Untangling the effects of biogeography and host plant associations: phylogenetic and phylogeographic studies in the *Exophthalmus* genus complex

by

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ABSTRACT

Phylogenetic as well as phylogeographic methods were implemented to assess the diversification of Caribbean entimine weevils. (1) A phylogeny of 62 species of entimine weevils (Coleoptera: Curculionidae: Entiminae) in the tribes Eustylini Lacordaire and Geonemini Gistel was inferred based on a combined analysis of mitochondrial COI and nuclear EF-1α markers. The analysis recovered a monophyletic ingroup with four major lineages. A TreeMap analysis yielded zero cospeciation events, due to an apparent lack of sufficiently narrow and stable host associations. In contrast, a DIVA optimization of ancestral areas revealed 15 inter-island and two intra-island splits along the internal nodes of the phylogeny. (2) A phylogeographic analysis was performed to assess the historical relationships of *Diaprepes abbreviatus* of the islands Dominica, Hispanola (Dominican Republic), Puerto Rico, and Mona. Also the patterns of genetic variation and diversity were examine within islands and between the main phylogenetic clades observed.

RESUMEN

Se implementaron métodos filogenéticos y filogeográficos para evaluar la diversificación de escarabajos picudos entiminos (Coleoptera: Curculionidae: Entiminae) del Caribe. (1) Se infirió una filogenia de 62 especies de escarabajos de las tribus Eustylini Lacordaire y Geonemini Gistel basado en los marcadores mitocondrial (COI) y nuclear (EF-1α). Se observó un grupo interno monofilético con cuatro linajes principales. Cero eventos de co-especiación fueron observados del análisis en TreeMap, debido a una aparente ausencia de asociaciones estrechas y estables entre escarabajos y plantas hospederas. En contraste, optimización de áreas ancestrales en DIVA reveló 15 divergencias inter-islas y dos intra-islas a lo largo de los nodos internos de la filogenia. (2) Un análisis filogeográfico fue realizado para evaluar las relaciones históricas de *Diaprepes abbreviatus* en las islas de Dominica, La Española (República Dominicana), Puerto Rico, y Mona. Patrones de variación y diversidad genética fueron examinados en las islas y entre los principales clados filogenéticos observados.

DEDICATION

Dedicated to two very important men in my life,

To my father, for being the best.

and

To my husband, for being my energy every morning and share all this process.

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I would like to thank Charles O'Brien and Jennifer Girón for their assistance in identifying the sampled entimine species. Jennifer Girón, Juliana Cardona, and Santo Navarro participated in the collecting events. Geidy Acevedo helped in the laboratory, and Hernán Torres provided insight into phylogenetic analyses. Field work in Colombia, Dominica, the Dominican Republic, Jamaica, and throughout Greater Puerto Rico was facilitated through permits issued by the following institutions (in the aforementioned sequence): Ministro de Ambiente, Vivienda y Desarrollo Territorial (MAVDT); Dominica's Forestry & Wildlife Division (DFWD); Secretaría de Estado de Medio Ambiente y Recursos Naturales (SEMARENA), Natural Resources Conservation Authority (NRCA), and the Departamento de Recursos Naturales y Ambientales (DRNA). The research on Caribbean entimine weevils was funded in part by the National Science Foundation, grant DEB-641231.

Thanks to all people from the Biology Department that helped me during my stay at UPRM. Finally, I would like to thank my family, for their unconditional support, inspiration and love.

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1 Introduction

The West Indian archipelago constitutes a system of heterogeneous islands that jointly represent a microcosm for developing and testing ecological and evolutionary hypotheses (Brown and Pestano, 1998; Emerson, 2002; Ricklefs and Bermingham, 2008). In comparison to other archipelagos – such as Hawaii, the Galapagos Islands, or the Canary islands – the West Indies offer special opportunities for research due to their unique biogeographic history. Specifically, their complex geological history is characterized by a close proximity to continental land masses as well as among islands (Iturralde-Vinent, 2006; Ricklefs and Bermingham, 2008). This proximity has facilitated different origins and colonization tracks for the islands' present-day biota. At the same time, many islands have been isolated for sufficient periods of time to accumulate endemic species or at least well-differentiated populations (Ricklefs and Bermingham, 2008), and therefore show high rates of endemism.

The *Exophthalmus* genus complex includes weevils of the genera *Compsus* Schoenherr, *Diaprepes* Schoenherr, *Exophthalmus* Schoenherr, *Exorides* Pascoe, and other genera like *Apotomoderes* Dejean, *Ischionoplus* Chevrolat, *Lachnopus* Schoenherr, and *Pachnaeus* Schoenherr (Kuschel, 1955; Vaurie, 1961; Woodruff, 1985; O'Brien and Wibmer, 1982; Morrone, 1999; O'Brien and Kovarik, 2000; Franz, 2010a, 2011). These genera are grouped mainly in two tribes Eustylini Lacordaire and Geonemini Gistel (Coleoptera: Curculionidae: Entiminae, *sensu* Alonso-Zarazaga and Lyal, 1999). However the taxonomic limits among these genera and even tribes remain unresolved. The main genera placed in this complex for the Caribbean include *Exophthalmus*, *Diaprepes* and *Lachnopus*, all with a Neotropical distribution (O'Brien and Wibmer, 1982).

Exophthalmus and related genera are placed in the subfamily Entiminae, which is the most diverse subfamily of the Curculionidae, with more than 12,000 described species (Oberprieler *et al*., 2007). The evolutionary success of the Curculionidae and of other phytophagous beetles is

typically explained in relation to their specialized host plant associations (Ehrlich and Raven, 1964; Anderson, 1993, 1995; Farrell, 1998; Marvaldi *et al*., 2002; Oberprieler *et al*., 2007; McKenna *et al*., 2009; Franz and Engel, 2010). Under this scenario, the varied yet specialized interactions of the beetles and plants have driven the diversification of both lineages via adaptive radiation and coevolution (e.g., Anderson, 1995; Farrell, 1998). However, for entimine weevils the host associations tend to be very broad and spatially and temporally unstable (Kuschel, 1995; Oberprieler *et al*., 2007). Therefore it is critical to examine up to what extent host plant associations can explain the species richness and patterns of endemism of *Exophthalmus* weevils in the Caribbean region.

This study uses phylogenetic as well as phylogeographic methods in order to address questions related to the patterns and process of diversification of eustyline and geonemine weevils in the Caribbean. Chapter two deals with the phylogenetic relations of the tribes Eustylini and Geonemini, which involve genera from the *Exophthalmus* complex. Along with the incorporation of geological, distributional and plant host information available, a hypothesis describing the observed patterns of species richness and endemism is presented. Specifically, this study examines whether the weevils' distributions are best explained by known host plant associations (Anderson, 1995; Farrell, 1998; Marvaldi *et al*., 2002) or by other biogeographic factors (e.g., Davies and Bermingham, 2002; Losos and Glor, 2003).

Chapter three focuses on phylogeographic patterns of *Diaprepes abbreviatus* (L.) populations in the Caribbean. This species is widely distributed in the Greater and Lesser Antilles (Lapointe, 2004). Since its accidental introduction in 1964 into the United States, the "citrus root weevil" is one of the most important pests on citrus crops in Florida, Texas and California (Woodruff, 1964; Lapointe *et al*., 2007). *Diaprepes abbreviatus* is also one of the most polymorphic species among those attacking citrus cultivars in Florida (Woodruff, 1985). Puerto Rico apparently presents the highest degree of phenotypic and genotypic variation in the Caribbean, and therefore is likely to be the center of origin of this species (Lapointe, 2004, Ascunce *et al*., 2008). Accordingly, the second goal of this study is to analyze the genetic structure of *D. abbreviatus* populations in Puerto Rico and thereby characterize its phylogeographic patterns.

1.1 Literature Review

1.1.1 Weevil natural history

Information on the natural history and distribution of Caribbean Eustylini and Geonemini species is scarce and typically restricted to ancillary data presented along with the original species descriptions (e.g., Hustache, 1929). Much of this information is summarized in O'Brien and Wibmer's (1982) annotated checklist of weevils of North America, Central America and the West Indies. Other general reference works include the catalogues published by Morrone (1999) and Alonso-Zarazaga and Lyal (1999).

Vaurie (1961) carefully revised the *Exophthalmus* species of Jamaica and O'Brien and Kovarik (2000) reviewed *Diaprepes* in the Caribbean region. Recently, redescriptions, new revisions of genera involved in the *Exophthalmus* genus complex and a phylogenetic analysis based on taxonomic characters have been published (Franz, 2010a, 2010b, 2011; Girón and Franz, 2010). These publications highlight the complex taxonomy of the group and in addition provide information on the species' polyphagous feeding habits as well as some cases of intraspecific variation correlated with specific localities on the same island. For example, Vaurie (1961) found that *E. similis* (Drury 1773) mainly occurs on the east side of Jamaica, having four varieties, one of them restricted to a sector of the island. A similar case is observed for *D. abbreviatus* in Puerto Rico (Virkki and Sepúlveda, 1990). Some authors have recognized distinctive morphological forms that are restricted to specific geographical areas in Puerto Rico (Virkki and Sepúlveda, 1990; O'Brien and Kovarik, 2000; Lapointe, 2004). For example, O'Brien and Kovarik (2001) mention seven to nine distinctive morphs, some widespread throughout the island and others limited to a single geographic area.

With regards to feeding habits, the adults of Entiminae are known to feed on leaves and young shoots of a range of host plants, as most species are generalists. The larvae live freely in the soil and feed on roots or root nodules without strict host-plant associations (Oberprieler *et al*., 2007; Bright and Bouchard, 2008). Different Central American species of *Exophthalmus* have been reported to feed on members of at least 15 plant families (Maes and O'Brien, 1990; Chaves and Fonseca, 1991; Rivas, 1992; Coto and Saunders, 2004). In the Caribbean region, some

Exophthalmus species have been observed feeding on more than 40 species of plants grouped in 21 families (Wolcott, 1929; Dixon, 1954 [cited by Vaurie, 1961]; Martorell, 1976). *Lachnopus* species have been reported feeding on close to 30 species of plants from 18 families (Martorell, 1976; Franz, 2010a). The remarkably polyphagous CRW has been found on approximately 270 species from 59 plant families (Simpson *et al*., 1996).

1.1.2 Analytical approach

Molecular tools were used in order to explore the mechanisms of diversification in Caribbean *Exophthalmus* genus complex. Specifically, mitochondrial gene cytochrome oxidase subunit I (COI) and the nuclear gene elongation factor 1α (EF-1α) were sequenced. The phylogenetic utility of these two genes has been shown in previous studies in which the combination of nuclear and mitochondrial genes has yielded well resolved topologies (Jordal, 2000, 2002; Caterino *et al*., 2000; Hughes and Vogler, 2004; Downie *et al*., 2008). EF-1α sequences have proven informative at intermediate phylogenetic levels in different groups of insects (Caterino *et al*., 2000; Cho *et al*., 1995; Jordal, 2002; Sequeira *et al*., 2000b; Cho *et al*., 1995; Djernaes and Damgaard, 2006).

On the other hand, the mtDNA gene (COI) is one of the most commonly sequenced regions in insect systematics (Simon *et al*., 1994; Kambhampati and Smith, 1995; Caterino *et al*., 2000) and it is well catalogued as a molecular marker for evolutionary studies (Lunt *et al*., 1996). COI sequences have been successfully used to differentiate between other island-bound beetle species (Sequeira *et al*., 2000a; Grobler *et al*., 2006). The mtDNA locus is particularly informative at the genus and species levels (Caterino *et al*., 2000; Palenko *et al*., 2004). In addition, COI sequences are being used to explore differentiation between populations of weevil species in relation to other factors such as host association, geographic separation, and patterns of dispersion (Kelly *et al*., 1999; Laffin *et al*., 2005; Ascunce *et al*., 2008).

2 Phylogenetics and diversification of Caribbean eustyline and geonemine weevils (Coleoptera: Curculionidae: Entiminae): untangling the effects of host plants and biogeography

2.1 Introduction

The West Indian archipelago is an extraordinary microcosm for assessing ecological and evolutionary hypotheses (Brown and Pestano, 1998; Emerson, 2002; Ricklefs and Bermingham, 2008). The complex history of this region is characterized by shifting grades of proximity to the North, Central, and South American continental land masses and among hundreds of islands with heterogeneous origins (Iturralde-Vinent, 2006; García-Casco *et al*., 2008; Ricklefs and Bermingham, 2008). These spatio-temporally shifting relationships have facilitated the distribution and diversification of the islands' present-day biota. At the same time, many islands were isolated for sufficient periods of time to accumulate endemic species or at least well-differentiated populations, and therefore display high rates of inter- and intra-island endemism (Ricklefs and Bermingham, 2008).

Weevils in the subfamily Entiminae (Coleoptera: Curculionidae) are well suited for analyzing the mechanisms of diversification in the West Indies. In particular, members of the Eustylini, Geonemini, and related tribes include several uniquely circumscribed lineages that are endemic to the Caribbean. One such lineage is the *Exophthalmus* genus complex, which contains *Exophthalmus* (86 species, approximately half of which are Caribbean endemics), *Diaprepes* (16 species, all are Caribbean endemics), and other genera such as *Pachnaeus* (Kuschel, 1955; Vaurie, 1961; Woodruff, 1985; O'Brien and Wibmer, 1982; Morrone, 1999; O'Brien and Kovarik, 2000). The taxonomic limits among these genera remain unresolved. In addition, *Exophthalmus* has been poorly delimited from other genera belonging to both the Eustylini, e.g.

Compsus and *Exorides*, and the Geonemini, e.g. *Apotomoderes* and *Lachnopus*, the latter with 58 species that are exclusive Caribbean endemics (see Franz, 2010a, 2010b; Girón and Franz 2010). The vast majority of these species is restricted to a single Caribbean island (O'Brien and Wibmer, 1982; Morrone, 1999; O'Brien and Kovarik, 2000), and consequently the complex holds much promise for quantitative historical biogeographic reconstructions (Page and Lydeard, 1994).

The most widely recognized paradigm explaining the evolutionary success of weevils and other phytophagous beetles holds that their high levels of diversity are causally grounded in their host plant associations (Ehrlich and Raven, 1964; Anderson, 1993, 1995; Farrell, 1998; Marvaldi *et al*., 2002; Oberprieler *et al*., 2007; McKenna *et al*., 2009; Franz and Engel, 2010). Under this scenario, the varied and often highly specialized interactions between beetles and plants have driven the diversification of both lineages via adaptive radiation and chemically mediated coevolution. However, it is questionable whether entimine weevils in particular present a good match with the coevolution paradigm since they tend to display broad and spatio-temporally unstable host ranges (Simpson *et al*., 1996; Machado, 2007; Oberprieler *et al*., 2007).

Here we assess the validity of the alternative hypotheses of coevolution versus historical biogeography as factors influencing the diversification of entimine species in a phylogenetic context. In particular, we present a molecular phylogenetic reconstruction for 62 species of primarily Caribbean eustyline and geonemine weevils, based on combined mitochondrial COI and partial nuclear EF-1α sequences (Caterino *et al*., 2000; Scataglini *et al*., 2005; McKenna *et al*., 2009). The areas sampled for this study include the islands of Dominica, Hispaniola, Jamaica, Mona, Puerto Rico, St. Lucia and Vieques. We use the phylogeny in combination with available host plant and distribution records to carry out quantitative tests for plant/insect cospeciation (Page, 1994) and dispersal-vicariance speciation (Ronquist, 1997), thereby determining which of the two best explains the inferred ancestral events of lineage splitting and diversification in the sampled taxa. This study constitutes the first molecular phylogenetic study of West Indian entimine weevils (though see Sequeira *et al*., 2000, 2008; and Scataglini *et al*., 2005, for analyses of South American lineages).

2.2 Materials and methods

2.2.1 Taxon sampling

The herein used concepts for entimine genera and species are adopted from O'Brien and Wibmer (1982) and Wibmer and O'Brien (1986). Species identifications were performed in consultation with Charles W. O'Brien (University of Arizona, Tucson, AZ); however, a large portion of the sampled taxa were identified only to the level of genus and morphospecies (Krell, 2004). Many of these species are likely new to science (cf. Franz and Girón, 2009; Franz, 2010b; Girón and Franz, 2010). A total of 59 species were sequenced anew, based on sampling efforts in a wide range of habitats in Colombia (primarily Cordillera Occidental), Dominica, the Dominican Republic (Hispaniola), Jamaica, Mona Island, Puerto Rico, St. Lucia, and Vieques Island (Fig. 2.1). The sampling scheme was supplemented with GenBank sequences of three non-Caribbean outgroup species (in phylogenetic sequence): *Scyphophorus* Schoenherr (Dryophthoridae; AY131110.1 and EF118297.1), *Sitona* Germar (Entiminae, Sitonini Gistel; AY131151.1 and EF577084.1), and *Galapaganus* Lanteri (Entiminae, Naupactini Gistel; AF015914.1 and EU748876.1). All taxa and collecting localities are listed in Table 2.1. Vouchers have been deposited in the University of Puerto Rico at Mayagüez Invertebrate Collection (UPRM-INVCOL; Franz and Yusseff Vanegas, 2009).

2.2.2 DNA extractions, PCR amplification and sequencing

Total genomic DNA was extracted from a single specimen of each weevil species. Depending on specimen size, either the entire specimen or just the thoracic musculature was used. The sampled material was ground individually with sterilized pestles in 1.7 ml Eppendorf tubes. DNA was extracted with DNeasy® Blood & Tissue Kit (Qiagen, USA). Genomic DNA was suspended in 200µl of RNAse-free ddH_20 and divided into aliquots of 1:10 in ddH_2O for PCR amplifications. DNA quality was evaluated by electrophoresis on 1% agarose gel.

Table 2.1. List of entimine weevil species and sampling localities of each individual used in this study.

