261	N/A	N/A	Dimitriou et al., 2019
Idotea chelipes (outgroup)	Italy	48	N/A	N/A	MN234 263	N/A	N/A	Dimitriou et al., 2019
Idotea chelipes (outgroup)	Italy	49	N/A	N/A	MN2342 64	N/A	N/A	Dimitriou et al., 2019
Sphaeroma serratum (outgroup)	Atlantic, Portugal	50	KU2482 78	N/A	N/A	N/A	37.07°N ~8.31°W,	Wetzer et al., 2018
Sphaeroma serratum (outgroup)	Siec island, France	51	AJ38807 4	N/A	N/A	N/A	N/A	Michel-Salzat and Bouchon, 2000
Idotea chelipes (outgroup)	N/A	52	GQ3026 89	N/A	N/A	N/A	N/A	Koutmos et al. (Unpublishe d)
Idotea chelipes (outgroup)	Ré island, France	53	AJ38808 0	N/A	N/A	N/A	N/A	Michel-Salzat and Bouchon, 2000
Idotea resecata (outgroup)	Monterey Bay, USA	54	N/A	AF2595 26	N/A	N/A	N/A	Wetzer, 2001

Model Selection

By using jModelTest 2.1.10 v. 20160303 (Darriba et al., 2012), we determined the most appropriate model of DNA substitution to calculate likelihood scores among 88 candidate models for NaK, 16s rDNA, 12s rDNA genes. jModelTest was only run only on each dataset.

Phylogenetic analyses

For ML analyses IQTree on web server (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al., 2016) were used with the simplest appropriate models for 16s rDNA, 12s rDNA and Nak genes with 100 bootstrap replicates. For Bayesian inferences, we used MrBayes on XSEDE 3.2.7a. This program was implemented in the CIPRES portal https://www.phylo.org/. All the tree files were

downloaded and visualized by FigTree v1.4.4 (Rambaut 2009) and the PDF files of the trees with

clade support were extracted from this software.

References

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Mitochondrial and nuclear datasets

Both mitochondrial and nuclear sequences showed that there are three different main haplotype groups in *Ligia* SNSM. Each haplotype corresponded to a certain elevation of the study area. The first haplotype's habitat is from 780-1445 meters above sea level (MASL), the second one's habitat is 1700-1817 MASL and the third haplotype's habitat starts at 1964 to 2198 MASL (Table 4, Figures 2, 3 and 4). Also, genetic divergences between these three haplotypes were calculated (Tables 5, 6 and 7). As there was no sequence divergence among individuals within each elevation, we retained one individual from each elevation in our phylogenetic analyses. For 16s rDNA, 41 sequences from different *Ligia* species and different localities were added to the dataset. *Sphaeroma serratum* and *Idotea chelipes* sequences were used as outgroups in the 16s rDNA dataset. However, for 12s rDNA, due to limited study, 33 sequences from other *Ligia* species of different localities were added to the dataset and *Idotea chelipes* was used as an outgroup. After alignment, the total characters of 16s rDNA were 561 and 431 characters were retained. For 12s rDNA, 487 characters were retained out of 518. The NaK gene was obtained from 41 individuals of *Ligia* SNSM which represent three different elevations exactly according to the mitochondrial genes. Similarly, there were no variabilities between individuals from each habitat for the NaK gene and the sequence of an individual from each elevation was chosen in our phylogenetic analyses. For this gene, 30 sequences of different *Ligia* species from different localities were added to the dataset. Also, *Sphaeroma serratum* and *Idotea chelipes* were considered as outgroups.

Table 5. Three different main haplotypes in Ligia SNSM.

Each haplotype corresponded to a certain elevation of the study area. The first haplotype's habitat (Green) is from 780-1445 meters above sea level (MASL), the second one's habitat (Blue) is 1700-1817 MASL and the third haplotype's habitat (Purple) starts at 1964 to 2198 MASL. The empty cells mean we did not PCR amplified the genes of that individuals.

Sample ID	16r DNA	12s rDNA	NaK	Sample ID	16s rDNA	12s rDNA	NaK
183-1	Green	Green		186-2	Green		
183-2	Green		Green	186-3	Green		Green
183-3	Green	Green		186-4	Green		Green
183-4	Green			186-5			
183-5	Green	Green		186-6			
183-6	Green	Green		186-7			
183-7	Green	Green		186-8			
183-8	Green	Green		186-9	Green		Green
183-9	Green	Green		186-10	Green		Green
184-1				186-11	Blue		
184-2	Green	Green	Green	186-12			
184-3	Green	Green	Green	186-13	Green		Green
184-4	Green	Green		188_1	Purple	Purple	Purple
184-5				188-2			
184-6	Green			188-3	Purple	Purple	Purple
184-7				188-4	Purple	Purple	Purple
184-8	Green			188-5	Purple		
184-9				188-6	Purple	Purple	Purple
184-10	Green	Green		188-7	Purple	Purple	
185-1	Green	Green		188-8	Purple		
185-2	Green	Green		188-9	Purple		
185-3	Green	Green		192-1	Blue	Blue	Blue
185-4	Green	Green		192-2	Blue	Blue	Blue
185-5				192-3		Blue	
185-6				192-4	Blue	Blue	
185-7	Green		Green	192-5	Blue	Blue	
185-8	Green			192-6	Blue		
pa-1	Green			192-7	Blue		
pa-2	Green			192-8			
pa-3	Green	Green		192-9	Blue	Blue	
pa-4	Green	Green	Green	192-10	Blue		
pa-5				193-1			
pa-6	Green	Green	Green	193-2	Blue		
pa-7	Green	Green		193-3	Blue		Blue
pa-8				193-4	Green		Green
pa-9				193-5			
pa-10				193-6			
pa-11				193-7			
pa-12				193-8	Green		Green
pa-13				185_?	Green		Green
pa-14	Green	Green	Green	186_?	Green		
pa-15	Green		Green	El Dorado Enterance	Purple		Purple
186-1							

