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Species delimitation of the North American orchard-spider *Leucauge venusta* (Walckenaer, 1841) (Araneae, Tetragnathidae)

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ABSTRACT

The orchard spider, *Leucauge venusta* (Walckenaer, 1841) is one of the most common and abundant orb-weavers in North America. This species has a broad geographic distribution extending across tropical and temperate regions of the Americas from Canada to Brazil. Guided by a preliminary observation of the barcode gap between sequences from specimens of *L. venusta* collected in Florida and other North American localities, we collected across a transect through the southeastern USA to investigate the observed genetic divide. The dataset, complemented with additional samples from Mexico, and Brazil was analyzed for species delimitation using STACEY and bGMYC based on sequences from one nuclear (ITS2) and one mitochondrial marker (COI). The analyses clearly separate USA samples into two deeply divergent and geographically structured groups (north–south) which we interpret as two different species. We generated ecological niche models for these two groups rejecting a niche equivalence hypothesis for these lineages. Taxonomic changes are proposed based on these findings, *Leucauge venusta* is restricted to denote the northern clade, and its known distribution restricted to the USA. *Leucauge argyrobapta* (White, 1841) is removed from synonymy to denote the populations in Florida, Mexico and Brazil. Although the delimitation analyses suggest each of these geographic clusters within the *L. argyrobapta* samples represent different species, more specimens from Central and South America are needed to properly test the cohesion of *L. argyrobapta* populations.

1. Introduction

The term “cryptic species” is used to refer to two or more effectively distinct species that, under the veil of common external morphology, are classified with the same name (Bickford et al., 2007). The use of DNA sequence data in general, and DNA barcoding efforts in particular (Robinson et al., 2009) have discovered many cryptic species in various groups of organisms revealing a hitherto uncharted diversity (Wheeler, 2005; Pfenninger and Schwenk, 2007; DeWaard et al., 2009; Adams et al., 2014; Telfer et al., 2015). At the same time, it has been recognized that gene sequence dissimilarity alone does not provide sufficient evidence for delimiting species (Desalle, 2006; DeWaard et al., 2009; Dasmahapatra et al., 2010; Kvist, 2013; Lohse, 2009). The problem of species delimitation is further compounded by a variety of confounding factors affecting the distribution of genetic differences in a group of individuals, such as migration, population size, local adaptation, geographic sampling, incomplete lineage sorting and introgression (Bergsten et al., 2012). In response to these difficulties, modern approaches to species delimitation favor an integrative approach combining the use of multi-locus sequence data, explicit evolutionary models in combination with ecological, morphometrics or behavioral data (Rissler and Apodaca, 2007; Leaché et al., 2009; Rittmeyer and Austin, 2012; Karanovic et al., 2015; Rannala, 2015).

The spider genus *Leucauge* White, 1841 (Tetragnathidae) is a species rich group of mostly tropical and subtropical orb-weaving spiders although some representatives occur in temperate regions in Asia, Australasia and the Americas. Some species of *Leucauge* are widely distributed and abundant but the vast majority of the described species are known from only a few records. The genus currently includes 174 valid species (World Spider Catalog, 2017) but many more remain undescribed. A much-needed taxonomic revision of *Leucauge* has been hampered by the sheer number of species and the lack of proper descriptions and illustrations for the described species.

The focal species of this paper, the orchard spider *Leucauge venusta* (Walckenaer, 1831), is one of the most common and abundant species of orb-weavers in the United States of America (USA) where only two species of *Leucauge* are known to occur. Geographically, *Leucauge venusta* has a broad continuous distribution from southern Canada to