Taxon	Distribution	Sampling locality	
Eudiagogini			
Promecops sp. 1	Dominica	N 15° 28' 42"	W 61° 23' 40"
Polydrusini			
Apodrosus argentatus Wolcott, 1924	Puerto Rico	N 18° 00' 40"	W 67° 06' 14"
Apodrosus epipolevatus Giron and Franz, 2010	Puerto Rico	N 18° 10' 00"	W 66° 35' 00"
Apodrosus quisqueyanus Giron and Franz, 2010	Dom. Rep.	N 18° 06' 45"	W 71° 36' 51"
Apodrosus sp. 1	Dom. Rep.	N 19° 02' 21"	W 70° 31' 05"
Apodrosus wolcotti Marshall, 1922	Puerto Rico	N 18° 10' 00"	W 66° 35' 00"
Tanymecini			
Pandeleteius nodifer Champion, 1911	Dominica	N 15° 28' 42"	W 61° 23' 40"
Pandeleteius sp. 1	Jamaica	N 17° 55' 45"	W 76° 53' 27"
Naupactini			
Litostylus sp. 1	Dominica	N 15° 25' 19"	W 61° 25' 31"
Litostylus sp. 2	Dominica	N 15° 23' 13"	W 61° 24' 06"
Eustylini I			
Eustylus hybridus Rosenschoeld, 1840	Dominica	N 15° 31' 35"	W 61° 27' 53"
Xestogaster sp. 1	Colombia	N 05° 04' 33"	W 75° 25' 56"
Xestogaster sp. 2	Colombia	N 05° 04' 33"	W 75° 25' 56"
Scelianoma elydimorpha	Puerto Rico	N 17° 58' 0"	W 66° 52' 0"
Geonemini			
Apotomoderes menecrater Franz, 2010	Dom. Rep.	N 18° 04' 32"	W 71° 39' 15"
Apotomoderes sotomayorae Franz, 2010	Mona Island	N 18° 05' 17"	W 67° 56' 16"
Artipus monae Wolcott, 1941	Mona Island	N 18° 05' 17"	W 67° 56' 16"
Artipus sp. 1	Puerto Rico	N 17° 58' 00"	W 66° 52' 00"
Artipus sp. 2	Dom. Rep.	N 18° 03' 47"	W 71° 08' 45"
Entiminae sp. 1	Dom. Rep.	N 17° 51' 44"	W 71° 38' 18"
Entiminae sp. 2	Jamaica	N 18° 08' 38"	W 77° 32' 16"
Entiminae sp. 3	Dom. Rep.	N 18° 04' 32"	W 71° 39' 15"
Entiminae sp. 4	Dom. Rep.	N 17° 51' 44"	W 71° 38' 18"
Entiminae sp. 5	Puerto Rico	N 17° 58' 00"	W 66° 52' 00"
Entiminae sp. 6	Vieques Island	N 18° 05' 42"	W 65° 25' 51"
Ischionoplus niveoguttatus Chevrolat, 1878	Dom. Rep.	N 19° 03' 41"	W 70° 51' 53"
Lachnopus coffeae Marshall, 1922	Puerto Rico	N 18° 10' 00"	W 66° 35' 00"
Lachnopus kofresi Wolcott, 1941	Mona Island	N 18° 05' 17"	W 67° 56' 16"
Lachnopus seini Wolcott, 1936	Puerto Rico	N 18° 10' 00"	W 66° 35' 00"
Lachnopus sp. 1	Dom. Rep.	N 17° 53' 02"	W 71° 15' 07"
Lachnopus sp. 2	Dom. Rep.	N 18° 21' 46"	W 70° 30' 50"
Lachnopus sp. 3	Dom. Rep.	N 18° 13' 53"	W 71° 09' 06"
Lachnopus sp. 4	Dominica	N 15° 28' 42"	W 61° 23' 40"
Eustylini II			
Compsus maricao Wolcott, 1924	Puerto Rico	N 18° 10' 00"	W 66° 35' 00"
Diaprepes abbreviatus Linnaeus, 1758	Puerto Rico	N 18° 27' 57"	W 67° 03' 09"

Diaprepes balloui Marshall, 1916	Dominica	N 15° 28' 42"	W 61° 23' 40"
Diaprepes boxi Marshall, 1938	St. Lucia		
Diaprepes famelicus Olivier, 1790	Dominica	N 15° 31' 35"	W 61° 27' 53"
Diaprepes maugei Boheman, 1840	Puerto Rico	N 18° 10 '00"	W 66° 35 '00"
Diaprepes sp. 1	St. Lucia		
Diaprepes sp. 2	Dominica	N 15° 28' 42"	W 61° 23' 40"
Exophthalmus quadrivittatus Schoenherr, 1823	Dom. Rep.	N 18° 29' 44"	W 71° 21' 30"
Exophthalmus marginicollis Chevrolat, 1880	Dominica	N 15° 28' 42"	W 61° 23' 40"
Exophthalmus quindecimpunctatus Olivier, 1807	Puerto Rico	N 18° 27' 00"	W 66° 36' 00"
Exophthalmus roseipes Chevrolat, 1876	Puerto Rico	N 18° 27' 28"	W 66° 26' 07"
Exophthalmus similis Drury, 1773	Jamaica	N 18°07' 07"	W 77° 01' 11"
Exophthalmus vittatus Schoenherr, 1823	Jamaica	N 18° 02' 15"	W 76° 23' 03"
Exophthalmus cinerascens Fabricius, 1792	Dom. Rep.	N 18° 32' 47"	W 68° 42' 27"
Exophthalmus sp. 1	Dom. Rep.	N 19° 02' 20"	W 70° 32' 40"
Exophthalmus sp. 2	Dom. Rep.	N 18° 03' 47"	W 71° 08' 45"
Exophthalmus sp. 3	Dom. Rep.	N 19° 02' 21"	W 70° 31' 05"
Exophthalmus sp. 4	Dom. Rep.	N 19° 02' 21"	W 70° 31' 05"
Exophthalmus sp. 5	Dom. Rep.	N 18° 50' 32"	W 70° 43' 30"
Exophthalmus sp. 6	Dom. Rep.	N 18° 58' 28"	W 70° 38' 54"
Exophthalmus sp. 7	Dom. Rep.	N 19° 2' 20"	W 70° 32' 40"
Exophthalmus sp. 8	Dom. Rep.	N 18° 54' 6"	W 69° 8' 21"
Exophthalmus sp. 9	Dom. Rep.	N 18° 50' 32"	W 70° 43' 30"
Pachnaeus sp.1	Jamaica	N 18° 3' 23"	W 76° 43' 29"
Pachnaeus marmoratus Marshall,	Jamaica	N 18° 10' 18"	W 76° 34' 8"

The primers used to amplify the targeted COI and EF-1α loci are listed in Table 2.2. Polymerase chain reactions were set up by mixing 8-10 µL of a 1:10 dilution of genomic DNA with a solution consisting of 0.2 µM of each primer, 0.2-0.3 µM of dNTPs, 2.5-3.5 mM of $MgCl_2$, 1x PCR Buffer, 0.2 µl of Go Taq® polymerase (Promega, USA) and deionized water to yield a total volume of 25-35 µl per reaction. The cycles for the COI locus were (1) 94°C for 30 seconds, (2) 47-51°C for 60 seconds, and (3) 72°C for 60 seconds; for a total of 35 cycles. The annealing temperature for the EF-1α locus varied from 55.0-55.8°C. In cases of ambiguous band amplification, sufficient quantities of PCR product were run on a 1.5% agarose gel from which the targeted band was cut out. The products were cleaned with a Wizard® SV Gel and PCR Clean-Op System Kit (Promega). Gene segments with low yield in the amplifications were cloned using the pGEM®-T Easy Vector System II (Promega). Plasmid DNA was isolated with Wizard® Plus SV Minipreps DNA Purification System (Promega); and 1% agarose gel was used to confirm the existence of the plasmid. The cloned band was then subjected to PCR reactions under the previous conditions, using 10 ng of plasmid DNA to confirm the presence of the targeted band.

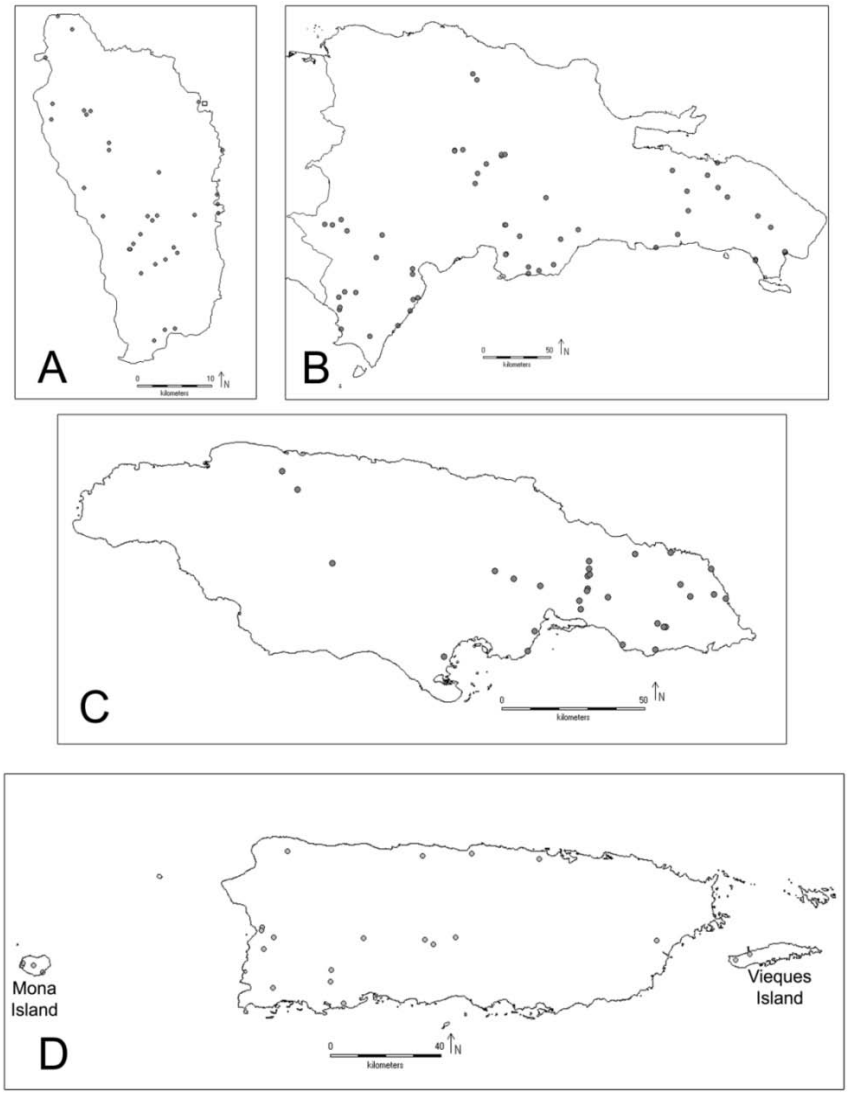


Fig. 2.1. Maps of collecting localities visited to acquire fresh weevil specimens for the DNA analyses. (A) Dominica; (B) Dominican Republic (Hispaniola); (C) Jamaica; (D) Greater Puerto Rico, including Mona, Culebra, and Vieques Islands.

The amplified and purified PCR products were processed at the Nevada Genomics Center (University of Nevada, http://www.cabnr.unr.edu/genomics/). The sequence chromatograms were examined and contiged in Sequencher™ 4.8 software (Gene Codes Corporation, USA). All sequences are deposited in GenBank under accession numbers: HQ891422-HQ891539.

Table 2.2. Primers used to amplify and sequence the COI and EF-1α genes of the targeted species, including primer reference publications.

Gene		Primer	Sequence
EF-1 α	Forward	Bo	5'– GCT GAG CGY GAR CGT GGT ATC AC –3' [a]†
	Reverse	Efa1106	5'– GTA TAT CCA TTG GAA ATT TGA CCN GGR TGR TT –3' [b]†
	Reverse	EfaV754	5'– CCA CCA ATT TTG TAG ACA TC –3' [b]†
	Reverse	Efa-923	5'– ACG TTC TTC ACG TTG AAR CCA A –3' [b]†
	Reverse	ER6	5'– AGG ATG GCA TCC AAA GCT TC –3' [f]‡
COI	Forward	K698	5'- TAC AAT TTA TCG CCT AAA CTT CAG CC –3' [c]†
	Forward	GF51940	5'– TAC ATA TAG CAG GTG TAT CAT C –3' [d]†
	Forward	s1541	5'– TGA KCY GGA ATA STA GGA ICA TC –3' [e]†
	Forward	CF7	5'– GCH ATT TTT AGH YTA CAH ATA GCH GG –3' [f]‡
	Reverse	a2771	5'– GGA TAR TCA GAR TAA CGT CGW GGT ATW C –3' [b]†
	Reverse	a2411	5'– GCT AAT CAT CTA AAA ACT TTA ATT CCW GTW G –3' [e]†
	Reverse	A3014	5'– TCC AAT GCA CTA ATC TGC CAT ATT A –3' [b]†
	Reverse	CR6	5'– CCD GCT ATD TGT ARD CTA AAA ATD GC –3' [f]‡

[a] Cho *et al*. (1995)
[b] Normark *et al*. (1999)
[c] Simon *et al*. (1994)
[d] Grobler *et al*. (2006)
[e] Ascunce *et al*. (2008)
[f] Present study
† Used to perform PCR and sequencing
‡ Used only to for sequencing

2.2.3 Phylogenetic analyses

Sequence fragments of COI and EF-1α were aligned using Muscle 3.6 (Edgar, 2004). The alignments were further subjected to eye inspection and translated into amino acids to check for the presence of an open reading frame. We used a comprehensive analytical approach in order to assess the strength and consistency of the molecular phylogenetic signal under a range of plausible evolutionary assumptions; viz. parsimony, maximum likelihood (ML) and Bayesian inference (Goloboff, 2003; Holder and Lewis, 2003). Partitioned parsimony analyses of COI and EF-1α were performed in light of apparent differences in the phylogenetic signal emitted from each gene (ILD test; P = 0.01; cf. Farris *et al*., 1995). In spite of these differences, we opted to combine matrices in order to obtain a consolidated phylogeny and explore the possibility of signal amplification across multiple loci (Wenzel and Siddall, 1999). Separate and combined parsimony topologies were obtained using the ratchet (Nixon, 1999) in TNT (Goloboff *et al*., 2008), spawned with ASADO, version 1.85 (Nixon, 2008).

Heuristic tree searches were conducted using 500 ratchet iterations, 10 rounds of tree-fusing, 100 cycles of tree-drifting (Goloboff, 1999a), 900 random addition sequences, and by finding the best scores three times. Maximum likelihood analyses were performed with RaxML,

version 7.2.2 (Stamatakis, 2006), thus permitting six independent optimizations of substitution rates according to each gene and codon position. The best scoring tree was found performing 1000 replicates of ML searches. In each case the analyses were started with a maximum parsimony tree (Stamatakis, 2006) and developed under the rapid bootstrapping algorithm (Stamatakis *et al*., 2008). Finally, Bayesian analyses were conducted with Mr Bayes, version 3.1.2, which treats different data partitions according to different stochastic models (Ronquist and Huelsenbeck, 2003). The most fitting evolutionary models for each of the six partitions (two genes x three codon positions) were estimated with jModelTest under the Bayesian Information Criterion (BIC) (Posada, 2008). Four simultaneous chains were run for four million generations while sampling every 100^{th} generation for a total of 40,001 trees per chain. A graphic representation of the likelihood values of each MCMC chain was obtained using the trace function of the software Tracer, version 1.5 (Rambaut and Drummond, 2009). Effective sample size (ESS) measures were recorded to test the efficiency of the sampling and convergence (Drummond *et al*., 2002). The first 1,000 trees were removed as burn-in as indicated by the results obtained through Tracer. The various phylogenetic results are summarized using either strict consensus and/or majority rule trees (Nixon and Carpenter, 1996a). Bootstrap support (Felsenstein, 1985) for individual branches on the final trees was calculated performing 1000 pseudoreplicates with NONA and ASADO (parsimony) and RaxML (ML). Posterior clade probabilities were generated from the Bayesian inference and interpreted as the frequencies of samples recovering particular clades (Huelsenbeck and Ronquist, 2001).

2.2.4 Testing for insect/plant cospeciation

Based on the preferred phylogenetic framework and a synthesis of host records and host phylogeny we attempted to test for the presence of cospeciation, or parallel cladogenesis, between the weevil species and their host plants (Brooks, 1981; Mitter and Farrell, 1991; Page, 2003). The test was limited to 25 of the 62 sampled weevil species for which such records were available. Host plant associations were extracted from various published sources (Wolcott, 1948; Dixon, 1954; Vaurie, 1961; Martorell, 1976; Maes and O'Brien, 1990; Simpson *et al*., 1996; Franz, 2010b; Girón and Franz, 2010) and supplemented with personal observations (Table 2.3). The often imprecise and unspecific records were represented at the lowest taxonomic level, i.e.

that of plant order, that could be applied consistently throughout the 25 weevil species. The host phylogeny was adopted from the Angiosperm Phylogeny Groug (APG III, 2009).

Table 2.3. List of host plant orders associated with 25 sampled entimine weevil taxa for which such information is available. See Appendix 1 for details.

Taxa	# orders	Plant order names
Eudiagogini		
Promecops sp. 1	1	Gentianales
Polydrusini		
Apodrosus argentatus	4	Brassicales, Fabales, Rosales, Zygophyllales
Apodrosus wolcotti	1	Fabales
Tanymecini		
Pandeleteius sp. 1	7	Boraginaceae, Caryophyllales, Fabales, Gentianales, Laurales, Malpighiales, Myrtales
Pandeleteius nodifer	3	Fabales, Lamiales, Malvales
Naupactini		
Litostylus sp. 1	2	Malpighiales, Sapindales
Litostylus sp. 2	1	Myrtales
Eustylini I		
Eustylus hybridus	2	Fabales, Myrtales
Geonemini		
Apotomoderes menecrater	2	Myrtales, Zygophyllales
Apotomoderes sotomayorae	1	Sapindales
Artipus monae	3	Fagales, Sapindales, Solanales
Lachnopus coffeae	2	Gentianales, Sapindales
Lachnopus kofresi	1	Solanales
Lachnopus seini	1	Ericales
Eustylini II		
Compsus maricao	3	Gentianales, Myrtales, Rosales
Diaprepes abbreviatus	19	Apiales, Arecales, Asparagales, Asterales, Brassicales, Dioscoreales, Ericales, Fabales, Fagales, Gentianales, Malpighiales, Malvales, Myrtales, Poales, Rosales, Sapindales, Solanales
Diaprepes balloui	2	Myrtales, Sapindales
Diaprepes famelicus	5	Fabales, Laurales, Myrtales, Poales, Sapindales.
Exophthalmus cinerascens	1	Solanales
Exophthalmus quadrivittatus	6	Ericales, Fabales, Gentianales, Sapindales, Solanales, Rosales
Exophthalmus quindecimpunctatus	1	Caryophyllales
Exophthalmus roseipes	7	Caryophyllales, Celastrales, Fabales, Malpighiales, Malvales, Myrtales, Sapindales
Exophthalmus similis	4	Gentianales, Malvales, Rosales, Sapindales
Exophthalmus vittatus	2	Fabales, Sapindales
Pachnaeus marmoratus	2	Dioscoreales, Sapindales

TreeMap, version 1.0 (Page, 1995) was used to reconcile the weevil and host plant phylogenies in an attempt to identify past events of parallel cladogenesis (Page, 1994; Franz, 2004). TreeMap generates a tanglegram and reconciled tree analysis that maximizes the number of cospeciation events while minimizing host switching and sorting events (extinction of hosts). In addition, a TreeMap randomization procedure is available to test whether the number of inferred cospeciation events is expected through chance alone.

2.2.5 Event-based historical biogeographic reconstructions

DIVA (dispersal-vicariance), version 1.1 (Ronquist, 1997) was used to optimize ancestral distributions on the internal branches of the weevil phylogeny and thereby infer occurrences of vicariance and colonization events. The program employs a step-matrix optimization algorithm that assigns specific costs for vicariance, colonization, and extinction events (Ronquist, 1997; Sanmartín, 2006; Morrone, 2009). Each of the 62 weevil species was assigned to one or more areas of endemism, which either correspond to widely recognized Latin American biogeographic regions or individual Caribbean islands (Morrone, 2006); i.e. (in alphabetical order) Central America, Dominica, Florida (southeastern United States), Guadeloupe, Hispaniola, Jamaica, Martinique, Mona Island (Puerto Rico), Nevis, Puerto Rico (main island), Saint Lucia, South America, and Vieques Island (Puerto Rico). Because DIVA requires a fully resolved input tree, we chose to utilize a consensus tree derived from the Bayesian analysis and subsequently edited with TreeAnnotator, version 1.5.4 (Rambaut and Drummond, 2010). This procedure was needed as an operational constraint to resolve all polytomies in the consensus; however, no evolutionary inferences were made regarding these unresolved nodes. The maximum number of ancestral areas was permitted to vary from 2-5 (Ronquist, 1997).

2.3 Results

2.3.1 Taxon sampling and molecular properties

The 62 entimine taxa included 57 species of Caribbean origin and represent at least 15 genera and seven tribes, including the provisionally named "Eustylini I" and "Eustylini II" (Table 2.1). Species identifications proved challenging in many cases given the scarcity of suitable identification resources and high likelihood that many of the sampled species represent genera or species new to science. Consequently, only 28 taxa (49.1%) were identified to species, whereas 23 taxa (40.4%) were classified to the level of genus and morphospecies (e.g. *Lachnopus* sp. 1), and six taxa (10.5%) were identified to the level of subfamily. Using the current classification (Alonso-Zarazaga and Lyal, 1999), *Exophthalmus* (16 species), *Diaprepes* (7 species), and *Lachnopus* (7 species) were the most diverse genera sampled. Select Caribbean members of the tribes Eudiagogini LeConte, Polydrusini Schoenherr, and Tanymecini Lacordaire are among the outgroup lineages. Fifty-three (93.0%) of the 57 species sampled in the Caribbean are purportedly endemic to a single island; of these, 21 species (39.6%) occur in Hispaniola and 13 species (24.5%) inhabit Puerto Rico, followed by the islands of Dominica (eight species; 15.1%), Jamaica (five species; 9.4%), Mona (three species; 5.7%), St. Lucia (two species; 3.8%), and Vieques (one species; 1.9%).