Figure 2. Unrooted MAXIMUM LIKELIHOOD TREE of 16s rDNA

Color coding corresponds to different altitudes. Green refers to 780 - 1445 MASL. Blue refers to 1700 to 1817 MASL and purple refers to 1964 to 2198 MASL.
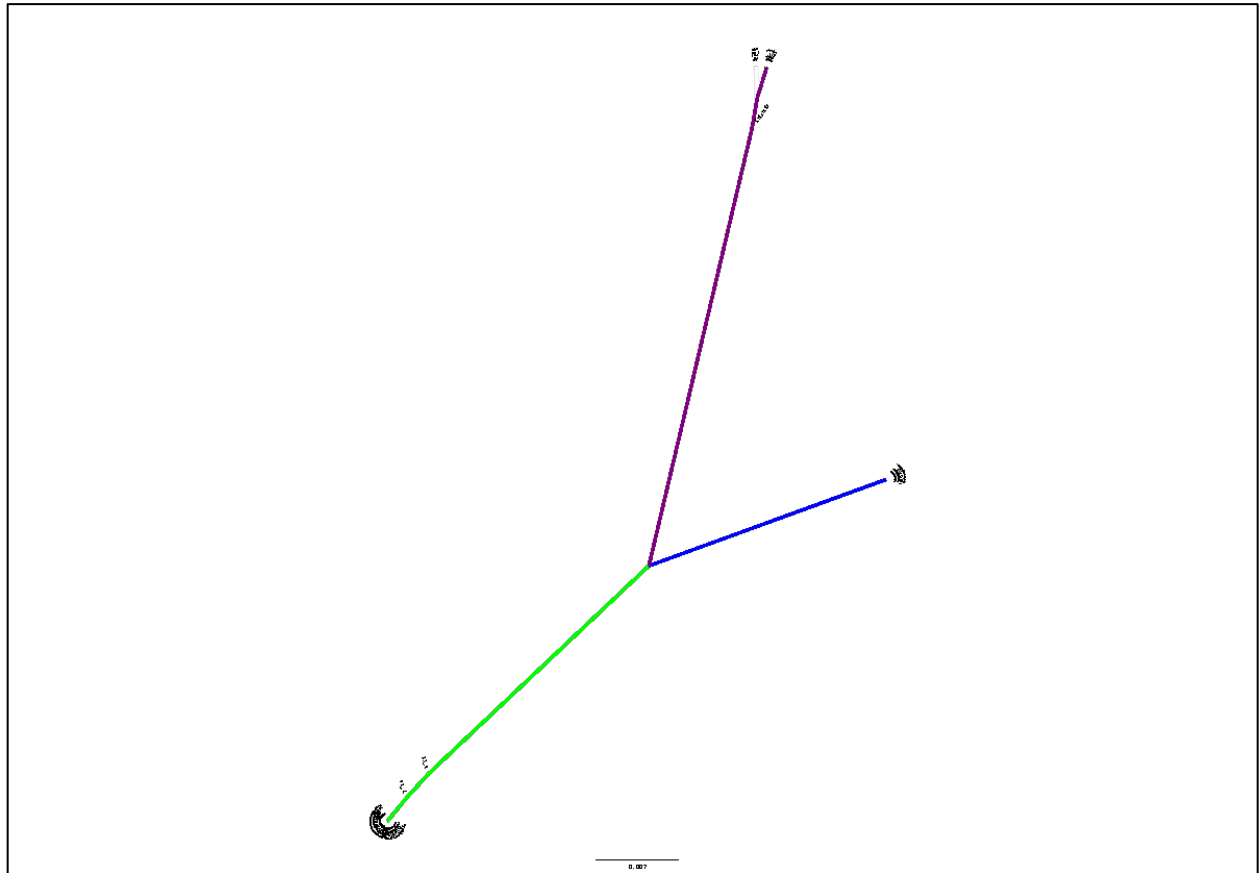

Table 6. Genetic divergences between different linages for 16s rDNA gene.