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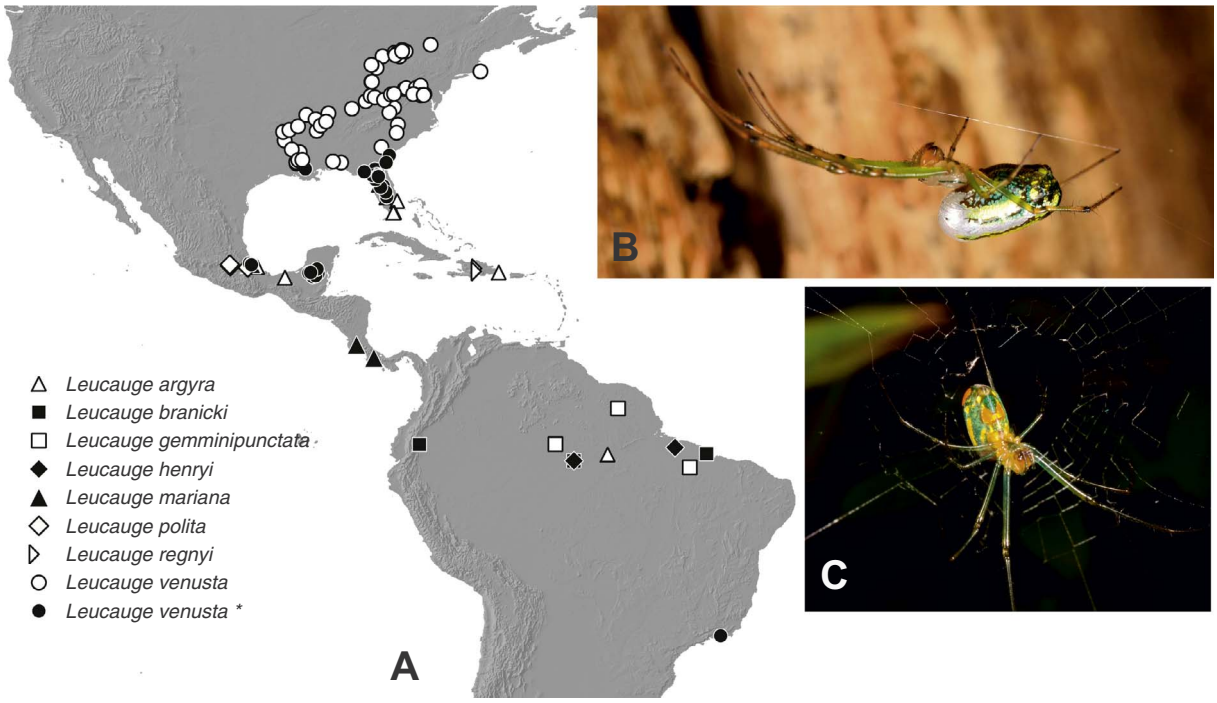

Fig. 1. (A) Distribution of the samples used in the species delimitation analyses. Black and white circles denote localities of *L. venusta* s. l.; dark circles indicate specimens removed from L. venusta in accordance with the results of this study. (B) Photo of a female of *L. venusta* from Arlington, Virginia, USA (DSC-0984-16249). (C) Photo of a female of L. venusta from Jekyll Island, Georgia (DSC-1485-16811).

Brazil. A disjunct population of *L. venusta* is known to occur in southern California where, unlike in the eastern USA, it is considered a rare species (Adams et al., 2014). In contrast *L. argyra* is restricted to tropical regions of the Americas (Walckenaer, 1831) and in the USA is only found in Florida. These two species are morphologically well defined and can be easily distinguished even where they occur in sympatry.

The nomenclatural history of *L. venusta* is inextricably intertwined with that of *L. argyrobapta*. The former species was described as *Epeira venusta* by Walckenaer in 1841 based on a drawing from John Abbot's unpublished series of watercolors on the spiders of Georgia (Abbot, ca. 1792–1804, plate 13, Fig. 113). The precise identity of several of the names proposed by Walckenaer and based on Abbot's work have been historically controversial (Chamberlin and Ivie, 1944; Levi and Levi, 1961). Although no precise record of the source of the specimens used by Abbot exists, the specimens likely came from Burke County (Georgia) where Abbot lived and collected (Chamberlin and Ivie, 1944).

Later the same year (1841), Adam White introduced the name *Leucauge* at the rank of subgenus and described its type species *Linyphia (Leucauge) argyrobapta* based on a female specimen collected by Charles Darwin in 1832 in the Rio de Janeiro area (Brazil) during the H.M.S. Beagle expedition. Unfortunately, Darwin's specimen was lost after White's description was published (Levi, 1980; Dimitrov and Hormiga, 2010). In the absence of the type specimen, the exact identity of *L. argyrobapta* remained a mystery for more than a century and a half, until Dimitrov and Hormiga (2010) searched for topotypical specimens of *L. argyrobapta* in Rio de Janeiro. Based on their own collections of *Leucauge* species as well as the study of specimens collected in the Rio de Janeiro area deposited at the Museu Nacional, Dimitrov and Hormiga (2010) were able to designate a neotype for *L. argyrobapta*. Based on the morphological examination of specimens of *L. venusta* (from Virginia, USA) and the neotype of *L. argyrobapta* (as well as other specimens collected in Rio de Janeiro along with the neotype) Dimitrov and Hormiga (2010) concluded these two represented the same taxon and proposed the synomymy of *L. venusta* = *L. agyrobata*, with priority favoring *L. venusta* as the valid name. Additionally, since *L. argyrobabpta* is the type species, *L. venusta* became the type species of the genus *Leucauge*. Based on their circumscription, *L. venusta* sensu lato (s. l. hereafter) extended from southeastern Canada, to southern Brazil. Such a broad distribution ranges are not particularly surprising in an orbweaver spider, many of which have remarkable air-mediated dispersal capabilities (e. g. Henaut et al., 2006; Bell et al., 2007; Eberhard, 1987), but few studies have investigated the genetic structure of such broadly distributed species (Su et al., 2007; Garb and Gillespie, 2009; Su et al., 2011; Kuntner and Agnarsson, 2011).