Descriptive data for each of the aligned genes are shown in Table 2.4. The aligned COI sequences had a length of 1229 base pairs (bp) without stop codons, yielding 496 parsimony informative characters. The aligned EF-1α matrix was limited to an exon sequence of 449 bp, with 122 parsimony informative characters. Thus the concatenated matrix had a length of 1678 bp, including 618 parsimony informative characters. The substitution model parameters for each gene and codon position under maximum likelihood and Bayesian inference are given in Table 2.5. Overall the COI gene showed more variation than the EF-1α gene. In accordance with the variation in the shape of the gamma (γ) distribution (Drummond *et al*., 2007), the third positions of each gene displayed relatively low and homogeneous rates of variation across sites, whereas the second positions showed significantly higher rates of among-site variation.

Table 2.4. Molecular and analytical properties of the sampled CO1 and EF-1α loci under parsimony, maximum likelihood, and Bayesian inference.

	COI	EF-1α	Combined
Sequence properties			
Sequence length	1229	449	1678
Average A+T ratio (%)	67.24	58.32	64.86
Number of constant sites	652	299	951
Analytical results			
Parsimony informative sites	496	122	618
Tree length	5895	615	6607
Number of MPT	5	38	1
CI	16	36	18
RI	38	68	41
ML –ln	-21555.19762	-3988.00026	-25219.30300
BI –ln	-23890.33900	-3975.09100	-28621.88900

2.3.2 Phylogenetic relationships

In spite of notable differences in the placement of select "floating" taxa, the composition and phylogenetic relationships of major clades were consistent among the total evidence topologies obtained under parsimony (Fig. 2.2), maximum likelihood (Fig. 2.3), and Bayesian inference (Fig. 2.4). In particular, all analyses recovered seven putative tribal lineages; viz. (in phylogenetic sequence) Eudiagogini, Polydrusini, Tanymecini, Naupactini, "Eustylini I", Geonemini, and "Eustylini II" (see details below). We retrieved as the ingroup a monophyletic Eustylini-Geonemini tribal complex, placed as sister to an outgroup clade representing the Naupactini and Tanymecini. However, the Eustylini in the present sense (Alonso-Zarazaga and Lyal, 1999) were not recovered as monophyletic, but instead are separated into two distinct lineages: the South American Eustylini I and the Caribbean Eustylini II (see, e.g., Fig. 2.4). The South American clade is strongly supported in all three analyses and is represented by *Eustylus hybridus* Rosenschoeld and the Colombian species of *Xestogaster* Marshall. The similarly well-supported Eustylini II, in turn, is exclusively of Caribbean origin. Depending on the method of inference, the Eustylini I are either placed as sister to a Eustylini II-Geonemini clade (parsimony; Fig. 2.2), or all three lineages and *Scelianoma* Franz & Girón form a polytomy (maximum

likelihood and Bayesian inference; Figs. 2.3 and 2.4). Several less inclusive lineages showed congruent results across all three analyses, including the *Lachnopus* complex, *Diaprepes*, and the "*Exophthalmus* I" clade (Fig. 2.4). The specific inferences derived from each type of analysis and relative support for clades are summarized in the following paragraphs.

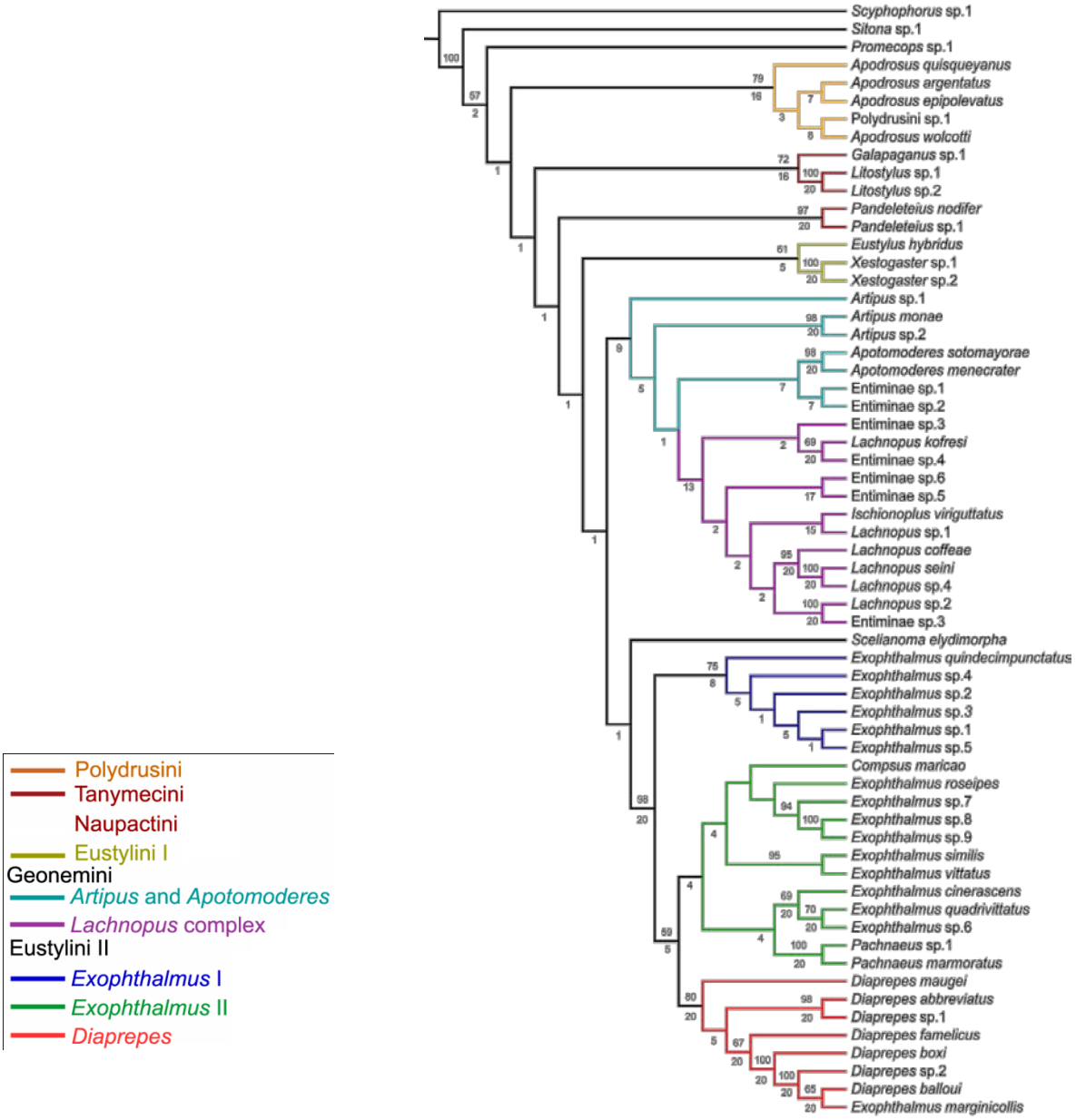


Fig. 2.2. Single most parsimonious tree of the combined COI and EF-1α matrix of entimine weevils. Bootstrap and Bremer support values are shown above and below each branch, respectively. See Table 2.4 for tree statistics. Color-codings indicate different focal lineages and are used consistently throughout Figs. 2.2 to 2.6.

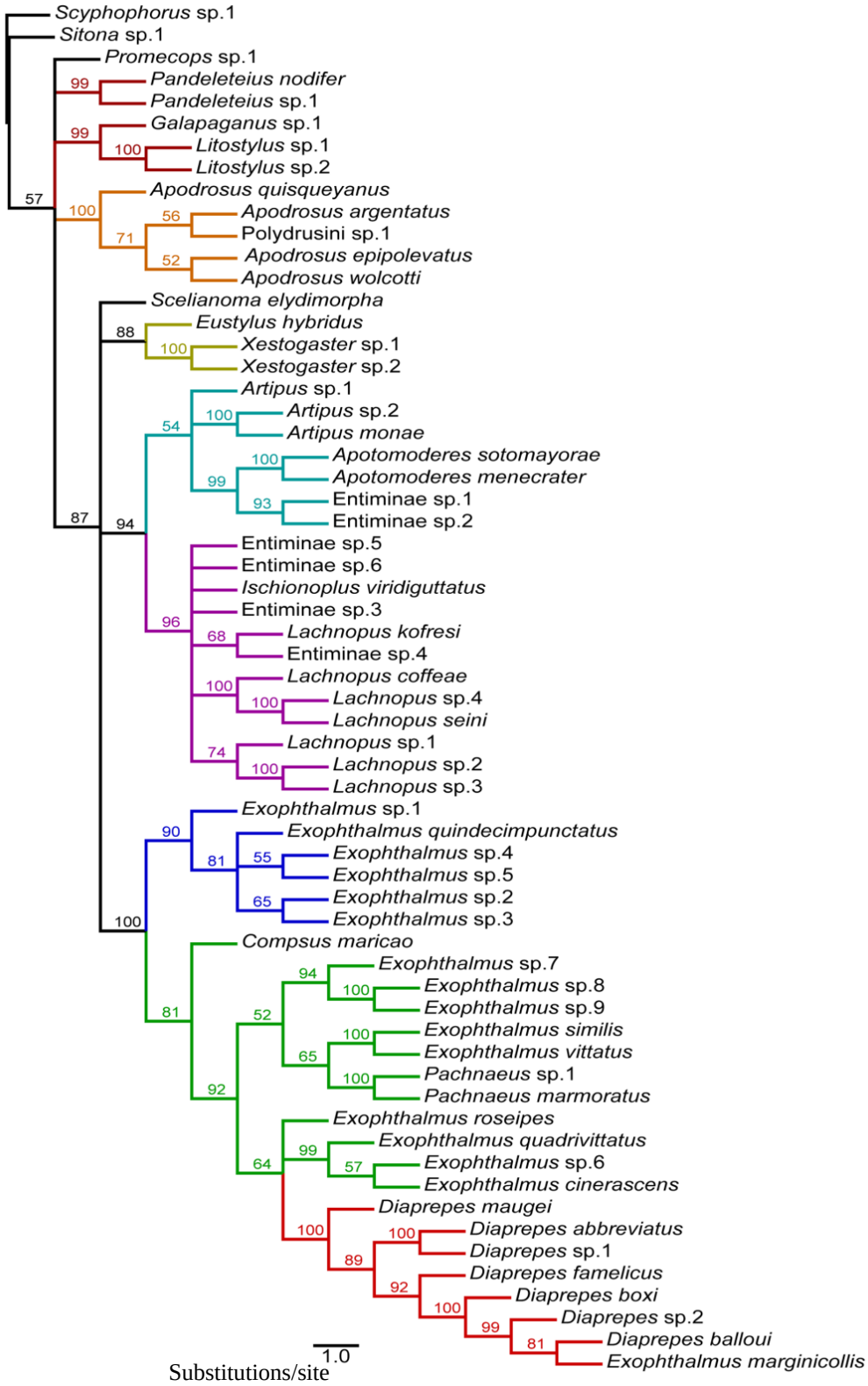


Fig. 2.3. Majority rule consensus tree resulting from combined COI and EF-1α analysis under maximum likelihood, including bootstrap values (threshold set at 50%).

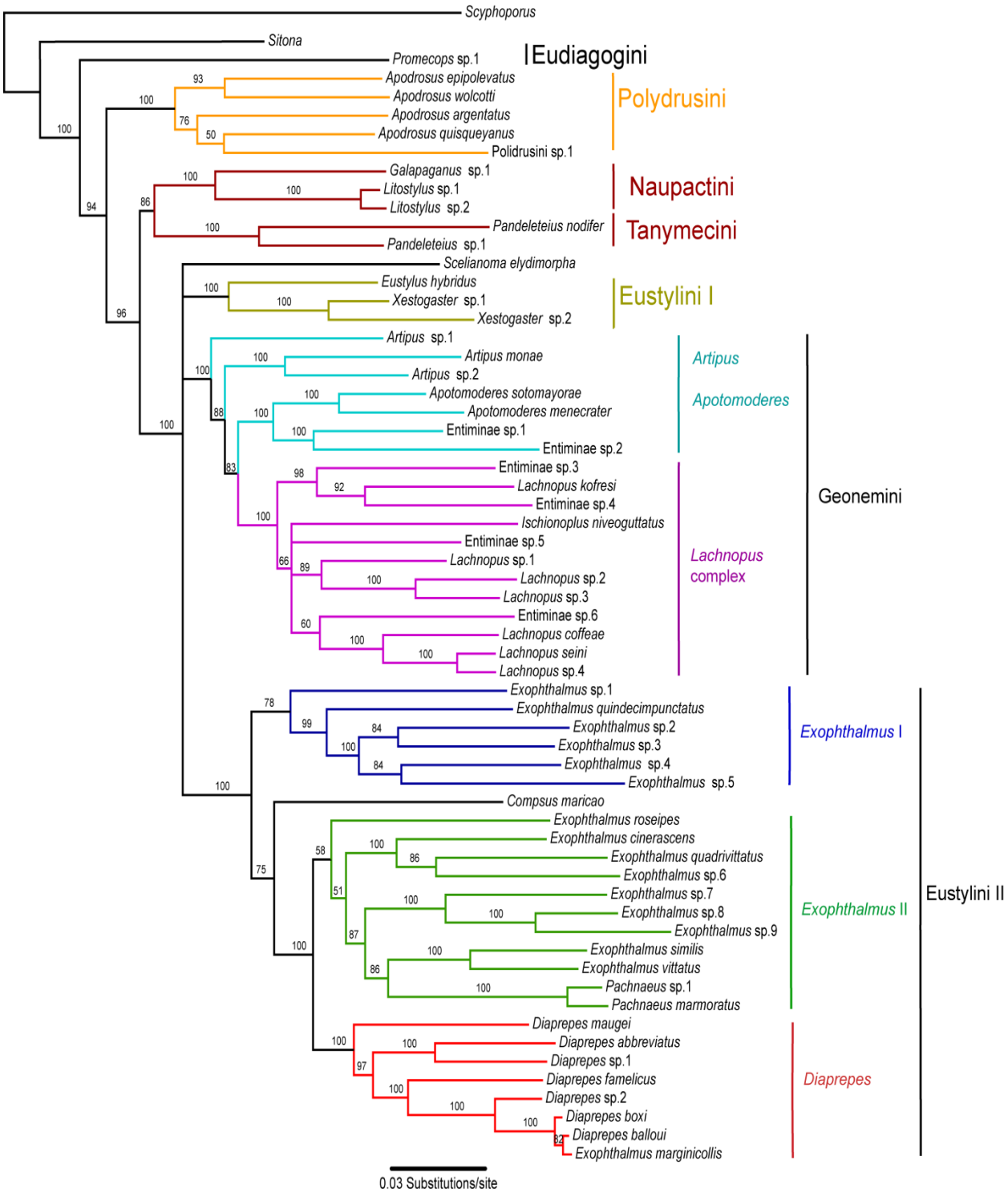


Fig. 2.4. Majority rule consensus resulting from COI and EF-1α analysis under Bayesian inference, including posterior probabilities (threshold set at 50%).

The partitioned parsimony analyses revealed gene-specific differences in the resolution of earlier versus later splitting events in the phylogeny (Figs. 2.5A and 2.5B). Overall the nuclear EF-1α shows higher consistency and retention indices in comparison to the mitochondrial COI (Table 2.4). Cytochrome oxidase I provides insufficient resolution and clade support among the most ancestral nodes, including the targeted Caribbean clades where taxon sampling was more comprehensive. For instance, COI resolves only eight nodes in the Geonemini, compared with 16 such nodes resolved by EF-1α. Conversely, COI yielded more resolution along the more recent nodes, including (e.g.) *Apodrosus* Marshall, *Diaprepes*, and more generally the Eustylini lineages where this gene recognizes 20 nodes as opposed to 13 nodes inferred by EF-1α. The placement of five species is strongly diverging between the two genes, and largely accounts for the apparent incongruence; i.e., *Compsus maricao* Wolcott, Entiminae sp. 4, *Ischionoplus viridiguttatus* Chevrolat, *Promecops* sp. 1, and *Scelianoma elydimorpha* Franz & Girón. Other groups within *Diaprepes* and *Lachnopus* were recovered consistently by the two genes (Figs. 2.5A and 2.5B).

The combined parsimony analysis yielded a single most parsimonious tree (L = 6607 steps, CI = 18, RI = 41; see also Table 2.4). While this topology is fully resolved (Fig. 2.2), it displays a lower number of branches with bootstrap support higher than 50% in comparison to the other analyses. Both the maximum likelihood (Fig. 2.3) and Bayesian topologies (Fig. 2.4) were less resolved yet showed more and higher levels of support for major clades than parsimony. Accordingly, the monophyly of members of a putative Geonemini clade is strongly supported, grouping together the genera *Artipus* Schoenherr, *Apotomoderes*, *Ischionoplus* Chevrolat, *Lachnopus*, and several additional taxa that are undescribed. *Artipus*, conventionally placed in the Naupactini (cf. Alonso-Zarazaga and Lyal, 1999), is herein inferred to represent the sister taxon of the remaining Caribbean Geonemini, whereas *Apotomoderes* and a closely related yet undescribed genus are sister to the species-rich *Lachnopus* genus complex. However, *I. viridiguttatus* and the undescribed Entiminae species 3, 5 and 6 have unstable placements among three well-supported clades within *Lachnopus* (Fig. 2.3). None of the analyses suggest an overlap among the *Lachnopus* complex and any eustyline genera, thus reaffirming their traditional tribal placements.

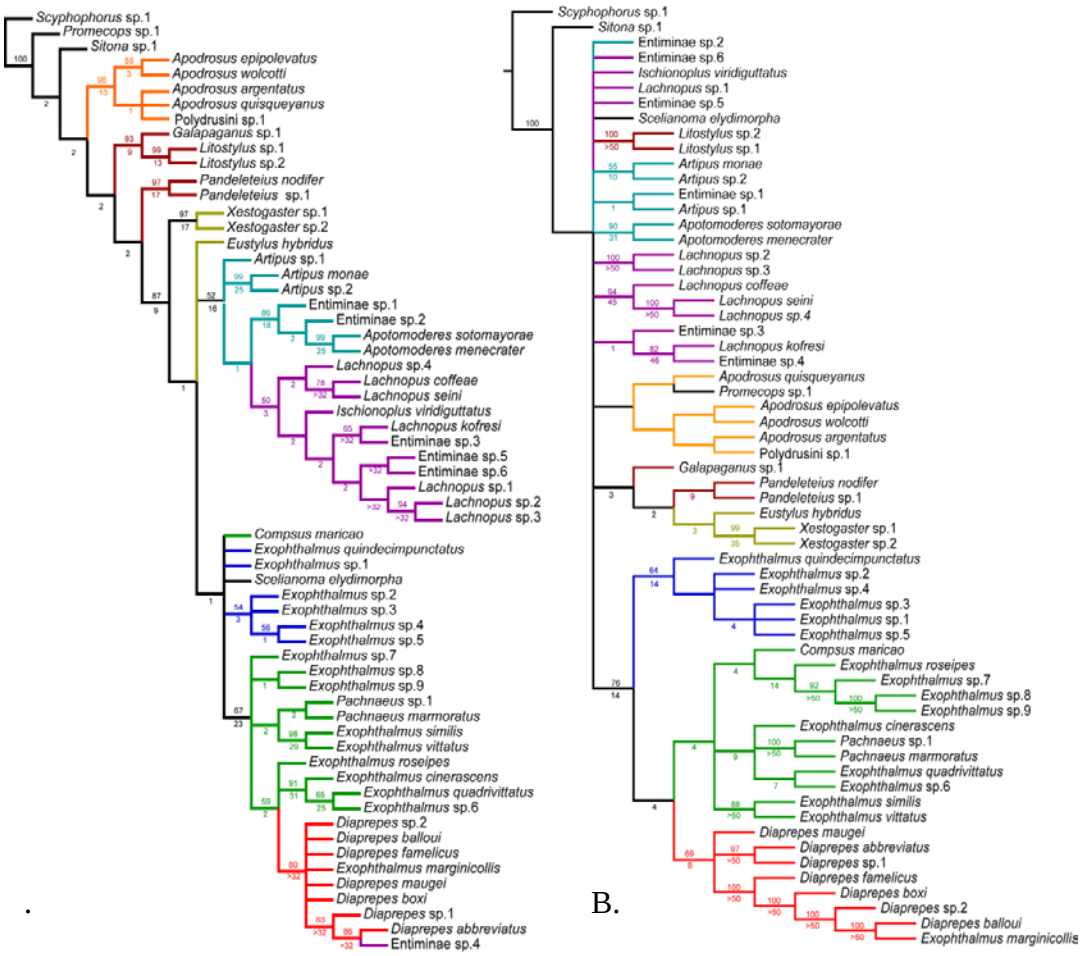


Fig. 2.5. Strict consensus trees of partitioned parsimony analyses of (A) EF-1α and (B) COI gene sequences. Bremer and bootstrap support as Fig. 2.2. See Table 2.4 for tree numbers and statistics for each partitioned analysis

Within the Caribbean Eustylini II clade, the large genus *Exophthalmus* is evidently not monophyletic. The genus is separated in two species groups, herein named here *Exophthalmus* I and *Exophthalmus* II (Fig. 2.4). The *Exophthalmus* I clade is sister to the remaining members of the Caribbean eustylines. This clade is supported in all three analyses; however, the relationships among its constituent species are not consistent among analyses (compare, e.g., Figs. 2.3 and 2.4). *Exophthalmus* II includes the genus *Pachnaeus*, which is traditionally placed in the Tanymecini, the Jamaican species *E. similis* Drury and *E. vittatus* Schoenherr, and numerous undescribed species. Both parsimony and Bayesian inference position *Diaprepes* as the sister group of *Exophthalmus* II, whereas maximum likelihood infers that a clade comprised of *E. cinerascens* Fabricius, the type species *E. quadrivittatus* Schoenherr, *E. roseipes* Chevrolat, and *Exophthalmus* sp. 6 is most closely related to *Diaprepes* (Fig. 2.3).