	Blue	Purple
Green	4.4%	5.9%
Blue	-	5.7%

Figure 3. Unrooted MAXIMUM LIKELIHOOD TREES of 12s rDNA

Color coding corresponds to different altitudes. Green refers to 780 - 1445 MASL. Blue refers to 1700 to 1817 MASL and purple refers to 1964 to 2198 MASL.
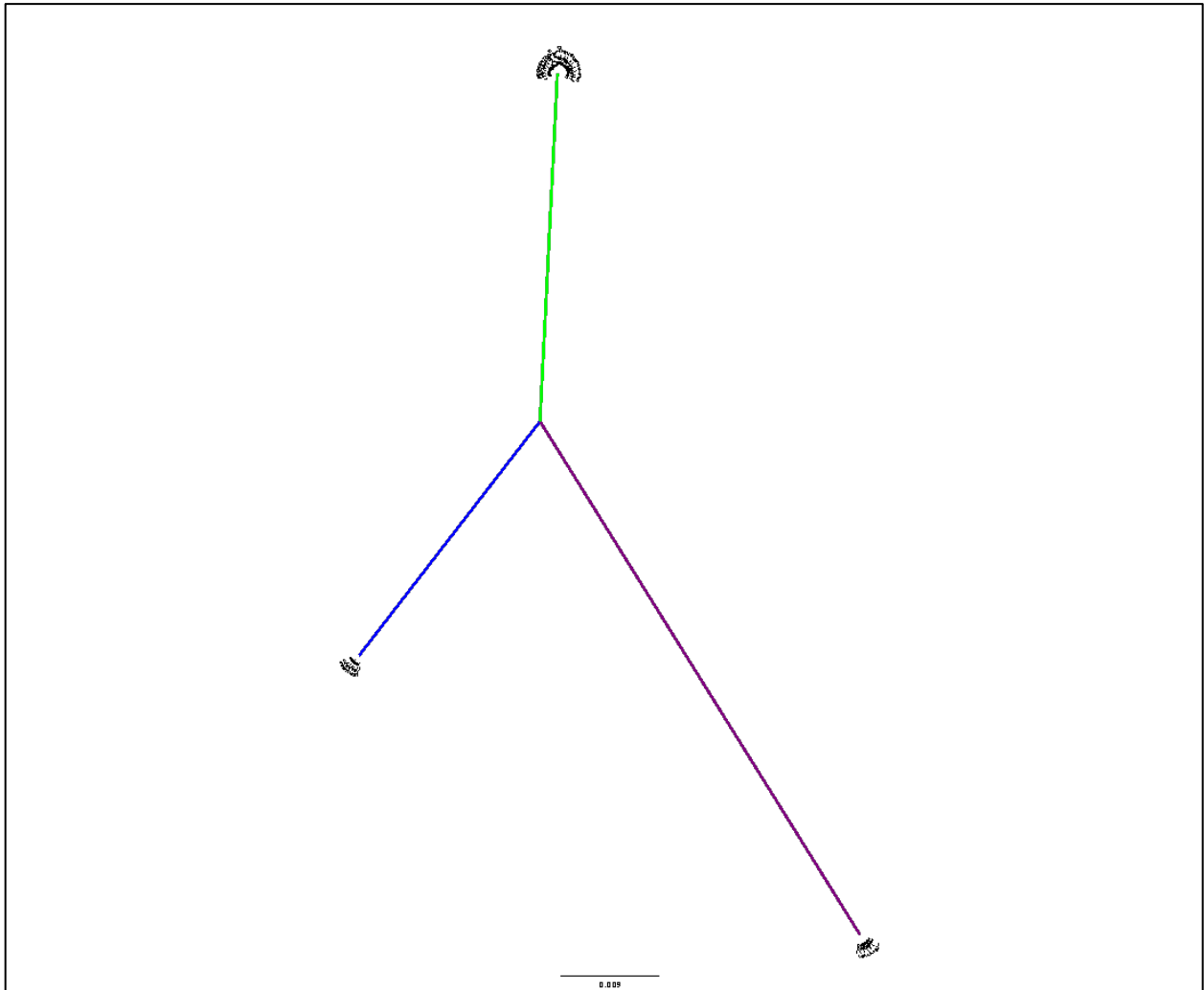

Table 7. Genetic divergences between different linages for 12s rDNA gene.

	Blue	Purple
Green	5.2%	8%
Blue	-	7.6%

24

Figure 4. Unrooted MAXIMUM LIKELIHOOD TREES of NaK gene

Color coding corresponds to different altitudes. Green refers to 780 - 1445 MASL. Blue refers to 1700 to 1817 MASL and purple refers to 1964 to 2198 MASL.
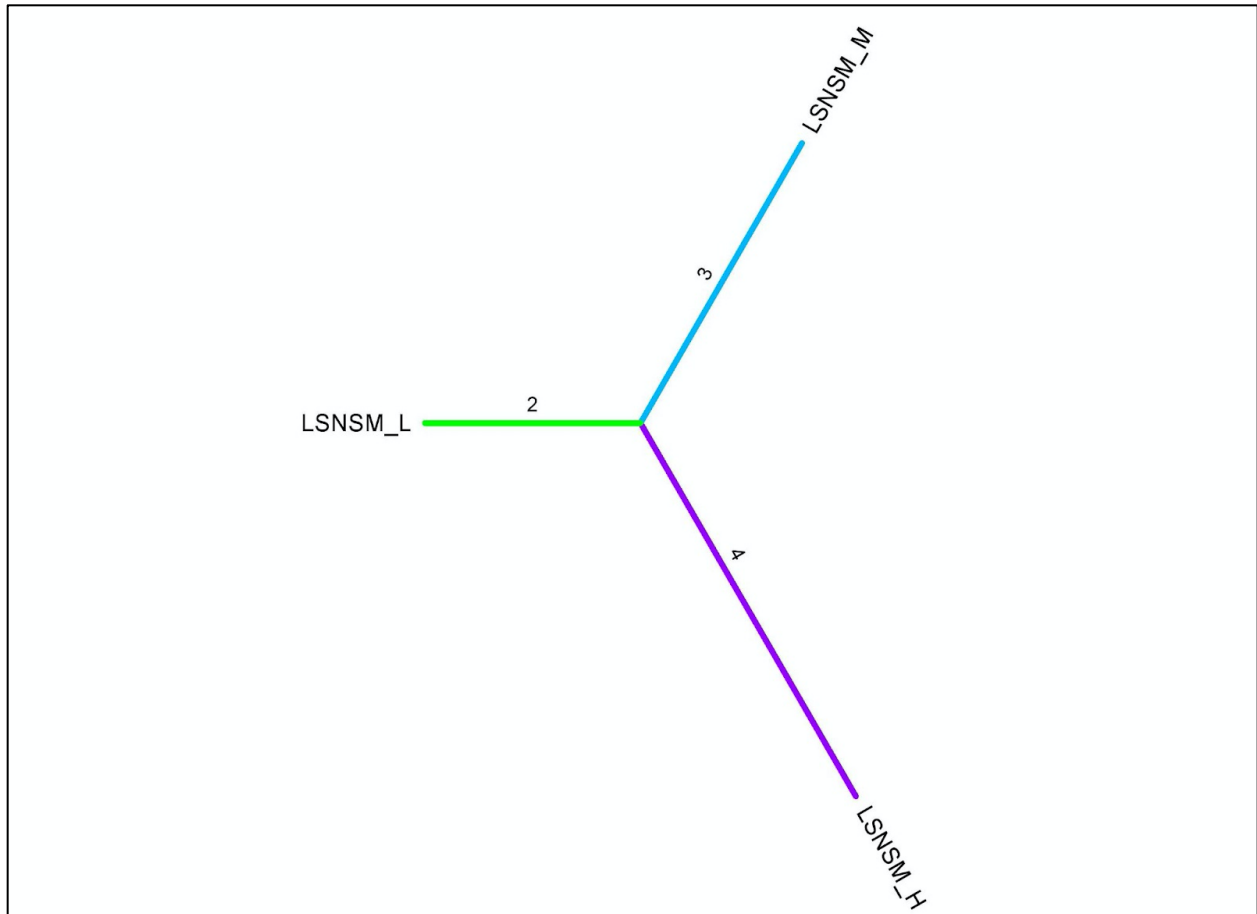