Table 2.5. Substitution model parameters for each position in the COI and EF-1α gene sequences. ML values were estimated using RaxML (Stamatakis, 2006) and the Bayesian parameters were computed with jModeltest (Posada, 2008). See Huelsenbeck and Crandall (1997) for descriptions of model properties.

Gene	**COI**						**EF-1α**					
Analysis	**ML**			**Bayesian**			**ML**			**Bayesian**		
Codon position	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**
Model	GTR	GTR	GTR	SYM+I+G	HKY+G	GTR+G	GTR	GTR	GTR	JC+G	JC+G	K80+G
Base frequencies												
A	0.302859	0.171450	0.434152	0.25	0.1789	0.4463	0.317591	0.323584	0.293287	-	-	-
C	0.158803	0.236035	0.113806	0.25	0.2400	0.1164	0.133478	0.248510	0.242776	-	-	-
G	0.286907	0.162331	0.024457	0.25	0.1583	0.0252	0.369555	0.154154	0.101309	-	-	-
T	0.251431	0.430184	0.427585	0.25	0.4228	0.4121	0.179376	0.273751	0.362629	-	-	-
Substitution rates												
A – C	0.449854	2.3657430	0.014110	0.3356	-	0.0293	0.231069	3.831093	0.822610	-	-	-
A – G	3.912354	13.063476	11.29091	4.9529	-	11.179	0.204946	1.567488	8.628227	-	-	-
A – T	1.135593	3.123616	0.000017	1.5861	-	0.0889	0.089237	0.000017	1.113260	-	-	-
C – G	0.000017	10.392684	0.935611	0	-	0.9664	0.543405	2.100176	1.616763	-	-	-
C – T	49.26019	8.2754660	7.562164	35.587	-	7.8238	3.175356	1.308800	8.934736	-	-	-
G – T	1	1	1	1	-	1	1	1	1	-	-	-
Gamma	0.136687	0.0200130	0.510554	0.1870	0.0110	0.3340	0.106494	0.020013	1.004626	0.011	0.016	1.02
Invsites	-	-	-	0.1760	0	0	-	-	-	0	0	0
tn/tv ratio	-	-	-	-	1.4854	-	-	-	-	-	-	4.4619

The intriguing position of *C. maricao* as sister to the *Diaprepes-Exophthalmus* II clade is supported by both maximum likelihood and Bayesian inference, whereas parsimony places this species within *Exophthalmus* II. *Diaprepes* itself shows high support and a stable internal topology in all analyses. Moreover, *E. marginicollis* Chevrolat is consistently nested within *Diaprepes.* Lastly, the enigmatic *Scelianoma elydimorpha,* provisionally assigned to the Eustylini by Franz and Girón (2009), occupies various sister group positions to either the Eustylini II (parsimony; Fig. 2.2) or the entire eustyline-geonemine complex (Figs. 2.3 and 2.4).

Among the outgroup lineages, the monophyly of the polydrusine genus *Apodrosus* is well supported (see also Girón and Franz, 2010). The Naupactini and Tanymecini are closely related tribes, although this clade was only recovered by Bayesian inference (Fig. 2.4). *Promecops* Sahlberg (Eudiagogini) is placed in a sister group relationships to the sampled Caribbean lineages (Figs. 2.2 and 2.4).

2.3.3 Insect/plant cospeciation

The following results are derived from the Bayesian topology (Fig. 2.4) which was selected primarily because of an apparent congruence with emerging morphological results (NMF, unpublished data), and furthermore because it shows more resolution and higher support values than the maximum likelihood tree (though see Randle and Pickett, 2010). The parsimony topology was not further considered because of the possibility of long branches (Bergsten, 2005) or insufficient sampling density affecting the variable placement of the species *C. maricao, E. roseipes, I. viridiguttatus, S. elydimorpha,* and taxa within the *Exophthalmus* I clade (compare Figs. 2.2 and 2.4). The main conclusions of this analysis will likely hold regardless of the preferred method of phylogenetic inference.

The available host plant records for 25 Caribbean entimine species are shown in Table 2.3 (see also Appendix 1). Establishing specific host plant association was challenging due to the high number of undescribed weevil species and the scarcity of natural history data available on the literature. The herein listed records may be regarded as a minimum baseline of hosts for each species (cf. Simpson *et al*., 1996). Only 25 of the 62 sampled species were demonstrably

associated with at least one host plant order according to the available data. In all, 24 orders of angiosperms are used by these weevil species, with little or no apparent specificity and consistency. Pest species such as *D. abbreviatus, E. roseipes,* and *E. quadrivittatus,* tended to have a higher and more taxonomically diverse number of records. On the other hand, *A. wolcotti* Marshall,, *A. sotomayorae* Franz, *E. cinerascens, E. quindecimpunctatus, L. kofresi* Wolcott, and *L. seini* Wolcott have been reported to occur on a single plant order (Table 2.3). Seven non-pest species were associated with two plant orders and three species with three plant orders.

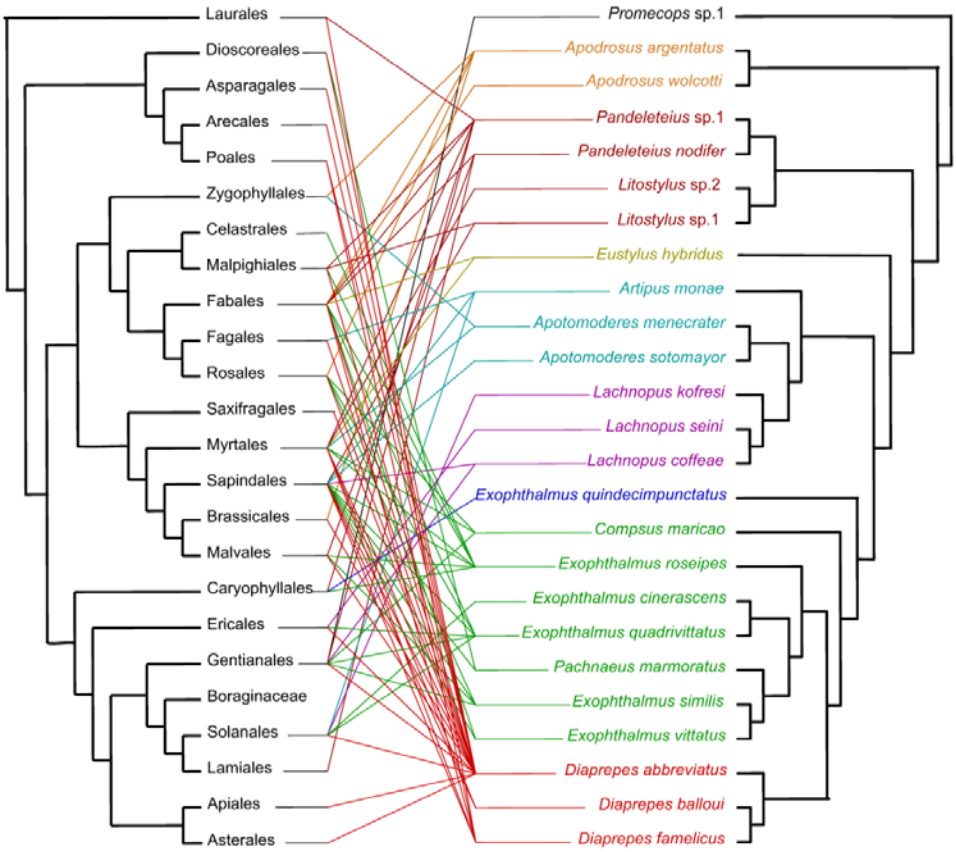


Fig. 2.6. Tanglegram juxtaposing host plant phylogeny (left) and weevil phylogeny (right), including lines that represent known associations. Color-codings as in Fig. 2.2.

The outcome of the reconciled tree analysis reveals a noisy pattern, with no events of cospeciation or phylogeny-tracking host switches (Fig. 2.6). Particular host plant orders such as the Sapindales, Fabales and Myrtales each harbor as many as 9-12 weevil species. In total, 24 duplications and 193 sorting events were required in order to fit the weevil tree onto the host

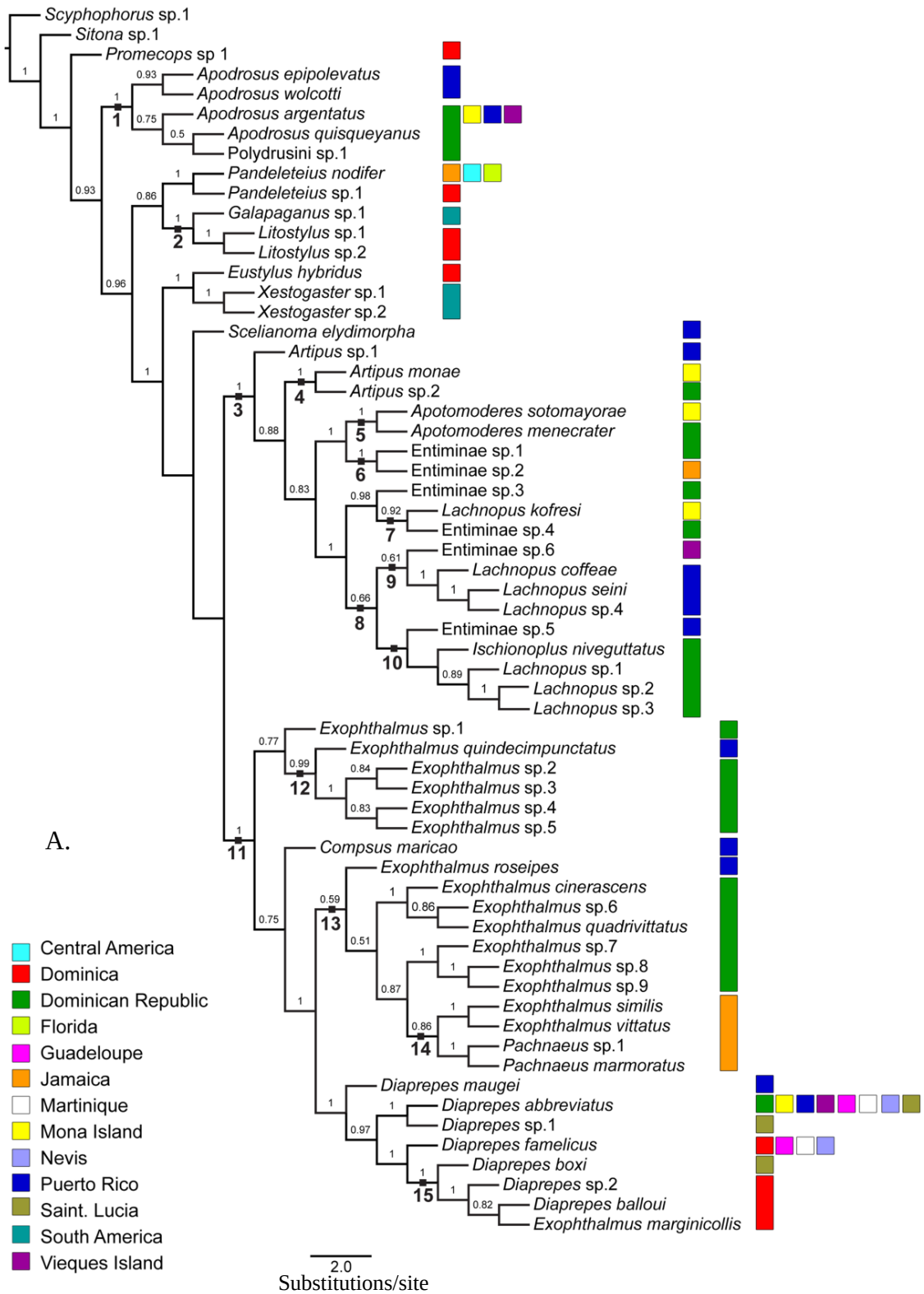
phylogeny (Fig. 2.6). Based on the TreeMap randomization test, we would expect 0-2 cospeciation events by chance alone in 986 out of 1000 cases. Thus the results strongly indicate that neither events of cospeciation nor host lineage tracking were represented in the analysis.

2.3.4 Historical biogeographic reconstructions

In contrast with the host plant analysis, DIVA reconstructions of ancestral areas under the commands "max areas 2, 3, and 4" revealed that minimally 15 splits among the 58 internal nodes are congruent with among-island separations (Fig. 2.7A). Different biogeographic patterns were obtained for each of the larger clades. Within the Geonemini alone, at least eight speciation events are correlated with the occupation of new areas. Three of these follow a shared directionality, with an origin in Hispaniola and dispersing to Mona Island, whereas one to Jamaica (Fig. 2.7.A). Hispaniola is inferred as the ancestral area for *Apotomoderes* (cf. Franz, 2010b) and its sister group including species of *Artipus* and *Lachnopus.* The Puerto Rican group of *L. coffeae* Marshall and *L. seini* has a sister species Entiminae sp. 6 on Vieques Island; however the ancestral area for this clade remains unclear.

The ancestral distribution of the Eustylini II clade is inferred to be either Hispaniola or Puerto Rico, or both. Within this group, at least four speciation events are correlated with a historical biogeographic separation. In particular, the *Exophthalmus* I clade is postulated to have originated in the Dominica Republic, but is furthermore represented by *E. quinquedecimpuntactus* Olivier in Puerto Rico. On the other hand, the ancestral area for the *Exophthalmus* II clade is ambiguously optimized as either Hispaniola or Puerto Rico. Most of the species in this clade occur on Hispaniola, although there is a single putative colonization event of Jamaica with multiple subsequent speciations within the latter island.

Finally, no unambiguous ancestral area was obtained for *Diaprepes* at the genus level given the confounding effects of the widespread species *D. abbreviatus* and *D. famelicus* Olivier (Fig. 2.7A). However, if Puerto Rico is assumed as the area of origin for *D. abbreviatus* (Lapointe, 2004), then this island may also represent the ancestral area of the genus, with subsequent colonization of and speciation within the Lesser Antilles.


Scyphophorus sp.1
Sitona sp.1
Promecops sp 1
Apodrosus epipolevatus
Apodrosus wolcotti
Apodrosus argentatus
Apodrosus quisqueyanus
Polydrusini sp.1
Pandeleteius nodifer
Pandeleteius sp.1
Galapaganus sp.1
Litostylus sp.1
Litostylus sp.2
Eustylus hybridus
Xestogaster sp.1
Xestogaster sp.2
Scelianoma elydimorpha
Artipus sp.1
Artipus monae
Artipus sp.2
Apotomoderes sotomayorae
Apotomoderes menecrater
Entiminae sp.1
Entiminae sp.2
Entiminae sp.3
Lachnopus kofresi
Entiminae sp.4
Entiminae sp.6
Lachnopus coffeae
Lachnopus seini
Lachnopus sp.4
Entiminae sp.5
Ischionoplus niveguttatus
Lachnopus sp.1
Lachnopus sp.2
Lachnopus sp.3
Exophthalmus sp.1
Exophthalmus quindecimpunctatus
Exophthalmus sp.2
Exophthalmus sp.3
Exophthalmus sp.4
Exophthalmus sp.5
Compsus maricao
Exophthalmus roseipes
Exophthalmus cinerascens
Exophthalmus sp.6
Exophthalmus quadrivittatus
Exophthalmus sp.7
Exophthalmus sp.8
Exophthalmus sp.9
Exophthalmus similis
Exophthalmus vittatus
Pachnaeus sp.1
Pachnaeus marmoratus
Diaprepes maugei
Diaprepes abbreviatus
Diaprepes sp.1
Diaprepes famelicus
Diaprepes boxi
Diaprepes sp.2
Diaprepes balloui
Exophthalmus marginicollis
1
2
3
4
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8
9
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11
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14
15
A.
Central America
Dominica
Dominican Republic
Florida
Guadeloupe
Jamaica
Martinique
Mona Island
Nevis
Puerto Rico
Saint. Lucia
South America
Vieques Island
2.0
Substitutions/site

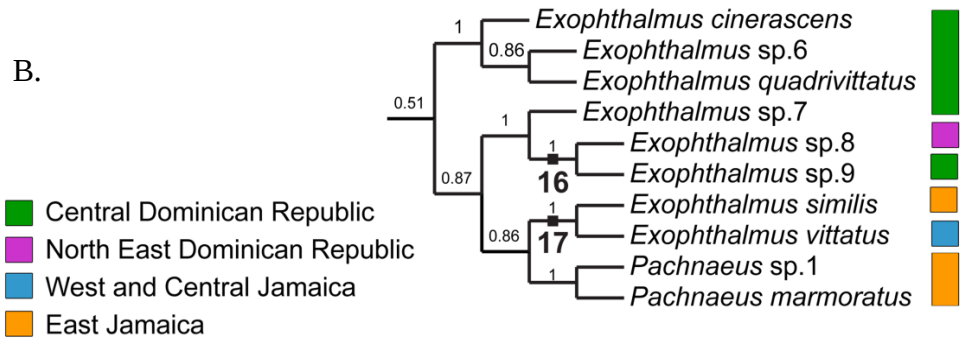

Fig. 2.7. DIVA reconstruction of ancestral areas optimized along the internal branches of the weevil reference phylogeny under Bayesian inference (see Fig. 2.4). (**A**) Caribbean region and surrounding source areas; (**B**) separate analysis of *Exophthalmus* II clade in Hispaniola and Jamaica. Color-codings represent proposed areas of endemism. Posterior probabilities are above branches. Split events are numbered in bold below the branches and represented by a black square.

Analyses of within-island patterns of the *Exophthalmus* II clade for Hispaniola and Jamaica reveals additional biogeographic patterns (Fig. 2.7B). Specifically, within Hispaniola, the Central Cordillera is inferred as the ancestral area of *E. cinerascens*, *E. quadrivittatus*, and *Exophthalmus* sp. 6, 7 and 9. Within this group, *Exophthalmus* sp. 8 has apparently colonized the northeastern region of the island. The entire subclade is sister to the Jamaican species group, which in turn shows a split of three eastern species and *E. vittatus* occupying the central and western region of Jamaica (Fig. 2.7B; see also Vaurie, 1961).

Due to limited outgroup representation; few biogeographic patterns are unambiguously resolved among and within the tribes Eudiagogini, Polydrusini, Naupactini and Tanymecini. However, Puerto Rico is resolved as the ancestral area of *A. epipolevatus* Girón & Franz and *A. wolcotti* Marshall, whereas Hispaniola is the inferred ancestral area for *A. argentatus*, *A. quisqueyanus* Girón & Franz and Polydrusini sp. 1.

2.4 Discussion

2.4.1 Phylogenetic relationships and taxonomic implications

Our results provide a new baseline for understanding the phylogenetic relationships of Neotropical broad-nosed weevils traditionally placed in the tribes Eustylini and Geonemini. In particular, the model-based reconstructions of the combined COI/EF-1α matrix (Figs. 2.3 and 2.4) support a monophyletic eustyline-geonemine complex consisting of Caribbean and northern South American taxa while largely (though not entirely) excluding members of the presumed outgroup tribes Eudiagogini, Naupactini, Polydrusini, and Tanymecini. The analyses further suggest that the Eustylini in the traditional sense (cf. Alonso-Zarazaga and Lyal, 1999) contain at least two separate lineages; viz. the South American Eustylini I clade – i.e. *Eustylus* Schoenherr, *Xestogaster,* and likely other genera such as *Compsus* and *Exorides* that were not sampled – and the Caribbean Eustylini II clade which includes *Diaprepes, Exophthalmus,* and *Pachnaeus;* the latter erroneously placed in the Tanymecini *sensu* Alonso-Zarazaga and Lyal (1999). Each of these two eustyline clades has considerable support under both maximum likelihood and Bayesian inference. However, based on the molecular data they form a polytomy with, and are possibly paraphyletic in relation to, the Geonemini clade. The latter, in turn, includes members of or near *Artipus* that are conventionally placed in the Naupactini, yet are not closely related to other naupactine genera such as *Litostylus* Faust which are widespread through Central and South America (O'Brien and Wibmer, 1982; Wibmer and O'Brien, 1986). The fourth and final element of the eustyline-geonemine complex is the enigmatic southwestern Puerto Rican genus *Scelianoma* (Franz and Girón, 2009). To our knowledge, *Scelianoma* has no apparent close relatives in the remaining Greater Antilles and may indeed represent an isolated surviving lineage of an Eocene/Oligocene colonization originating in South America (cf. Iturralde-Vinent, 2006) – a proposition that could be corroborated through inclusion of additional South American entimine taxa.

Both the limited sampling of described species within the larger genera *Diaprepes* (16 species), *Exophthalmus* (43 species) and *Lachnopus* (57 species; all numbers referring to Caribbean representatives only; see Morrone, 1999; O'Brien and Kovarik, 2000), and the

inclusion of minimally 15 species that appear to fall within their limits yet were not assignable to described species (Table 2.1), severely limit our ability to propose taxonomic changes within each of these genera. While the need for such monographic revisions is clear, particularly for the *Exophthalmus* genus complex (Vaurie, 1961; Woodruff, 1985; O'Brien and Kovarik, 2000; Franz, 2010a), this lies outside the scope of the present work. Nevertheless, numerous higher-level groupings inferred from the combined molecular matrix (Figs. 2.2 to 2.4) are congruent with emerging results from an ongoing morphological analysis of this complex (Franz, unpublished data). These elements of molecular/morphological congruence and incongruence are briefly discussed here to underscore their combined effect and illustrate the taxonomic implications of our results. We note, however, that the taxon sampling scheme of the unpublished morphological analysis is too divergent from that of the present study to permit a full integration (cf. Nixon and Carpenter, 1996b).

The main ingroup clade representing the eustyline-geonemine complex (Figs. 2.3 and 2.4) is largely congruent with morphological results, with the exception of *Scelianoma,* which is placed outside this clade. Putative diagnostic features include a special configuration of the lamina of the male spiculum gastrale and the presence of a central, multi-part endophallic sclerite of the male aedeagus (cf. Franz, 2010a). The latter structure is increasingly complex and "contorted" in the South American Eustylini I, yet simpler and multi-laminate to tubular in the Geonemini and Eustylini II, which are inferred as sister lineages by morphology. The taxonomic composition of each of the latter two clades is generally congruent with the molecular signal, with the exception of *Apotomoderes* and *Artipus* being placed outside of the Geonemini, which may be due to more extensive outgroup sampling. Morphology also congruently supports a sister group relationship of *Diaprepes* (including the misplaced Lesser Antillean *E. marginicollis*) and a strictly Caribbean analogue to the *Exophthalmus* II which includes the type species *E. quadrivittatus.* The monophyly of each of these clades (Figs. 2.3 and 2.4) is reaffirmed by morphological characters of the rostrum (tricarinate versus glabrate), pronotum (with irregular foveae in *Diaprepes*), elytra (with interrupted stripes of suberect scales in *Exophthalmus*), and endophallus of the male aedeagus. The present molecular analysis therefore adds support to the recognition of a monophyletic *Diaprepes* within the exceedingly paraphyletic concept of *Exophthalmus* which had been elusive for decades (O'Brien and Kovarik, 2000). On the other hand, morphology suggests that species of the herein postulated *Exophthalmus* I clade, along

with *E. roseipes*, *C. maricao* (which is not closely related to the South American members of *Compsus* = Eustylini I) and *Pachnaeus*, are grouped together with Cuban and Central American members of *Exophthalmus* and additional related genera such as *Tetrabothynus* Labram & Imhoff and *Tropirhinus* Schoenherr (= *Callizonus* Schoenherr, a junior synonym; see O'Brien and Wibmer, 1982). Again, this discrepancy may be due to the more comprehensive sampling of Cuban and Central American eustylines in the morphological study.

In summary, emerging lines of molecular and morphological evidence on the eustyline-geonemine complex show a high degree of overall congruence, especially at intermediate taxonomic levels as labeled in Fig. 2.4. Apparent discrepancies must be resolved through expanded molecular taxon sampling and subsequent integration of the two sources of data (Nixon and Carpenter, 1996b; Graybeal, 1999; Mitchell *et al*., 2000). Until then, we refrain from making classificatory changes within this diverse and heterogeneous complex.

2.4.2 Cospeciation versus historical biogeographic processes driving diversification

A comparison of the TreeMap and DIVA analyses (Figs. 2.6 and 2.7) strongly suggests that the diversification of the sampled entimine lineages was not mediated by coevolutionary interactions with their host plant lineages (i.e., zero cospeciation events), but was instead driven by historical biogeographic and other non-coevolutionary processes. This result may seem surprising in light of the nearly five-decade prevalence of the escape-and-radiation paradigm as an explanation for the success of plant associated insects and weevils in particular (e.g. Ehrlich and Raven, 1964; Jermy, 1976; Mitter and Brooks, 1983; Mitter *et al*., 1988; Mitter and Farrell, 1991; Anderson, 1993; Farrell, 1998; Marvaldi *et al*., 2002; Franz, 2004; Oberprieler *et al*., 2007; McKenna *et al*., 2009; Franz and Engel, 2010). Yet aside from general concerns of plausibility (Yoder and Nuismer, 2010) and testability (Mayhew, 2007), the coevolution hypothesis is apparently not a good candidate for explaining specifically the success of weevils in the 12,000+ species-rich subfamily Entiminae, given that they often have taxonomically and spatiotemporally variable host associations.

The failure to recover any cospeciation events is directly related to the inability to establish sufficiently narrow and stable host associations for the sampled weevil species (Table 2.3, Fig. 2.6, Appendix 1). This outcome differs dramatically from other weevil lineages where host associations are narrow and phylogenetically conserved (e.g. Franz and Valente, 2005). Many entimines do not show strong and spatiotemporally persistent feeding preferences for a particular plant lineage and are considered oligo- to polyphagous (Wolcott, 1929, 1936; Vaurie, 1961; Martorell, 1976; Woodruff, 1985; Simpson *et al*., 1996; O'Brien *et al*., 2006; Machado, 2007; Oberprieler *et al*., 2007; Bright and Bouchard, 2008). The adults typically feed on younger leaves and shoots that have greater nitrogen content, less allelochemicals, and are generally easier to ingest (Wright *et al*., 2003). The larvae, in turn, are soil inhabiting external feeders on roots and root nodules. While this life strategy does not avoid interaction with, and possible adaption to, plant chemistry (cf. Velázquez de Castro *et al*., 2007), it differs markedly from the more exposed or endophagous habits of (e.g.) lepidopteran larvae (Ehrlich and Raven, 1964) or other herbivorous coleopterans (Futuyma and McCafferty, 1990; Farrell and Mitter, 1998). Indeed, one of the more effective strategies to collect entimine weevils in a wide range of Caribbean habitats is to simply survey the locally occurring citrus and legume shrubs (personal observation). Therefore we should not assume parity of evolutionary processes driving the diversification of the sampled entimine weevils.

In contrast to the cospeciation analysis, the DIVA reconstructions (Figs. 2.7A and 2.7B) suggests that at least 17 lineage divergence events in the Caribbean eustyline-geonemine complex were correlated with biogeographic shifts, both among and within islands. Of these, seven splits (41.2%) have occurred among Hispaniola and Puerto Rico, three (17.6%) among Hispaniola and Mona Island, and two splits (11.8%) among Hispaniola and Jamaica; jointly accounting for 70.6% of the inferred splits. Although these numbers are clearly influenced by taxonomic and geographic emphasis (see Section 3.1.), they are also a plausible correlate of the high rate of single island endemism in the sampled Caribbean species (i.e. 53/57 species). Such high rates of endemism are especially common in entimines of the Greater Antilles; for instance, more than 50 species of *Lachnopus* are reported as single island endemics, eight of 10 species of *Exophthalmus* are endemic of Cuba and five out six species are endemic to Jamaica, etc. (Vaurie, 1961; O'Brien and Wibmer, 1982; Peck, 2005). The rates of endemism are comparatively lower in the Lesser Antilles (e.g. O'Brien and Kovarik, 2000), and are expected to drop further as

distributional information is published. On the other hand, recent revisionary work on Caribbean entimines shows evidence of marked patterns of intra-island endemism in the Greater Antilles. For instance, the low elevation dry forest habitats in southwestern Hispaniola (Jaragua National Park), Mona Island, southwestern Puerto Rico (Guánica Dry Forest), and Vieques Island (Lighthouse Peninsula) are known to harbor numerous narrowly endemic entimine species (Franz and Girón, 2009; Franz, 2010b; Girón and Franz, 2010; Franz, unpublished data). Thus there is growing evidence that events of inter- or even intra-island colonization are common in these weevils, and that instead ancestral geographic distributions have been a determining factor in the isolation and splitting of lineages. Until more specific data on their natural history are available, we can only suggest that "geo-ecological specializations" to certain types of habitats – including but not limited to host plant composition, elevation, precipitation patterns, and geographic location and connectedness – are better determinants of diversification than coevolution with specific host lineages. In this sense, the studied eustyline and geonemine weevil lineages are similar to other non-herbivorous beetles of the West Indies (e.g. Liebherr 1988a, 1988b; Nichols, 1988). Such historical biogeographic mechanisms are also thought to have driven the radiation of entimine weevils in other island regions, thus suggesting the presence of a more general island-related pattern (Spanton, 1992; Claridge, 2006; Machado, 2007, Machado *et al*., 2008; Sequeira *et al*., 2008).

2.4.3 Historical biogeographic patterns

The sequence of vicariance and colonization events that have led to the present-day West Indian weevil fauna can only be understood in light of the region's complex and still controversial geology (e.g. Ricklefs and Lovette, 1999; Iturralde-Vinent and MacPhee, 1999; Hedges, 2001, 2006; Iturralde-Vinent, 2006; Ackerman *et al*., 2007; García-Casco *et al*., 2008; Ricklefs and Bermingham, 2008). A critical component of such a reconstruction is the ability to produce fossil-calibrated clade divergence times (Ware *et al*., 2010) and to reconcile these with the geological sequence of island connections and separations. Unfortunately, the lack of properly classified Neotropical entimine fossils is a severe limitation in this sense (though see Gratshev and Zherikhin, 2003; Rheinheimer, 2007; Poinar and Brown, 2010). In particular, there are no descriptions of fossil species of Eustylini and Geonemini from Dominican amber (S.R. Davis, personal communication). Thus at present our historical biogeographic reconstructions

(Figs. 2.7A and 2.7B) must remain without a time line that would allow comparisons across clades. We consequently refer to the distributional divergences in our reconstructions as "splits", leaving it ambiguous whether they are the result of vicariance or colonization events with subsequent speciation. We would expect that different weevil lineages have radiated during different historical periods, resulting in separate cenocrons *sensu* Morrone (2009), as is likely the case in the entimine genus *Apodrosus* (Girón and Franz, 2010). Nevertheless, several of the observed taxon-area splits are discussed in more detail in the following sections, starting with the western-most regions and progressing towards the Lesser Antilles.

Two independent splits were inferred between Hispaniola and Jamaica. The first of these (# 6 in Fig. 2.7A) concerns *Apotomoderes* and the (as of yet undescribed) sister clade including Entiminae spp. 1-3. According to Franz (2010b), *Apotomoderes* originated during the Oligocene-Miocene transition. This would suggest that the *Apotomoderes*/Entiminae spp. 1-3 split occurred previously in the Eocene-Oligocene period during which the eastern Jamaican Blue Mountains formation and the southern Hispaniola peninsula were separated by a shorter distance and possibly connected via a land bridge (Iturralde-Vinent and MacPhee, 1999; Iturralde-Vinent, 2006; García-Casco *et al*., 2008). The second Hispaniola/Jamaica split (# 14) occurred within the *Exophthalmus* II lineage. The clade composed of *Exophthalmus* spp. 7-9 is distributed in the Central Cordillera and northern region of Hispaniola (# 16), and represents the putative sister group of the Jamaican species of *Exophthalmus* and *Pachnaeus* (Fig. 2.7B). It is unclear whether this split represents a vicariance or colonization event. The subsequent intra-Jamaican split of *E. similis* and *E. vittatus* (# 17) was recognized by Vaurie (1961). The latter species is distributed in the western and central parts of Jamaica, whereas the former species is present in the eastern Blue Mountains block and also more sparingly in the west, which may have resulted from a colonization event with subsequent speciation. However, *E. similis* and *E. vittatus* are distributed sympatrically in the St. Andrew Parish, localized between the Blue Mountains block and the central-western karst region of Jamaica. This pattern may have resulted from a coalescence of the two regions in the Middle Miocene (~ 5-10 mya; Iturralde-Vinent, 2006). Similar partly disjunct distributions are found among species of *Eleutherodactylus* Duméril & Bibron (Anura: Eleutherodactylidae) (Hedges, 1989).

The reconstruction furthermore posits three independent colonization events from Hispaniola to Mona Island (Fig. 2.7A; #'s 4, 5, and 7; see also Franz *et al*., 2009; Franz, 2010b; Girón and Franz, 2010). Mona Island emerged above sea level during the Pliocene-Pleistocene period (~ 5-7 mya) and has never been in contact with any of the Greater Antillean islands (Gonzalez *et al*., 1997).

Resulting in part from the sampling emphasis, many of the inferred splits involve Hispaniola and Puerto Rico, which were last connected during the Late Oligocene to Middle Miocene transition period when the Mona Passage was established (~ 12-28 mya; Iturralde-Vinent, 2006). The split within *Apodrosus* (# 1) likely reflects a vicariance event, considering the widespread ancestral range of the genus (Girón and Franz, 2010). Two similarly timed vicariance events may have occurred in the *Lachnopus* complex (#'s 8 and 10). Moreover, the undescribed Entiminae sp. 6 is inferred as the sister group to the *L. coffeae-L. seini* clade (Fig. 2.7A), and this putative new species occurs only on Vieques Island. Given that Puerto Rico, Culebra, Vieques, and the Virgin Islands were connected above sea level until ~ 6-10 kya (Heatwole *et al*., 1981), the Puerto Rico/Vieques Island split (# 9) likely represents a recent vicariance or colonization event with subsequent speciation. Three additional Hispaniola/Puerto Rico splits are inferred in the *Exophthalmus* I and II clades, respectively (#'s 11, 12, and 13). They require further study, particularly since the variably placed Puerto Rican species *C. maricao, E. quindecimpunctatus,* and *E. roseipes* are involved (see Section 3.2.).

Diaprepes largely replaces *Exophthalmus* in the Lesser Antilles (cf. O'Brien and Kovarik, 2000). The widespread distributions of *D. abbreviatus* and *D. famelicus* make it difficult to optimize ancestral splits; nonetheless, Puerto Rico has been hypothesized as the area of origin for *D. abbreviatus* (Lapointe, 2004; Ascunce *et al*., 2008; Mazo-Vargas *et al*., unpublished data). Limiting the number of ancestral areas to one in DIVA yields Puerto Rico as the area of origin for *Diaprepes,* with several subsequent splits among the Lesser Antilles (e.g. # 15; Fig. 2.7A). Oversea colonization is the most plausible process in these cases, possibly during the Miocene or later (cf. Ricklefs and Bermingham, 2008).

3 The case of *Diaprepes abbreviatus* in the Caribbean

3.1 Introduction

The citrus root weevil *Diaprepes abbreviatus* (Linnaeus 1758: 386) (Coleoptera: Curculionidae: Entiminae: Eustylini Lacordaire *sensu* Alonso-Zarazaga and Lyal, 1999) is a well known pest of citrus trees, sugar cane, and numerous other cultivars (Lapointe, 2004). Following its introduction during the 1960s from the Caribbean region (Woodruff 1964, 1968), this rapidly colonizing species now occurs on more than 100,000 acres in the southern United States, causing annual losses in crop production of more than US $70 million in the state of Florida alone (e.g. Hall, 1995; Grafton-Cardwell *et al*., 2004; Weissling *et al*., 2009).

Diaprepes abbreviatus was first studied in detail by Wolcott (1936) in Puerto Rico, and has since been the subject of a wide range of evolutionary and applied studies (e.g. Hall *et al*. 2001; McCoy *et al*. 2003; Sirot *et al*., 2007). These studies have jointly revealed an immense reproductive capacity and ecological adaptability. For instance, females mate multiple times (Sirot and Lapointe, 2008) and lay up to 11,000 eggs in their lifetime (Nigg *et al*., 2004). Simpson *et al*. (1996) summarized records of *D. abbreviatus* on nearly 270 species and 60 genera of host plants, making it one of the most polyphagous beetle species known to date (cf. Anderson, 2000). Furthermore, citrus root weevils are more widely distributed throughout the Caribbean archipelago than any other species of entimine weevil; including Antigua, Barbados, Culebra Island, Dominica, Grenada, Guadeloupe, Hispaniola, Martinique, Mona Island, Montserrat, Puerto Rico, Saint Kitts, Saint Lucia, Saint Vincent, and Vieques Island (O'Brien and Wibmer, 1982; O'Brien and Kovarik, 2001; Lapointe 2004). Within the United States, *D. abbreviatus* is well established in California, Florida, and Texas, yet has the potential to colonize additional regions (Lapointe *et al*., 2007). The species' considerable ecological amplitude is matched by a high diversity of regional morphs with varying patterns of carinae along the strial

and scale coloration (Fig 3.1, also see, Pierce, 1915; Woodruff, 1985; Hantula *et al*., 1987; O'Brien and Kovarik, 2001; Franz, 2010a).