Table 8. Genetic divergences between different linages for NaK gene.

	Blue	Purple
Green	0.9%	1.9%
Blue	-	1.8%

For the 16s rDNA dataset, jModelTest selected GTR model with six substitution parameters (AC, AG, AT, CG, CT, GT) +I and, +G according to the Akaike Information Criteria

(AIC) (weight = 0.7386) and AICc (weight = 0.5794). The selected model according to the Bayesian Information Criterion (BIC) was a simpler model e.i. TVM (weight = 0.7205) with five substitution parameters (AC, AT, CG, GT, AG = CT) +I and, +G. For the 12s rDNA dataset, the selected model based on AIC was GTR (weight = 0.4491) with six substitution rates +G and +I, based on the AICc the selected model was TIM2 (weight = 0.4027) with for substitution rates (AC = AT, CG = GT, AG, CT) +I +G. Under BIC, the selected model was TrN (weight = 0.3276) with three substitution rates (AC = AT = CG = GT, AG, CT) +I and, +G (Table 8). For each dataset, we considered the simplest model according to the substitution rate. The selected model for the 16s rDNA was TVM+I+G and for the 12s rDNA was TrN+I+G. We applied these models for both Bayesian and Maximum likelihood analyses.

For the NaK dataset, jModelTest selected the TIM3 model (weight = 0.1708) with four substitution rates (AC = CG, AT = GT, AG, CT) +G based on AIC. Models TIM3ef+G (weight = 0.1967) with four substitution parameters (AC = CG, AT = GT, AG, CT) and K80+G (weight = 0.3227) with two substitution rates (AC = AT = CG = GT, AG = CT) were selected in order for AICc and BIC. For this dataset, we considered the simplest model according to the substitution rate e.i. K80+G for both Bayesian and Maximum likelihood analyses.

Table 9. Best model selected by jModeltest

Gene	No. of Character	Best Model AIC	Best Model AICc	Best Model BIC
NaK	347	TIM3+G Partition: 012032 Weight: 0.1708	TIM3ef+G Partition: 012032 Weight: 0.1967	K80+G Partition: 010010 Weight: 0.3227
16s rDNA	431	GTR+I+G Partition: 012345 Weight: 0.7386	GTR+I+G Partition: 012345 Weight: 0.5794	TVM+I+G Partition: 012314 Weight: 0.7205
12s rDNA	487	GTR+I+G Partition: 012345 Weight: 0.4491	TIM2+I+G Partition: 010232 Weight: 0.4027	TrN+I+G Partition: 010020 Weight: 0.3276

Phylogenetic results

Although there are some discrepancies among different analyses and genes, that are reflected in the support values and topologies of trees; the phylogenetic analyses of both mitochondrial and nuclear datasets indicated that *Ligia* from SNSM is a divergent group from all other species of *Ligia* and corresponded to a well-supported monophyletic clade (Bootstrap Support (BS): 90-100; Posterior Probability (PP): 97-100) (Figures 5, 6, 7, 8, 9 and 10).

Relationships of *Ligia* SNSM to other *Ligia* were not well resolved, and inferences differed according to both gene and analysis. From all phylogenetic trees for three genes and both analyses we can say that *Ligia* SNSM did not diverge from the one of Caribbean that is represented here. The position of *Ligia* SNSM in relation to other species is unknown because its position changed depending on different genes and analyses. In ML analysis for 12s rDNA gene, *Ligia* SNSM is the sister clade to Caribbean *Ligia*. However, it is not supported by other genes and analyses. The main result from these analyses is that *Ligia* SNSM did not diverged within Caribbean lineage.

Figure 5. Bayesian tree for 16s rDNA gene

The model applied for this gene was TVM+I+G. This tree is rooted with *S. serratum* from Portugal and France and *I. chelipes* from France.
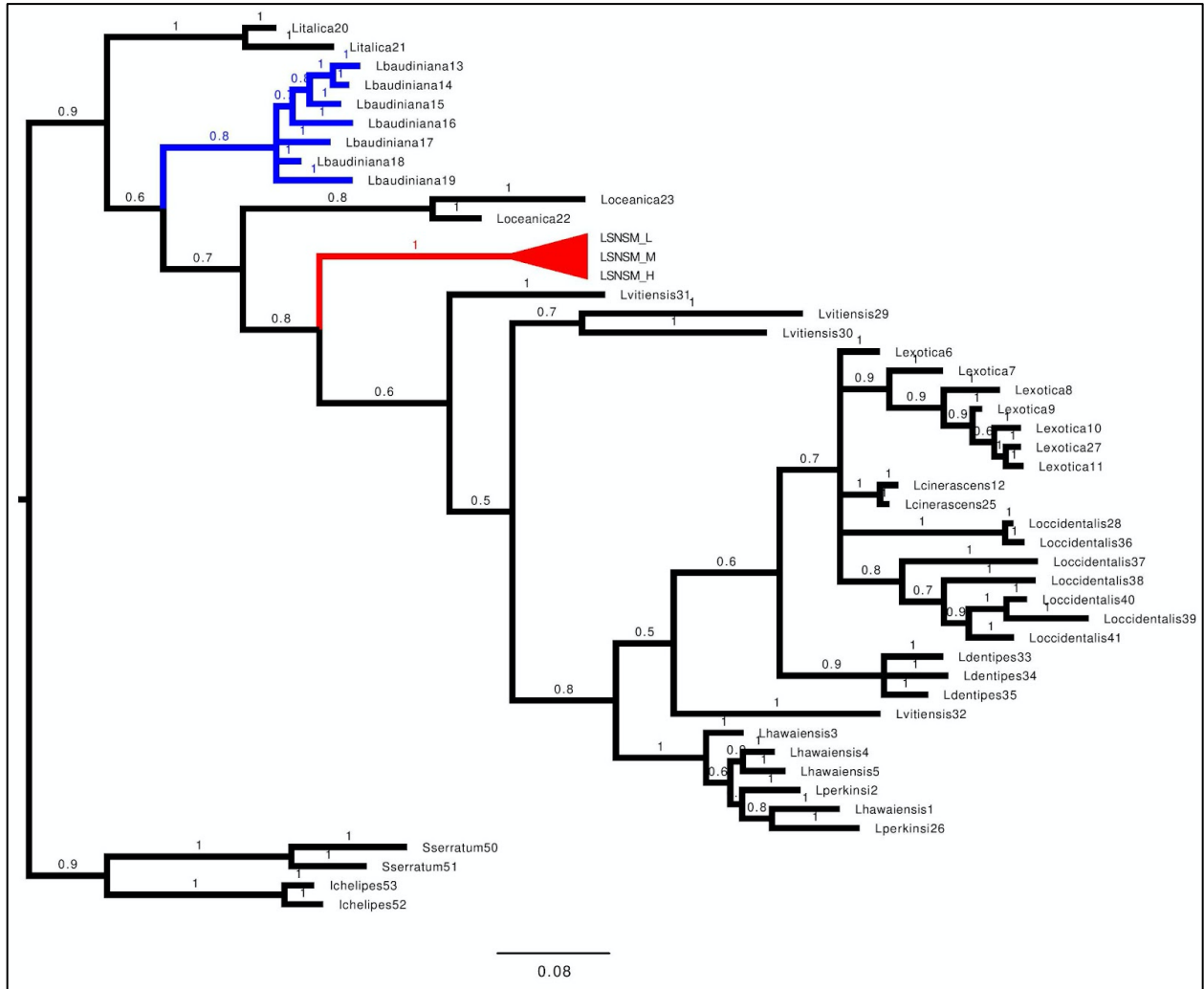
Figure 6. Bayesian tree for NaK gene