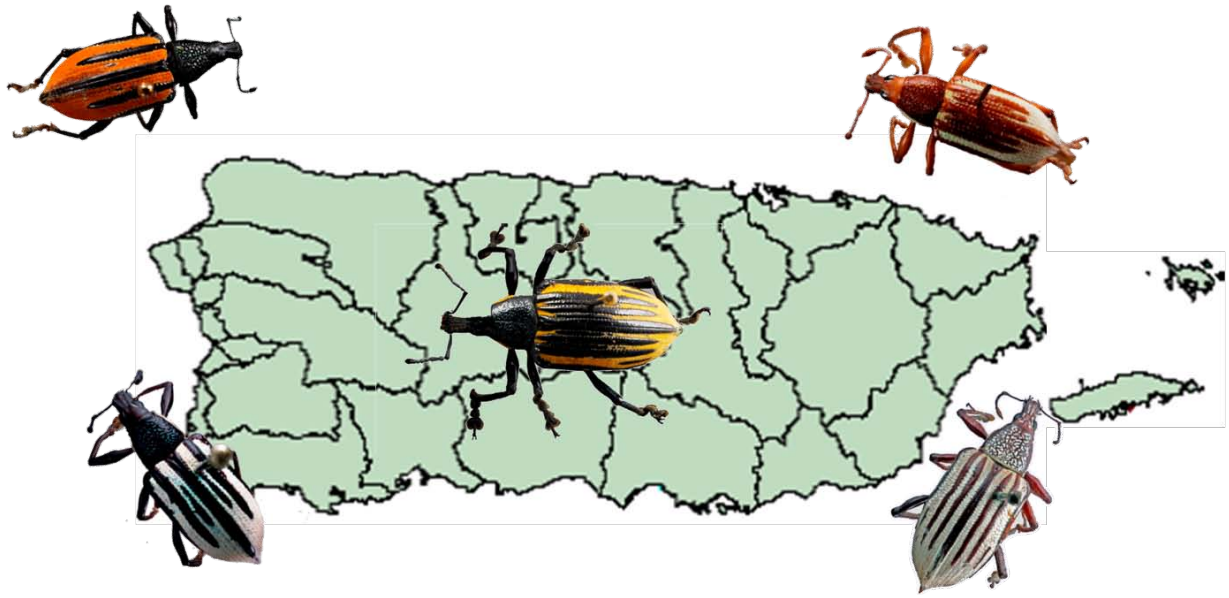

Fig. 3.1. Examples of color variation of *Diaprepes abbreviatus* in Puerto Rico.

Compared to the vast amount of research published on *D. abbreviatus* in relation to its pest status, relatively little information is available regarding its evolutionary origins and phylogeographic relationships. O'Brien and Kovarik (2001) reviewed the taxonomic status of 16 species of *Diaprepes* Schoenherr recognized as valid, all of which occur in the Caribbean region. Franz (2010a, 2011) provided a detailed redescription of *D. abbreviatus* and an exemplar-based phylogenetic framework in which seven additional species of *Diaprepes* were analyzed. Accordingly, *D. abbreviatus* is part of a monophyletic complex that also includes the widespread species *D. comma* Boheman (Hispaniola, Puerto Rico, Trinidad and Tobago, Venezuela) and *D. doublierii* Guérin (Hispaniola, Puerto Rico) – all of which present glabrous elevations along (minimally) the elytral strial interval IV-V (geographic records according to O'Brien and Kovarik, 2001). At the population level, several studies indicate that Puerto Rico harbors the highest levels of phenotypic and genotypic variation of *D. abbreviatus* in the Caribbean, with distinctive morphological forms being restricted to specific intra-island regions (Jones, 1915; Hantula *et al*., 1987; O'Brien and Kovarik, 2001). Such observations have led to the hypothesis

that Puerto Rico represents the center of origin of *D. abbreviatus* (Lapointe, 2004; Ascunce *et al*., 2008).

The widespread occurrence of the citrus root weevil in the Caribbean region opens up possibilities to correlate its population structure with intra- and inter-island geographic patterns using mitochondrial DNA data (cf. Kelley *et al*., 1999; Laffin *et al*., 2005). Such an approach was recently taken by Ascunce *et al*., (2008) giving emphasis on haplotype diversity in Florida, although this study included one specimen of *D. abbreviatus*, four *D. balloui* Marshall and one unidentified species of *Diaprepes* from Dominica. Expanding the mtDNA sampling to the Caribbean islands promises new insights into the historical differentiation of *D. abbreviatus* populations vis-à-vis the Caribbean region's complex geological history (Iturralde-Vinent, 2006). Such studies are also likely to shed further light on the subject of insect diversification in the Caribbean archipelago, one of the world's premier biodiversity hotspots (Liebherr, 1988; Mittermeier *et al*., 2004; Ricklefs and Bermingham, 2008). Lastly, most Caribbean entimines are remarkable for their relative lack of host plant specificity – and thus limited potential for host-mediated chemical coevolution – but high responsiveness to biogeographic factors (cf. Franz and Engel, 2010; Girón and Franz, 2010; Mazo-Vargas *et al*., 2011). A focus on Caribbean populations of *D. abbreviatus* will help understand how these factors operate at presumably shorter time scales below the species level.

Here we expand the phylogeographic analyses of *D. abbreviatus* through inclusion of 68 specimens pertaining to numerous populations from the islands of Dominica, the Dominican Republic, Mona, and Puerto Rico. Specifically, we utilize information of the mitochondrial cytochrome oxidase I (COI) locus to correlate *D. abbreviatus* population structure within and among islands. Genetic diversity and demographic history are assessed using Tajima's D statistic (Tajima, 1989) and an analysis of molecular variance (AMOVA; Excoffier *et al*., 1992). The herein presented data thus represent an important building block to understanding both the evolutionary history of citrus root weevils in the Caribbean and origins of the United States populations.

3.2 Materials and Methods

3.2.1 Insect Sampling

A total of 68 specimens of *D. abbreviatus* were collected, originating from relatively unaltered to agricultural habitats in Dominica, the Dominican Republic, Mona Island, and Puerto Rico (Table 3.1). The majority of specimens were taken at Hg or UV lights and by beating or sweeping their host plants during day time. The specimens were subsequently preserved in 95% ethanol and stored at -20°C until further processing. One specimen from Saint Lucia (courtesy of M. A. Ivie, Montana State University, Bozeman, Montana) and four specimens from the United States Horticultural Research Laboratory (USHRL) in Fort Pierce, Florida, were also included in the analysis, the latter representing Floridian haplotypes. In addition, published sequences of the haplotypes COI-1, COI-2, and COI-3 as described in Ascunce *et al*. (2008) were used (GenBank accession numbers EF042129–EF042134). Sequences of the following five species were added to root the phylogeny (in alphabetical order): *D. balloui*, *D. boxi* Marshall 1938, *D. famelicus* (Olivier), *D. maugei* (Boheman), and *Exophthalmus quadrivittatus* (Olivier) (taxonomic concepts as in O'Brien and Kovarik, 2001; GenBank accession numbers: HQ891433–HQ89143336 and HQ891448).

3.2.2 DNA Isolation and Sequencing

Genomic DNA isolations were achieved by grinding the thoracic musculature of individual weevils with sterilized pestles in 1.7 ml Eppendorf tubes. DNA extractions were made using DNeasy® Blood & Tissue Kits (QIAGEN, USA) as indicated by manufacturer, resulting in samples that were suspended in 200µl of RNAse-free ddH20. DNA quality was assessed via electrophoresis on a 1% agarose gel. A 1208 base pair fragment of COI was amplified using combinations of the following primers: s1541, 5'– TGAKCYGGAATASTAGGAICATC –3' (B. Crespi, personal communication; cited in Ascunce *et al*., 2008); a2411, 5'– GCTAATCATCTAAAAACTTTAATTCCWGTWG –3' (Normark *et al*., 1999); K698, 5'– TACAATTTATCGCCTAAACTTCAG CC –3' (Simon *et al*., 1994); and a2771, 5'– GGATARTCAGARTAACGTCGWGGT ATWC –3' (Normark *et al*., 1999). PCR reactions for

each specimen were prepared with 0.2 µM of each primer, 0.25 µM of dNTPs, 2.5mM of MgCl2, 1x PCR Buffer, 0.2 µl of Go Taq® polymerase (Promega, USA), and deionized water; yielding a total volume of 25-35 µl per reaction. The thermal profile for the PCRs consisted of an initial denaturalization step at 94 °C for 5 minutes; a total of 35 cycles at 94 °C for 30 seconds, 51 °C for 60 seconds, and 72 °C for 60 seconds; and a final extension at 72 °C for 5 minutes. All amplified and purified PCR products were sent to the Nevada Genomics Center (Reno, Nevada) for sequencing (see http://www.cabnr.unr.edu/ genomics/). The returned sequence chromatograms were examined and edited in Sequencher™ 4.8 (Gene Codes Corporation, Ann Arbor, MI). The obtained *D. abbreviatus* sequences were deposited in GenBank under accession numbers JF302898–JF302968. Voucher specimens were deposited in the University of Puerto Rico at Mayagüez Invertebrate Collection (UPRM–INVCOL).

Table 3.1. List of haplotypes of *Diaprepes abbreviatus* observed, including frequencies and sampling localities in the Caribbean.

Haplotype	Frequency	Island	Region/Place	Coordinates	
Group A					
Hap 1	1	Dominica	St. Peter Parish	N15°30'47"	W61°25'20"
Hap 2	4	Dominica	St. Peter Parish	N15°30'47"	W61°25'20"
Hap 3	1	Dominica	St. Peter Parish	N15°31'6"	W61°25'31"
Hap 4	1	Dominica	St. Joseph Parish	N15°25'19"	W61°25'31"
Hap 5	1	Dominica	St. Joseph Parish	N15°25'19"	W61°25'31"
Hap 6	1	Dominica	St. Patrick Parish	N15°14'50"	W61°18'41"
Hap 7	1	Dominica	St. Patrick Parish	N15°14'50"	W61°18'41"
	3	Dominica	St. Patrick Parish	N15°14'45"	W61°19'25"
Hap 8	1	Dominica	St. David Parish	N15°23'18"	W61°17'13"
Hap 9	2	Dominica	St. Joseph Parish	N15°21'52"	W61°21'15"
	1	Dominica	St. David Parish	N15°23'26"	W61°15'25"
Hap 10	1	Dominica	St. Joseph Parish	N15°28'8"	W61°23'40"
Hap 11	1	Dominica	St. David Parish	N15°28'9"	W61°15'5"
Hap 12	1	Dominica	St. David Parish	N15°28'9"	W61°15'5"
Hap 13	1	Dominica	St. Paul Parish	N15°20'46"	W61°22'8"
Hap 14	1	Dominica	St. Paul Parish	N15°23'13"	W61°24'6"
Hap 15	1	Dominica	St. George Parish	N15°18'58"	W61°21'13"
Hap 16	1	Saint Lucia			
Total	**24**				

Group B					
Hap 17	3	Florida	Fort Pierce -USDA-LAB Colony		
Hap 18	1	Florida	Fort Pierce -USDA-LAB Colony		
Hap 19	1	Puerto Rico	Mona Island	N18°5' 19"	W67°56'17"
Hap 20	1	Puerto Rico	Mona Island	N18°5 19	W67°56'17"
Hap 21	1	Puerto Rico	Adjuntas	N18°10'40"	W66°47'52"
Hap 22	2	Puerto Rico	Adjuntas	N18°10'40"	W66°47'52"
Hap 24	1	Puerto Rico	Isabela	N18°27'57"	W67°3'8.97"
Hap 25	2	Puerto Rico	Isabela	N18°27'57"	W67°3'8.97"
Hap 26	1	Puerto Rico	Isabela	N18°27'57"	W67°3'8.97"
Hap 32	1	Puerto Rico	Sabana grande	N18°2'5.13"	W66°54'34"
Hap 34	1	Puerto Rico	Toa baja	N18°26'22"	W66°12'44"
Hap 35	1	Puerto Rico	Toa baja	N18°26'22"	W66°12'44"
	1	Dom. Rep.	El Seybo	N18°48'57"	W69°3'50"
	5	Dom. Rep.	Hato Mayor	N18°47'26"	W69°16'32"
Hap 36	1	Puerto Rico	Toa baja	N18°26'22"	W66°12'44"
Hap 37	2	Puerto Rico	Tortuguero	N18°27'28"	W66°26'7"
Hap 40	4	Dom. Rep.	La Vega – Manabao	N19°4'18"	W70°48'18"
	4	Dom. Rep.	La Vega – Constanza	N18°58'28"	W70°38'54"
Hap 43	1	Dom. Rep.	Villa Altagracia	N18°44'51"	W70°14'19"
Total	**34**				
Group C					
Hap 23	1	Puerto Rico	Hormigueros	N18°8'33"	W67°7'50"
Hap 27	1	Puerto Rico	Mayaguez	N18°12'12"	W67°8'21"
Hap 28	1	Puerto Rico	Mayaguez	N18°10'45"	W67°5'55"
	1	Puerto Rico	Mayaguez	N18°12'50"	W67°8'18"
Hap 29	1	Puerto Rico	Mayaguez	N18°12'48"	W67°8'16"
Hap 30	1	Puerto Rico	Mayaguez	N18°12'45"	W67°8'19"
	1	Puerto Rico	Mayaguez	N18°12'50"	W67°8'18"
Hap 31	1	Puerto Rico	Mayaguez	N18°12'48"	W67°8'16"
Hap 33	1	Puerto Rico	Susua	N18°4'17"	W66°54'21"
Hap 38	2	Dom. Rep.	San Cristobal	N18°17'53"	W70°11'16"
Hap 39	1	Dom. Rep.	La Vega – Manabao	N19°4'18"	W70°48'18"
Hap 41	1	Dom. Rep.	La Vega – Constanza	N18°58'28"	W70°38'54"
	1	Dom. Rep.	El Seybo	N18°48'57"	W69°3'50"
Hap 42	1	Dom. Rep.	Pico Diego de Ocampo	N19°34'55"	W70°44'23"
Total	**15**				

3.2.3 Phylogenetic and Phylogeographic Analyses

The COI sequence fragments were aligned using Muscle 3.6 (Edgar, 2004). The alignments were subjected to eye inspection and translated into amino acids to check for the presence of open reading frames. All sequences were surveyed to identify different haplotypes among the sampled *D. abbreviatus* populations. The phylogenetic relationships among mtDNA haplotypes were inferred under parsimony, maximum likelihood (ML), and Bayesian inference (BI), and a median joining algorithm was used to estimate a network (Bandelt *et al*., 1999). Parsimony topologies were obtained using the parsimony ratchet, (Nixon 1999) as implemented in TNT (Goloboff *et al*., 2008), and spawned with ASADO (Nixon 2008). Heuristic tree searches were conducted using 500 ratchet iterations, 10 rounds of tree-fusing, 100 cycles of tree-drifting (Goloboff, 1999a), 900 random addition sequences, and finding the best scores three times. Maximum likelihood analyses were performed with RaxML, version 7.2.2 (Stamatakis, 2006), thus permitting independent optimizations of substitution rate models according to each codon position. The best scoring tree was found performing 1000 replicates of ML searches under the rapid bootstrapping algorithm (Stamatakis *et al*., 2008). Bayesian phylogenetic inference was performed with Mr Bayes, version 3.1.2, which similarly allows different data partitions to be optimized according to different stochastic models (Ronquist and Huelsenbeck, 2003). The best-fitting evolutionary models for each of codon positions were estimated with jModelTest under the Bayesian Information Criterion (BIC; see Posada, 2008). Four simultaneous chains were run for one million generations while sampling every 100^{th} generation, for a total of 40,001 trees per chain. A graphic representation of the likelihood values of each MCMC chain was obtained using the trace function of the software Tracer, version 1.5 (Rambaut and Drummond, 2009). The first 1000 trees were deemed burn-in. Finally, a median-joining network (MJN) was estimated in Network 6.0 (Fluxus Technology, 2011). Where necessary, posterior processing was performed using the MP algorithm in order to delete all superfluous median vectors not contained in the shortest network trees (Polzin and Daneschmand, 2003).

The phylogenetic results were summarized using a 50% majority rule Bayesian tree. Node support was estimated using the bootstrap procedure (Felsenstein, 1985), based on 1000 pseudoreplicates as performed with ASADO and NONA (Goloboff, 1999b) and RaxML, respectively. Similarly, posterior clade probabilities (PP) were generated from the Bayesian

inference and interpreted as the frequencies of samples recovering particular clades (Huelsenbeck and Ronquist, 2001). The Network output was assessed through comparison with geographic maps of Dominica, the Dominican Republic, Mona Island, and Puerto Rico. The coordinates of the sampled sites were located on the GIS Caribbean map (Twichell *et al*., 2005) with the help of ArcGIS, version 9.3 (ESRI, 2008). Lastly, the sequences obtained in this study were trimmed to 611 base pairs for facilitate comparison with the Floridian haplotypes (COI-1, COI-2, and COI-3) identified in Ascunce *et al*., (2008).

3.2.4 Population Diversity

Diversity indices were calculated using DnaSP, version 5.10 (Librado and Rozas, 2009); including the number of polymorphic sites (S), the number of haplotypes (h), nucleotide diversity (π), and haplotype diversity (H*d*) (Nei, 1978). The data were partitioned in accordance with the three major groups recovered from the phylogenetic analyses and the three main Caribbean islands (Dominica, Hispaniola, and Puerto Rico). Genetic differentiation between the three major clades was estimated through the calculation of Fst values with Arlequin, version 3.5 (Excoffier *et al.*, 2010). The statistical significance of the differences was assessed through 80,088 permutations. In addition Tajima's D statistic was estimated in order to infer population demographic history (Tajima, 1989). In cases where the statistics was negative, a frequency graph of pair-wise differences between the alleles or mismatched distributions was generated in Arlequin suite ver 3.5 (Excoffier *et al.*, 2010), thus permitting the detection of signatures of population growth or decline (Rogers and Harpending, 1992). The smoothness of the observed distribution was estimated with the raggedness statistics, under the null hypothesis of an expanding population (Harpending, 1994). Finally, analyses of molecular variance (AMOVA; Excoffier *et al*,. 1992) were performed on the sequence data to explore the amount of genetic variability within and among the major groups.

3.3 Results

3.3.1 Phylogenetic and Phylogeographic Analyses.

A total of 73 mtDNA sequences of *D. abbreviatus* individuals and pertinent outgroup taxa were analyzed, each with a length of 1208 nucleotides. Of these, 212 sites were parsimony informative and 236 sites were segregating. In all, 43 haplotypes of *D. abbreviatus* were observed, of which ten haplotypes were shared among at least two localities (Table 3.1, Fig. 3.2). There is a high number of unique haplotypes, 32 out of 73 have a frequency of one. The most common haplotype h40 (8 out of 73 sequences) was found in two localities of the Dominican Republic. The second most frequent haplotype (h35 = 7 out of 73 sequences) was the only haplotype present in more than one island, the Dominican Republic and Puerto Rico.

The resulting phylogenetic relationships were highly consistent across the three methods used for phylogenetic inference, and generally yielded very well supported clades. The results are summarized here based on Bayesian topology (Fig. 3.2). Accordingly, the haplotypes of *D. abbreviatus* formed three highly supported groups, as follows. Group A represents all individuals from Dominica and, nested within this group, the single analyzed specimen from St. Lucia. The phylogenetic reconstruction indicates a large genetic divergence gap between this Dominica/St. Lucia group and the Greater Antillean populations. On the other hand, Groups B and C are more closely related to each other, and each contains primarily haplotypes from the Dominican Republic and Puerto Rico. In addition, Group B includes individuals of *D. abbreviatus* from Mona Island as well as Florida (Fig. 3.2).

The primarily Dominican Group A comprises sixteen distinct haplotypes (Table 3.1; Fig. 3.2). The spatial distribution of these haplotypes appears strongly correlated with the geography of Dominica, as indicated by plotting the nested analysis onto a map of the island (Fig. 3.3). Accordingly, five major lineages are distinguished, of which two are more widespread than the remaining three lineages. The first of these widespread lineages (A1) is represented by haplotypes ranging from the north to the central-eastern coast, whereas the second group (A2) includes haplotypes from the central-eastern region. A third haplogroup (A3) from the south is closely related with another group (A4), which occurs along the southwestern coast and both

closely related to the unique representative from St. Lucia, which is localted futher south. Finally, a fifth and highly divergent haplogroup (A5) is present in the area of Mero along the central-western coast of the island (Fig. 3.2).