The best model was applied for this gene was K80+G. This tree is rooted with *S. serratum* from Italy.
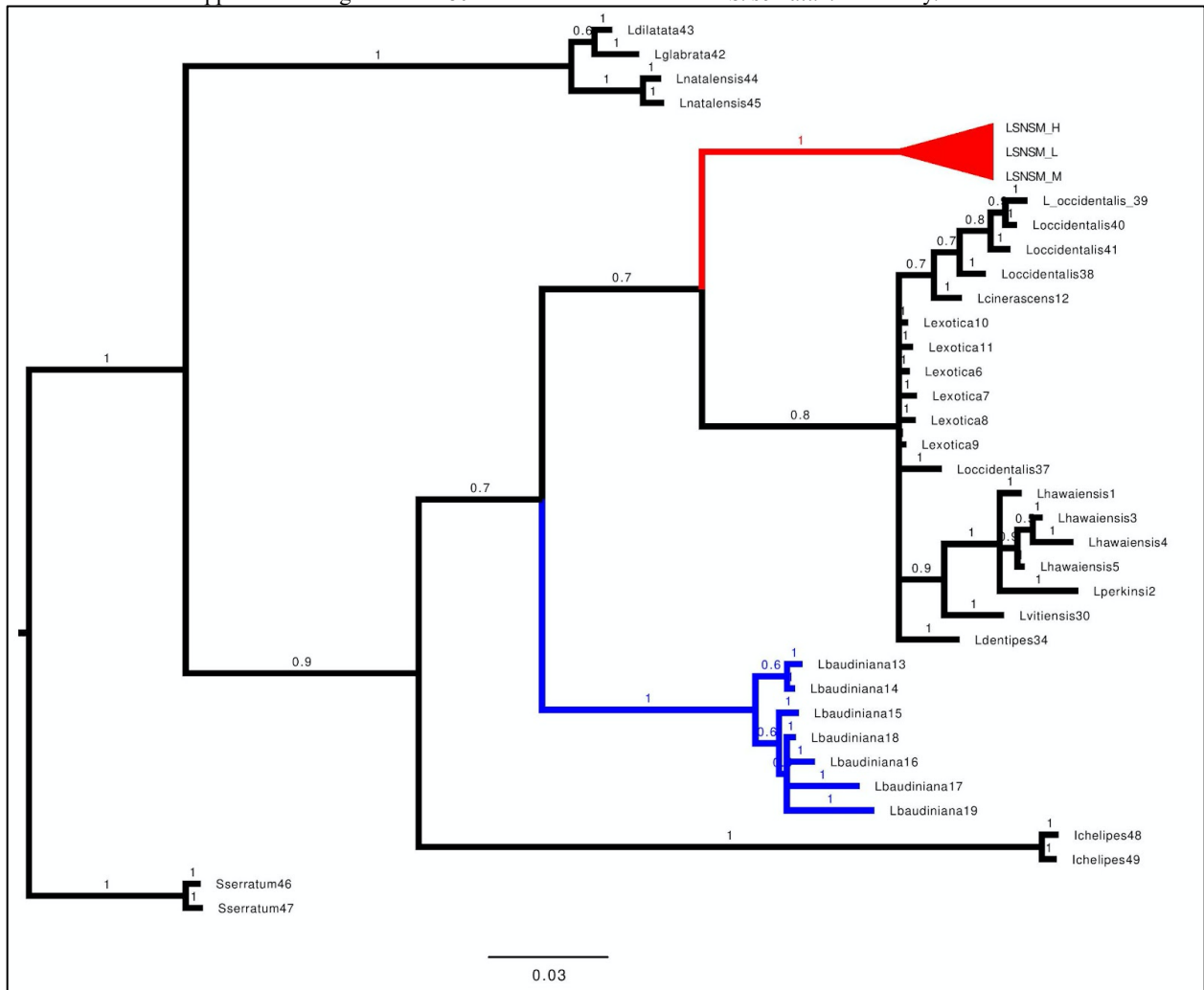
Figure 7. Bayesian tree for 12s rDNA gene

The best model was applied for this gene was TrN+I+G. This tree is rooted with *I. chelipes* from USA.
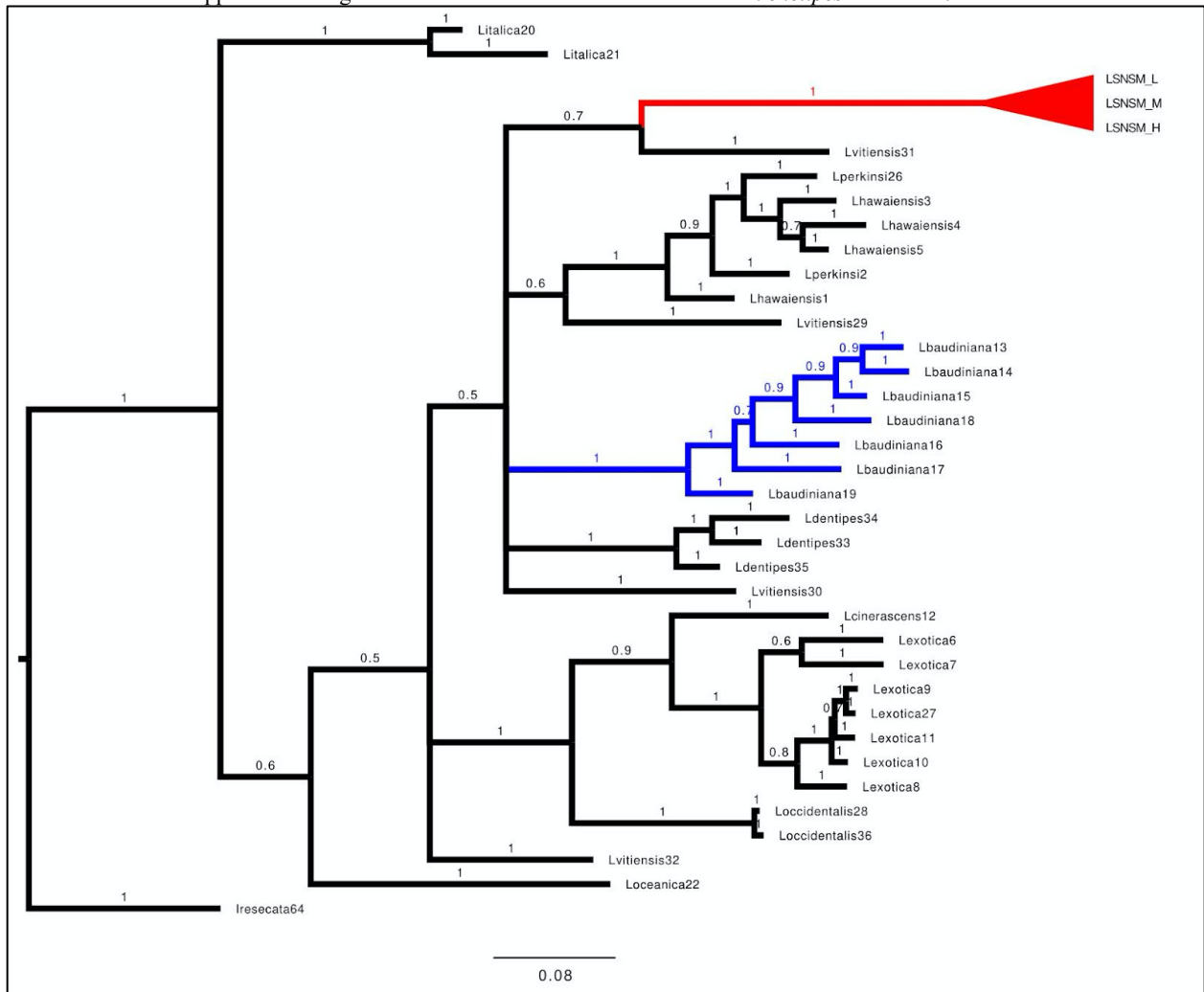
Figure 8. Maximum Likelihood tree of 16s rDNA gene