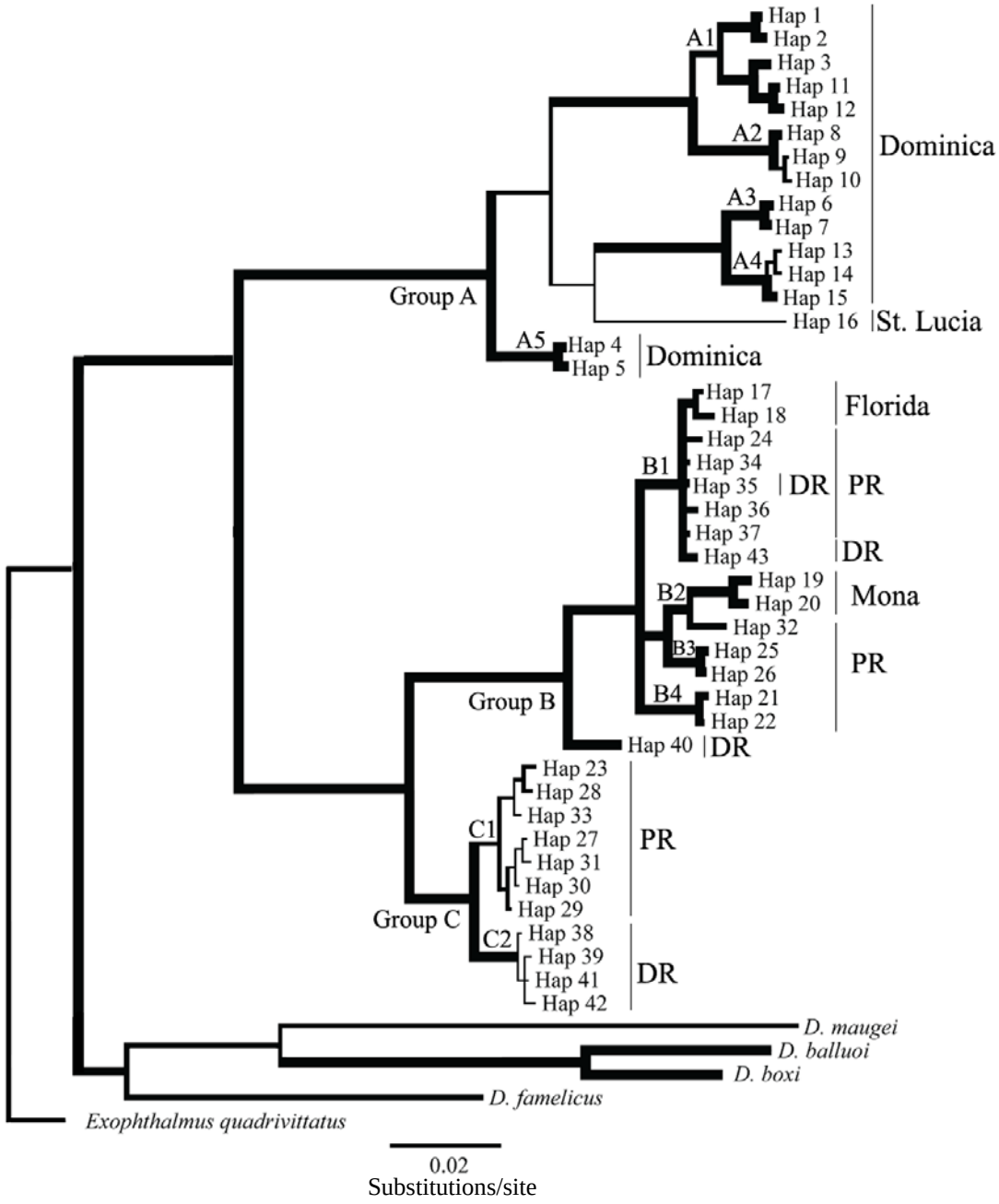

Fig. 3.2. Bayesian phylogenetic tree of the COI gen haplotypes of *Diaprepes abbreviatus*. The thickest branches represent bootstrap values from parsimony and maximum likelihood >90% and posterior probabilities >0.9 from the Bayesian inference. DR, Dominican Republic; PR, Puerto Rico.

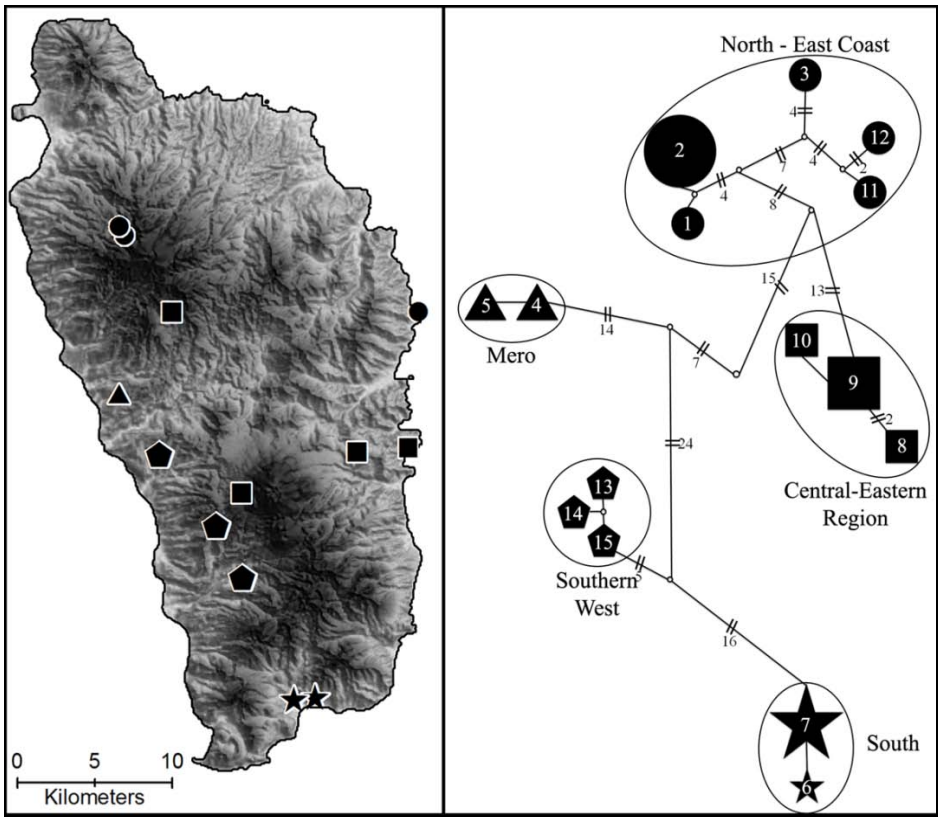

Fig. 3.3. Collecting localities of *Diaprepes abbreviatus* in Dominica with corresponding haplotype network. Each symbol represents different haplogroups and the size is proportional to the number of individual per haplotype. Numbers on branches indicate number of mutational steps. Branches without number imply one change.

Group B is the most widespread lineage, containing 16 haplotypes and 34 samples from four distinct regions (Table 3.1). The majority of these samples originate from agricultural areas, in particular citrus plantations with varying levels of intense to minimal management, with the exception of individuals from Mona Island and Tortuguero, northern-central Puerto Rico. Most of the observed haplogroups within Group B are well supported; however, in some of the inter-haplogroup relationships remain ambiguous (Fig. 3.2, B1). Specifically, the two represented haplotypes from Florida are clustered in a polytomy with other lineages from northern Puerto Rico and the eastern and central regions of the Dominican Republic (Fig. 3.4a). The sister group of this internally poorly resolved lineage (B1) is unclear according to both parsimony and Bayesian inference. The maximum likelihood analysis, in turn, shows a weakly supported relationship (77% bootstrap support) with haplotypes 21 and 22 (B4) of central Puerto Rico. This unresolved clade B1 also encompasses haplotype 35, which is the only haplotype in the analyses

shared among the two Greater Antillean islands, i.e. among the eastern regions of the Dominican Republic and northern Puerto Rico (Fig. 3.4a). The sequenced individuals from Mona Island are closely related with samples from Sabana Grande (B2), a primarily dry forest region in southwestern Puerto Rico. Three additional haplotypes are represented in the region around Isabela, along the northwestern coastal region of Puerto Rico: haplotype 24 is closely related to the Florida populations, whereas haplotypes 25 and 26 (B3) show closer affinities to the Mona Island/Sabana Grande groups. The last element of Group B is haplotype 40, which occurs in the Central Cordillera of the Dominican Republic and is sympatric with the haplotypes 39 and 41 of Group C.

Group C contains 11 haplotypes; four of these are from the Dominican Republic (C2) and are jointly well differentiated from the remaining seven Puerto Rican haplotypes (C1) (Figs. 3.2 and 3.4b). The haplotypes from C1 were found mainly in state forests and other non-agricultural habitats, whereas only haplotype 42 (Pico Diego de Ocampo) was observed outside of cultivated areas in the Dominican Republic. The inter-haplotype relationships for each Greater Antillean island are generally weakly supported. Nevertheless, several locally restricted haplotypes may be recognized; i.e. in C1 the haplotype 33 from the Susúa State Forest, and haplotype 23 from Hormigueros. In the Dominican Republic haplogroups within C2 are less genetically diverse and more widespread (Fig. 3.4b).

After trimming the sequences to 611 base pairs, the herein identified haplotypes 34, 35, and 37 collapsed into haplotype COI-2 sensu Ascunce *et al*. (2008), now with a total of 11 samples, and haplotypes 17 and 18 collapsed into COI-1, now with seven samples. None of the sequences obtained in this study are assignable to Ascunce *et al*.'s (2008) haplotype COI-3. Accordingly, haplotype COI-2 is shared between Florida, the Dominican Republic, and Puerto Rico. All three haplotypes of Ascunce *et al*. (2008) fall within Group B (Fig. 3.4c).

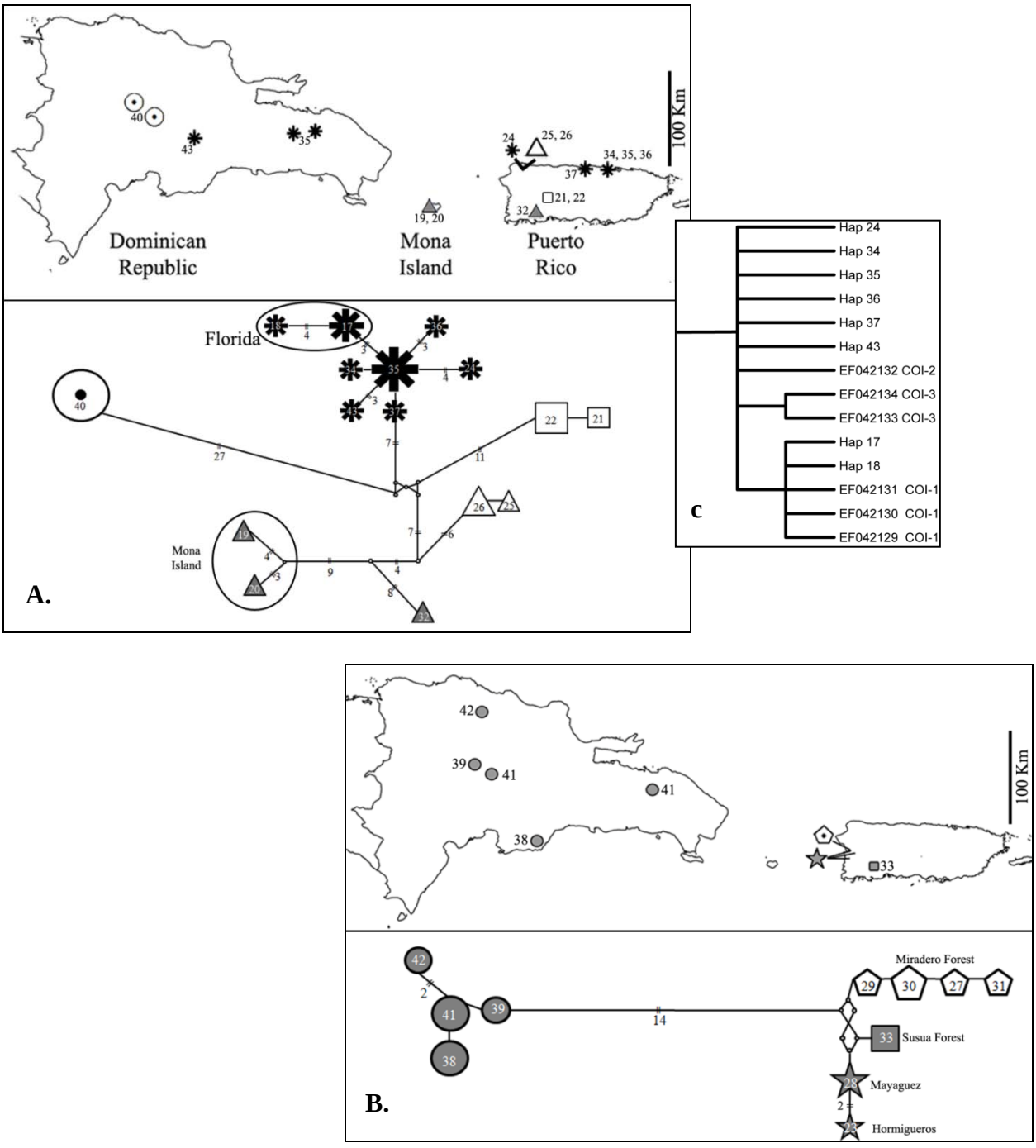

Fig. 3.4. Collecting localities and corresponding haplotype network of *Diaprepes abbreviatus* from (a) the Group B and (b) Group C in Dominican Republic, Mona Island and Puerto Rico. Each symbol represents different haplogroups and the size is proportional to the number of individual per haplotype. (c) Phylogenetic relations of haplotypes COI-1, COI-2, and COI-3 with the subgroup B1.

3.3.2 Population Diversity

The sampled *D. abbreviatus* populations represent a high diversity of segregating sites, haplotypes, and nucleotides (Table 3.2). Inter-island comparison of diversity indices reveals that the Puerto Rican populations are the most diverse in terms of haplotypes diversity (Hd = 0.978), whereas the samples from Dominica have the highest nucleotides diversity (π = 0.043). Populations from the Dominican Republic presented the lowest diversity values (Hd = 0.786, π = 0.034). Data partitioning according to the three main phylogenetic groups indicates that Group A has the highest values of segregating sites and diversity of nucleotides (S=144, π = 0.045). Group B shows the lowest diversity of haplotypes, and Group C presents the lowest diversity of nucleotides, but it has the highest diversity of haplotypes (Hd = 0. 962, π = 0. 009) (Table 3.2).

The demographic history of populations as inferred via Tajima's D values fails to show significant differences between the three phylogenetic groups or between islands (Table 3.2). Posterior analysis in each of the subgroups revealed only a significant Tajima's D for the subgroup B (-1.74, p-value = 0.02) and furthermore, showed a relatively high diversity of haplotypes yet a low diversity of nucleotides (Hd = 0.816, π = 0.003). The mismatch analysis provides evidence of a rapid range expansion in subgroup B1 (Fig. 3.5). However, neither the raggedness statistics (r) nor the Sum of Squared Deviation (SSD) were significant under the sudden expansion model (r = 0.12, P-value = 0.19, SSD= 0.04, p-value = 0.26), thus lending no support for a stable (non-expanding) population (Harpending 1994).

The AMOVA analyses reveal significant genetic variation among groups as well as islands. In particular, 72% of the observed variation was related to among-group differences ($p < 0.001$) and 28% was represented by within-group differences ($p < 0.001$). However, data partitioning according to the three islands explained 56.1% of the variation, whereas 43.9% of the genetic differences were found within each island. Pair-wise comparisons show significant genetic differentiation between (1) the three groups, (2) Dominica versus Puerto Rico (Fst= 0.637, $p < 0.001$), and (3) Dominica versus the Dominican Republic populations (Fst= 0.648, $p < 0.001$; Table 3.3). Differences in genetic variation between Puerto Rico and the Dominican Republic were not significant (Fst= 0.099, p >0 .05; Table 3.3).

Table 3.2. Summary statistics for COI gene sequences from *Diaprepes abbreviatus* by phylogenetic groups and island. Number of individuals (*n*), Number of haplotypes (*h*), Number of segregating sites (*S*), Haplotype diversity (*Hd*), Nucleotide diversity (π), Tajima's D test (D, p>0.10).

Partition	***n***	***h***	***S***	H_d	**π**	**D**
Groups						
Group A	24	16	144	0.946+/- 0.029	0.045+/- 0.023	1.317
Group B	34	16	93	0.902+/- 0.033	0.020+/- 0.010	0.128
Group C	15	11	28	0.962+/- 0.034	0.009+/- 0.005	1.501
Total	73	43	236	0.972+/- 0.008	0.069+/- 0.033	-
Islands						
Dominica	23	15	120	0.941+/- 0.031	0.043+/- 0.021	2.049
Puerto Rico	22	17	101	0.978+/- 0.019	0.036+/- 0.018	1.929
Dom. Republic	21	7	89	0.786+/- 0.064	0.034+/- 0.017	2.351
Total	66	38	222	0.967+/- 0.011	0.070+/- 0.033	-

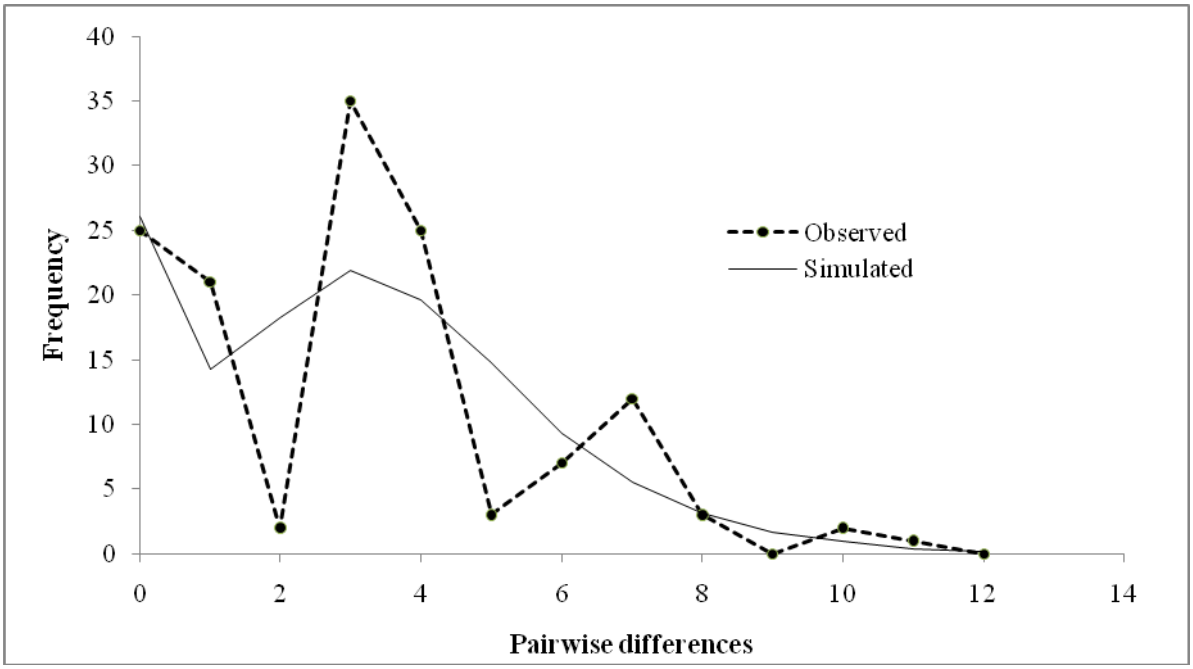

Fig. 3.5. Mistmach distribution of mithocondrial DNA sequences based on pairwise differences among haplotypes of the subgroup B1 of *D. abbreviatus.* Solid lines in the curves indicate the expected distributions under population expansion and dotted lines indicate the observed distributions.

Table 3.3. Comparisons of pairs of population samples (below the diagonal) and significant Fst p- values (above the diagonal) (+) =significant, (-) =non-significant, α= 0.05.

Partition	**Group A**	**Group B**	**Group C**
Group A	*	+	+
Group B	0.723	*	+
Group C	0.713	0.727	*

	Dominica	**Puerto Rico**	**Dom. Republic**
Dominica	*	+	+
Puerto Rico	0.637	*	-
Dom. Republic	0. 648	0. 099	*

3.4 Discussion

Our phylogeographic analyses of Caribbean *D. abbreviatus* populations provide strong support for the presence of three haplotype lineages with distinct historical and distributional traits (Fig. 3.2). Of these, Group A is the most geographically isolated and corresponds primarily to the populations of Dominica, which may however be due in part to sampling focus (see also Ascunce *et al*. 2008). Groups B and C, in turn, are partially sympatric and both occur in the Dominican Republic and Puerto Rico. In general, the mitochondrial COI sequences of *D. abbreviatus* revealed a high genotypic diversity which is matched by a high phenotypic diversity as previously reported for the species (Pierce, 1915; Hantula *et al*., 1987; O'Brien and Kovarik, 2001; Lapointe, 2004). Nevertheless, this diversity is not homogeneously distributed across samples and localities, suggesting that different factors and time scales have operated to produce the observed differences in population structure. In particular, we propose that the population structure of *D. abbreviatus* in Dominica and western Puerto Rico has been strongly influenced by long-term, geological processes; whereas populations in the Dominican Republic, Mona Island, and northern Puerto Rico are more strongly impacted by recent ecological events including human activity.

According to the F_{ST} values for pair-wise population comparisons (Table 3.3), Group A has experienced reduced gene flow and is well differentiated from the other two Antillean groups. The Dominican populations furthermore exhibit a strong phylogeographic structure (Fig. 3.3) that is seemingly aligned with the island's topography and history of volcanism (cf. Maury *et al*., 1990). Similar phylogeographic patterns – i.e. a general congruence between lineage divergence and major pyroclastic flows in the past 50,000 years – have been reported for the Dominican anole *Anolis oculatus* Cope (Malhotra and Roger, 2000; Stenson *et al*., 2002). Other beetle populations show similar range disruptions in response to intensive volcanic activity, e.g. in the Mexican bark beetle *Dendroctonus mexicanus* Hopkins (Anducho-Reyes *et al*., 2008). The documented tendency of *D. abbreviatus* populations to disperse slowly and locally (Bas *et al*., 2000) would facilitate differentiation in response to such geological events.

The more widespread Greater Antillean Groups B and C occur sympatrically in the Dominican Republic but not in Puerto Rico, which suggests that each has had a distinct historical trajectory. According to our phylogenetic reconstruction (Fig. 3.2), there are minimally two (B1, C2) and possibly three (h40) independent colonizations events directed from Puerto Rico to the Dominican Republic. Within Group B in particular, as many as four inter-island dispersal events may be postulated as follows: (1) from southern Puerto Rico to Mona Island (h20, h21); (2) from northern Puerto Rico to Florida (h17, h18); (3) from northern Puerto Rico to the eastern Dominican Republic (h42); and (4) from the presumed ancestral area of Groups B and C to the Dominican Republic (h40). Given the widespread occurrence and comparatively low genetic variation among the majority of the respective haplogroups, we consider that up to three of the aforementioned colonization events (1-3) may be recent and facilitated by human agricultural activity (see also Lapointe *et al*., 2007; Ascunce *et al*., 2008).

Group C contains two well-differentiated lineages from Puerto Rico (C1) and from the Dominican Republic (C2), respectively (Figs. 3.2 and 3.4b). Five out of the seven C1 haplotypes obtained from Puerto Rico are present in the central west-coast municipality of Mayagüez, where they occur mainly in forest remnants such as the Miradero forest adjacent to the University of Puerto Rico at Mayagüez campus (Fig. 3.4b). This perhaps surprisingly high diversity of *D. abbreviatus* populations in an area dominated by fragmented and secondary habitats is nevertheless congruent with an overall high diversity of beetle species found in forest remnants in the vicinity of Mayagüez (Martínez *et al*., 2009). The distinct haplotypes from Hormigueros and from the Susúa State Forest are apparently restricted to just these regions. On the other hand, the C2 lineage is widely distributed throughout the Dominican Republic (Fig. 3.4b), with little congruence between population identity and geographic distribution within the island. Virtually all specimens were taken in smaller-scale agricultural habitats, with the exception of haplotype 42, which was sampled in habitats surrounding the Pico Diego de Ocampo Biological Reserve, Santiago. The lower genetic diversity and wider distribution of the Dominican Republic haplotypes may reflect (1) relative recent divergences within the C2 clade or (2) the presence of a bottle-neck effect (cf. Scataglini *et al*., 2006; Ruiz *et al*., 2010). Either process must have occurred in a sufficiently distant past to allow for the observed divergences in the mitochondrial DNA of the populations. The inclusion of samples from well preserved areas in the Dominican

Republic and incorporation of reliable molecular time-calibrations are needed to provide further insights into the divergence between the C1 and C2 lineages.

The phylogeographic structure among and within the three main lineages (Fig. 3.2) provides some indication as to the relative timing of the inferred colonization events. In general, a higher genetic variability is expected in ancient populations (Hewitt, 2004; Ruiz *et al*., 2010). Accordingly, the populations from Dominica and Puerto Rico, which show the highest values of diversity (see H*d* and π values; Table 3.2) may have originated from an early split of the ancestral population, possibly during the configuration of the Lesser Antilles between the late Miocene and early Pliocene (Maury *et al*., 1990; Peck, 2006). At this time the Lesser Antilles no longer had subaerial connections to either the Greater Antilles or to South America (Iturralde-Vinent, 2006; Ricklefs and Bermingham, 2008). Under these premises *D. abbreviatus* should have arrived to Dominica no earlier than 10 mya. The relatively high diversity of haplotypes and nucleotides within Group A could also imply a higher mtDNA substitution rate, possibly facilitated by the mode of inter-island diversification, i.e. (1) reductions in effective population size, which occur due to a bottleneck at the time of colonization, and/or (2) long-term restrictions in range size which can increase the substitution rate values (Woolfit and Bromham, 2005; Papadopoulou *et al*., 2010).

Mona Island presents another interesting case regarding the timing of dispersal of *D. abbreviatus* populations. This island is located between the southwestern and southeastern tips of Puerto Rico and the Dominican Republic, respectively; it was lifted above sea level in the Late Miocene/Early Pliocene (i.e. 5-7 mya; see González *et al*., 2007). The island harbors two exclusive haplotypes, which are most closely related to that of Sabana Grande, southwestern Puerto Rico. Such east-to-west dispersals from this region of Puerto Rico to Mona Island are known to have occurred in other insect groups, and may have been facilitated by a prevailing wind asymmetry (Smith *et al*., 1994; Rodríguez-Robles *et al*., 2007; Franz *et al*., 2009). Mona Island has experienced only limited subsistence agriculture over the past centuries (Wadsworth, 1973); nevertheless, the island's *D. abbreviatus* haplotypes are related to populations that occur predominantly on citrus crops in Puerto Rico.

Our results regarding citrus root weevil population diversity further underscore the dynamic history of this species in the Caribbean region. A negative Tajima's D statistic indicates an excess of recent mutations, as is expected when populations have undergone a demographic expansion or positive selection (Tajima, 1989). Such an expansion is supported by the relative low nucleotide diversity, high haplotype diversity (H*d* = 0.816, π = 0.003), and the star-like clustering of nodes around a "founder population" – i.e. haplotype 35 (Fig. 3.4a) which corresponds in the trimmed version (see methods) to haplotype COI-2 of Ascunce *et al*., (2008). The shape of the mismatch distribution furthermore suggests that there were two differently timed expansions (Fig. 3.5), thus implying separate colonization events from Puerto Rico to the Dominican Republic and to Florida. Previous studies have reported the establishment of three distinct *D. abbreviatus* populations in Florida (Bas *et al.*, 2000; Ascunce *et al.*, 2008). The present analyses show no haplotypes of the COI-1 and COI-3 groups in Puerto Rico; at least the CO-I haplotype is present in eastern Puerto Rico (M. S. Ascunce, personal communication). The CO-2 haplotype inhabits agricultural as well as more conserved habitats in northern Puerto Rico, and has evidently spread to the United States, most recently to Texas and California (Skaria and French, 2001; Lapointe *et al*., 2007; Jetter and Godfrey, 2009). Recent and human-induced movements of *D. abbreviatus* populations also seem to have shaped the distributional patterns of haplotypes observed in the Dominican Republic and in northern Puerto Rico.

The results of our study provide new insights into the evolutionary origins of *D. abbreviatus*. A recent phylogenetic analysis of Caribbean entimine weevils based on morphological data (Franz, 2011) indicates that *D. abbreviatus* is part of a monophyletic group that also includes the widespread and similar colored species *D. comma* (Hispaniola, Puerto Rico, Trinidad and Tobago, Venezuela) and *D. doublierii* (Hispaniola, Puerto Rico). In addition, Mazo-Vargas *et al*.'s (2011) molecular phylogenetic analysis positions *D. abbreviatus* in close relationship with other Lesser Antillean species. The ancestral populations of *D. abbreviatus* may thus have occupied the eastern parts of the Greater Antilles, and particularly Puerto Rico where the putative sister taxon the "striped, yellowish" complex *D. maugei* occurs. Under this scenario, subsequent colonization events towards the Lesser Antilles are required to explain the present-day distribution of *D. abbreviatus*. On the other hand, neither Cuba nor Jamaica harbors *D. abbreviatus* populations, suggesting that the species diverged after the formation of Mona Passage in the Late Oligocene/Mid Miocene period (Iturralde-Vinent, 2006).

Our results add a new phylogeographic dimension to previous studies which have focused on the origins and genetic variation of citrus root weevils in the southeastern United States (Bas *et al*., 2000; Ascunce *et al*., 2008). Specifically, the inclusion of specimens from both the Greater and Lesser Antilles reveals a complex inter- and intra-island population structure influenced by different regionally and temporally operating factors. For instance, the apparent long standing and isolated evolution of *D. abbreviatus* populations on Dominica has produced several narrowly restricted haplotypes with distinct properties from those of the Greater Antilles and United States. Unpublished data of genetic distance analyses (M. A. Ascunce, personal communication) furthermore show that populations from the Dominican Republic, Puerto Rico, and the United States pertain to two main clusters: (1) eastern Puerto Rico and the United States, and (2) central, northern, and western Puerto Rico, and the Dominican Republic. In contrast, the herein inferred Groups B and C show a geographically mixed pattern which suggests the existence of multiple independent colonization events.

4 Conclusions and Future Work

Our results provide a basis for a more balanced understanding of the diversification of Neotropical weevils in the subfamily Entiminae, where the evolution of inter- and intra-island endemism and ecological specialization may ultimately deserve more explanatory weight than the paradigmatic escape-and-radiation hypothesis (Franz and Engel, 2010). These results furthermore demonstrated that, in spite of grave taxonomic challenges regarding both described and undescribed species and genera, weevils in the *Exophthalmus* genus complex represent an excellent system to study diversification at the Caribbean/Neotropical mainland interface. Accordingly, suitable directions for future research in this system include: (1) testing for the possible influence of GAARlandia (Iturralde-Vinent, 2006) in the Greater Antilles and particularly in Puerto Rico (e.g. *Scelianoma elydimorpha*), through inclusion of additional South American taxa; (2) expanding the taxon sampling to Cuba and Mesoamerica in order to establish phylogenetic connections to the Caribbean taxa and infer the number and directionality of dispersal events; (3) describing and incorporating fossil taxa (e.g. from Dominican and Mexican amber) into the phylogenetic reconstructions in order to infer the timing clade divergences; and (4) reconciling the expanded and time-calibrated phylogeny with known geological relationships to ascertain the sequence and relative importance of mainland-to-island, inter-island, and intra-island diversification in the main entimine lineages. Such comparisons of Caribbean and Neotropical mainland clades may also improve our understanding of the potential effects of long-term habitat stability on the diversity and host specificity of entimine weevils. On one hand, host specificity is likely to increase in high-elevation habitats and well-preserved and xeric low-elevation habitats. On the other hand, the dynamic history of the Caribbean archipelago, including rapid habitat and climate shifts (Iturralde-Vinent, 2008; Ricklefs and Bermingham, 2008), may have limited specialization to host plants. Multiple phylogenetically independent comparisons of the diversity and host specificity among Neotropical mainland and Caribbean entimine lineages will allow us to systematically refine and test these propositions (cf. Antonelli *et al*., 2009; Rull, 2009)

The phylogeographic analysis of *D. abbreviatus* significantly expands our knowledge of the genetic diversity of this species in the Caribbean region. Future research on the phylogeography of *D. abbreviatus* should focus on adding samples from agricultural and well preserved habitats throughout the entire distributional range of this species. Such an approach promises to resolve ambiguities apparent from our study regarding the number, direction, and timing of *D. abbreviatus* range expansions within and among islands. Moreover, the inclusion of nuclear markers such as intron sequences and/or microsatellites, will permit assessments of the amount of population admixture and historical dispersal tendencies of citrus root weevils in the Caribbean region. Lastly, a comprehensive analysis of the evolutionary tendencies of this species must incorporate and contrast information on the external and internal morphological variation that characterizes different local populations of *D. abbreviatus* (cf. Hantula et al., 1987).

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APPENDIX 1.

Detailed list of host plant orders associated with 25 of the sampled entimine weevil taxa for which such information is available. Primary resources: Wolcott, 1929, 1936, 1948; Dixon, 1954; Vaurie, 1961; Martorell, 1976; Woodruff, 1985; Maes and O'Brien, 1990; Chaves and Fonseca, 1991; Rivas, 1992; Coto and Saunders, 2004; Peck, 2005, 2006, 2009; Franz, 2010b).

Weevil taxon	Order	Family	Host taxon
Eudiagogini			
Promecops sp. 1	Gentianales	Rubiaceae	*Coffea* L.
Polydrusini			
Apodrosus argentatus	Brassicales	Capparaceae/Brassicaceae	*Quadrella indica* Iltis & Cornejo
	Fabales	Fabaceae	*Dalbergia ecastaphyllum* (L.) Taub.
	Fabales	Fabaceae	*Prosopis* L.
	Rosales	Rhamnaceae	*Colubrina colubrina* Millsp.
	Zygophyllales	Zygophyllaceae	*Guaicum sanctum* L.
Apodrosus wolcotti	Fabales	Mimosaceae	*Inga fagifolia* L.
Tanymecini			
Pandeleteius sp. 1	Boraginaceae	Boraginaceae	*Cordia* L.
	Caryophyllales	Nyctaginaceae	*Pisonea aculata* L.
	Fabales	Fabaceae	*Inga* Mill.
	Gentianales	Rubiaceae	*Psychotria* L.
	Laurales	Lauraceae	
	Malpighiales	Malpighiaceae	
	Myrtales	Melastomataceae	
Pandeleteius nodifer	Fabales	Fabaceae	*Cassia* L.
	Fabales	Fabaceae	*Pithecellobium* Mart.
	Fabales	Fabaceae	*Prosopis* L.
	Lamiales	Verbenaceae	*Lantana* L.
	Malvales	Malvaceae	*Gossypium* L.

Weevil taxon	Order	Family	Host taxon
Naupactini			
Litostylus sp. 1	Malpighiales	Malpighiaceae	*Heteropteris* Fée
	Sapindales	Rutaceae	*Citrus* L.
Litostylus sp. 2	Myrtales	Myrtaceae	*Pimenta racemosa* (Mill.) J.W.Moore
Eustylini I			
Eustylus hybridus	Fabales	Fabaceae	*Lathyrus* L.
	Myrtales	Myrtaceae	*Syzygium* Gaertn.
Geonemini			
Apotomoderes menecrater	Myrtales	Myrtaceae	*Pimenta* sp.
	Zygophyllales	Zygophyllaceae	*Guaiacum officinale*
Apotomoderes sotomayor	Sapindales	Rutaceae	*Citrus* sp.
Artipus monae	Fagales	Casuarinaceae	*Casuarina equisetifolia* L.
	Sapindales	Rutaceae	*Amyris elemifera* L.
	Solanales	Solanaceae	*Solanum melongena* L.
Lachnopus coffeae	Gentianales	Rubiaceae	*Coffea arabica* L.
	Sapindales	Rutaceae	*Citrus paradisi* Macfad.
	Sapindales	Rutaceae	*Citrus sinensis* Osbeck
Lachnopus kofresi	Solanales	Solanaceae	*Solanum melongena* L.
Lachnopus seini	Ericales	Primulaceae	*Rapanea ferruginea* (Ruiz & Pav.) Mez.
Eustylini II			
Compsus maricao	Gentianales	Rubiaceae	*Coffea* L.
	Myrtales	Myrtaceae	*Eugenia* L.
	Rosales	Moraceae	*Cecropia pelpata* L.
	Rosales	Rosaceae	*Prunus occidentalis* Swartz
Diaprepes balloui	Myrtales	Myrtaceae	*Psidium* L.
	Sapindales	Rutaceae	*Citrus* L.
Diaprepes famelicus	Fabales	Fabaceae	*Cajanus cajan* (L.) Millsp.
	Laurales	Lauraceae	*Persea americana* Mill.
	Myrtales	Myrtaceae	*Psidium* L.

Weevil taxon	Order	Family	Host taxon
	Poales	Poaceae	*Saccharum officinarum* L.
	Sapindales	Rutaceae	*Citrus* L.
Exophthalmus cinerascens	Solanales	Convolvulaceae	*Ipomea* All.
Exophthalmus quadrivittatus	Ericales	Sapotaceae	*Chrysophyllum cainito* L.
	Fabales	Fabaceae	*Sesbania* Scop.
	Fabales	Fabaceae	*Sesbania sericea* (Willd.) Link
	Gentianales	Rubiaceae	*Coffea* L.
	Rosales	Rosaceae	*Fragaria* L.
	Sapindales	Rutaceae	*Citrus* L.
	Solanales	Solanaceae	*Solanum* L.
Exophthalmus quindecimpunctatus	Caryophyllales	Nyctaginaceae	*Guapira fragans* (Dum.-Cours) Little
Exophthalmus roseipes	Caryophyllales	Polygonaceae	*Coccoloba uvifera* L.
	Caryophyllales	Cactaceae/Caesalpiniaceae	*Hymenaea courbaril* L.
	Celastrales	Celastraceae	*Cassine xylocarpa* Vent.
	Celastrales	Celastraceae	*Elaeodendron xylocarpum* (Vent.) DC.
	Fabales	Fabaceae	*Andira inermis* (Wright) Kunth ex DC.
	Fabales	Fabaceae	*Andira jamaicensis* Urb.
	Fabales	Fabaceae	*Dalbergia ecastaphyllum* (L.) Taub.
	Fabales	Mimosaceae	*Inga fagifolia* (L.) Willd. ex Benth
	Fabales	Mimosaceae	*Inga vera* Willd.
	Malpighiales	Chrysobalanaceae	*Chrysobalanus icaco* L.
	Malpighiales	Ochnaceae	*Ouratea litoralis* Urb.
	Malvales	Malvaceae	*Gossypium barbadense* L.
	Myrtales	Combretaceae	*Conocarpus erectus* L.
	Myrtales	Combretaceae	*Terminalia catappa* L.
	Sapindales	Rutaceae	*Citrus* L.
	Sapindales	Rutaceae	*Citrus sinensis*
Exophthalmus similis	Gentianales	Rubiaceae	*Coffea* L.
	Malvales	Malvaceae	*Hibiscus* L.

Weevil taxon	Order	Family	Host taxon
	Rosales	Rosaceae	*Malus* Mill.
	Sapindales	Rutaceae	*Citrus* L.
Exophthalmus vittatus	Fabales	Fabaceae	*Gliricidia sepium* (Jacq.) Kunth
	Sapindales	Rutaceae	*Citrus medica* L.
	Sapindales	Rutaceae	*Citrus paradisi* Macfad.
	Sapindales	Rutaceae	*Citrus* L.
Pachnaeus marmoratus	Dioscoreales	Dioscoreaceae	*Dioscorea* L.
	Sapindales	Rutaceae	*Citrus* L.