The tree was obtained from IQTree for 16s rDNA (model TVM+I+G) rooted with *S.serratum* from Portugal and France and *I. chelipes* from France. Values are SH-aLRT support (%) / aBayes support / ultrafast bootstrap support (%)
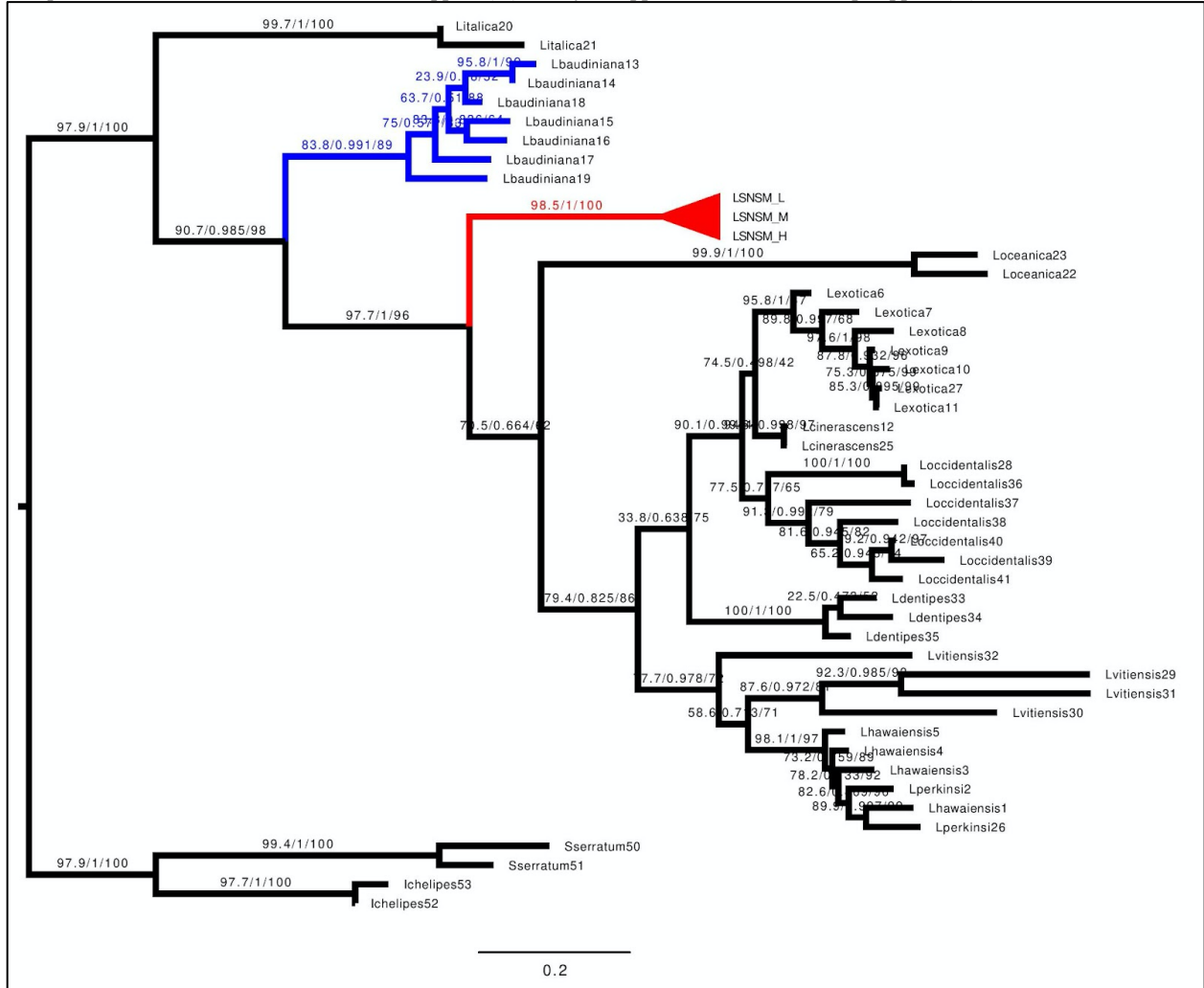
Figure 9. Maximum Likelihood tree of 12s rDNA gene

The tree was obtained from IQTree for 12s rDNA (model TrN+I+G) rooted with *I. chelipes* from USA. Values are SH-aLRT support (%) / aBayes support / ultrafast bootstrap support (%)

Figure 10. Maximum Likelihood tree of NaK gene

The tree was obtained from IQTree for NaK (model K80+G) rooted with *S. serratum* and *I. chelipes* from Italy. Values are SH-aLRT support (%) / aBayes support / ultrafast bootstrap support (%)
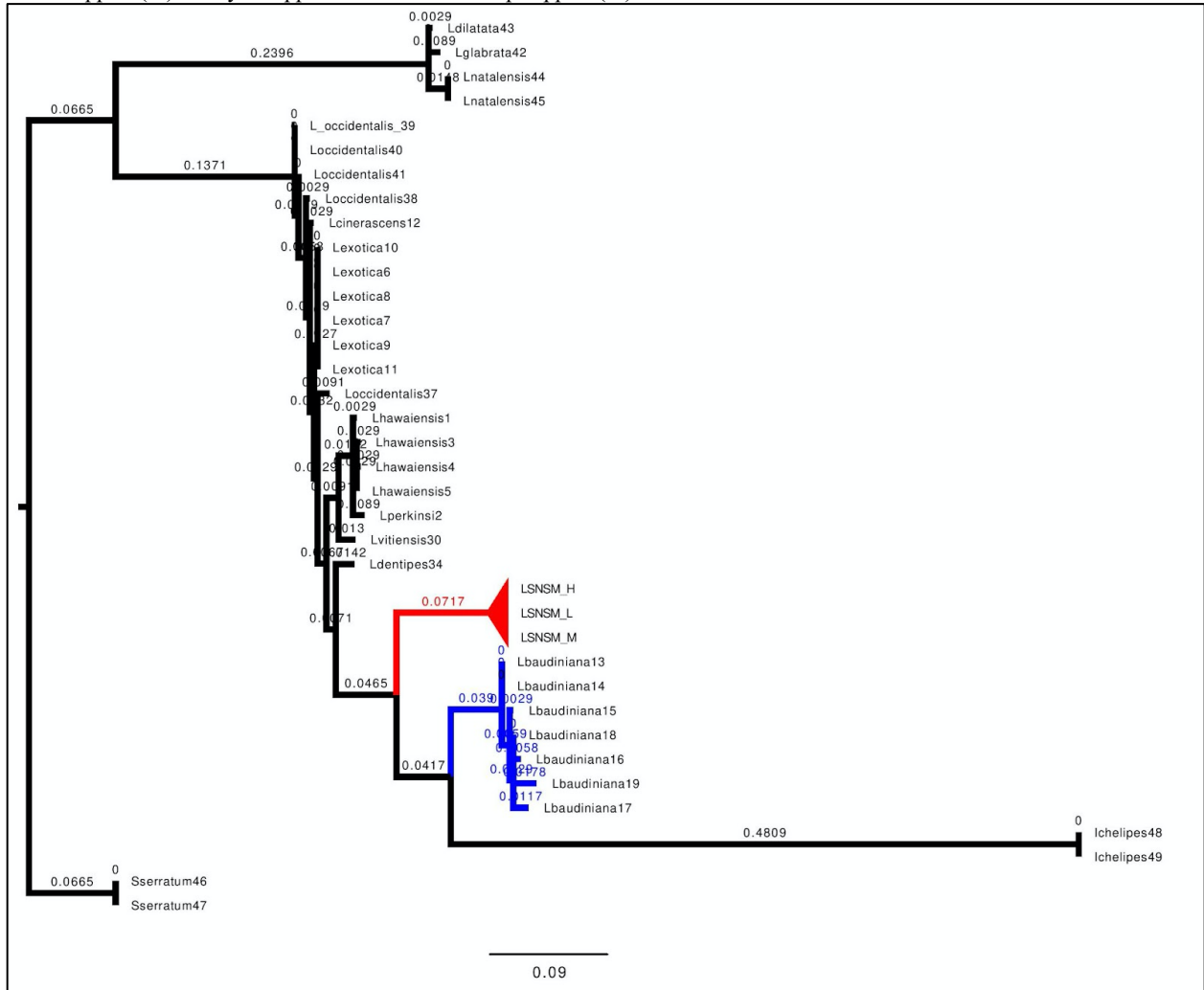
In this study for the first time we showed that *Ligia* SNSM corresponded to a well-supported monophyletic group and also included three divergent haplotypes in *Ligia* SNSM. We found three lineages of *Ligia* in the Sierra Nevada de Santa Marta, which were distributed according to elevation, One from 780 to 1,445 MASL, the second one from 1,700 to 1,817 MASL and the third one from 1,964 to 2,198 MASL. We showed that the altitude is a factor contributing to divergence of this isopod in the SNSM. All three genes showed a clear divergence between individuals in different heights. It is possible that each of the three clusters corresponds to a different species, which would add to the extraordinary levels of endemisms in this region. The high cryptic diversity observed for *Ligia* SNSM emphasizes the high level of biodiversity and endemisms in this region and highlights the importance of conservation studies, efforts and strategies.

Despite all the discrepancies that were observed in values and topologies of mitochondrial and nuclear genes, our study showed that the terrestrial *Ligia* in SNSM is a divergent group from all other *Ligia* species all around the world. Although there is uncertainty about the phylogenetic position of *Ligia* SNSM within the genus *Ligia*. We conclude, however, that the divergence of SNSM *Ligia* was not within the present-day Caribbean *Ligia*.

Implications and future work

The finding of three new *Ligia* lineages endemic to the SNSM adds to the extraordinary diversity of this region and highlights the need for similar studies in other invertebrate groups that have been poorly studied. It also highlights the needs for proper conservation of the unique endemics of this

megadiverse region. Phylogenetic analyses with representatives of all *Ligia* species are needed to establish the evolutionary relationships among members of this genus.

According to our results, *Ligia* from SNSM represents a good system to study adaptation to terrestrial life, and also to different altitudes, vegetation, microbial communities and temperature.