Preliminary comparisons of publicly available sequences of the barcoding gene, cytochrome *c* oxidase subunit 1 (COI), of *L. venusta* s. l. from Florida, Canada and Northeastern USA show a clear gap (10%) between the specimens from Florida and the rest of the samples, while all northern samples are all very similar in their COI sequences (less than 1%, see supplementary Fig. 8 for prelimary barcode analyses). Notably, the genetic distances between the Florida samples and our sequences of *L. venusta* from Rio de Janeiro (Brazil) are surprisingly low (1.6%) in spite of the large geographic distance. In previous spider barcoding surveys the reported average intraspecific difference is 1.4 % and congeneric interspecific 16% (Barrett and Hebert, 2005; Robinson et al., 2009; Čandek and Kuntner, 2015; Hamilton et al., 2014). This barcode gap suggests the possibility of at least one cryptic species within *L. venusta* s. l. and, more importantly, places the geographic scenario for such divide in the southeast region of the USA.

In this study we investigate this cryptic species hypothesis using sequences of two molecular markers commonly used at shallow evolutionary scale for spider phylogenetics and phylogeography: the mitochondrial COI and one nuclear gene, the internal transcriber spacer 2 (ITS2) (e. g. Chang et al., 2007; Agnarsson, 2010; Peres et al., 2015; Mammola et al., 2015; Planas and Ribera, 2015; Planas et al., 2014). Our sampling of specimens of *L. venusta* is centered on the southern USA, including specimens in the vicinity to the probable type locality of *Epeira venusta* in the state of Georgia. Additionally, this study includes some samples from localities in Mexico and Brazil, including the type locality of *L. argyrobapta* in Rio de Janeiro (Brazil) (Fig. 1).

The results from the species delimitation analyses are

complemented with models of potential distribution based on occurrence records and environmental variables associated with the collection data and subject to ecological niche equivalence tests. These analyses aim to explore if the groups recognized by the species delimitation analyses differ in their habitat preferences.

The concept of isolation by distance (IBD) was introduced by Wright (1943) as a part of a population genetics model to explain the relation of genetic differences (F_{st}) as a function of population sizes and their spatial distribution. In the context of species delimitation, IBD is used to account for cases where spatial distribution may be the cause for observed genetic structure between populations (Queiroz, 2007; Zapata and Jiménez, 2012; Kozak et al., 2008; Rissler and Apodaca, 2007; Bergsten et al., 2012; Rittmeyer and Austin, 2012). The most common approach to disentangling geographic and genetic correlation is through a simple Mantel test (Mantel, 1967). Recent works have questioned the validity of Mantel test as a general tool to evaluate the response of variables of interest with spatial or spatially autocorrelated matrices (Meirmans, 2012; Guillot and Rousset, 2013; Harmon and Glor, 2010; Legendre and Fortin, 2010; Legendre et al., 2015). The critique centers on the lack of power of the Mantel test to characterize the relationship of multi-dimensional variables (such as both genetic and geographic data), when the data has been transformed into unidimensional values (distances). Unfortunately, there is no standard alternative to the Mantel test and its adequacy for species delimitation has not been assessed. In this study we explored the IBD scenario as an explanation for the observed genetic structure using bivariate plots, Mantel tests and redundancy analysis (RDA), an ordination method following the recommendations by Legendre et al. (2015).

2. Material and methods

2.1. Specimens and taxon sampling

Most of the specimens for this study were collected by hand and preserved in 95% ethanol along a transect throughout the Southeastern USA in the summer of 2014. These specimens are deposited in the spider tissue collection at The George Washington University and identified by a unique catalog number (see supplementary Table 4 for specimen metadata and GenBank accession numbers); voucher specimens will be deposited at the Museum of Comparative Zoology, at Harvard University. The sampling effort aimed to collect across known biogeographic barriers in the region, such as the Appalachian Mountains and the Mississippi river, and was complemented with specimens from museum collections and donations. Additionally, specimens of *L. mariana* (Taczanowski, 1881), *L. polita* (Keyserling, 1893), *L. argyra* (Walckenaer, 1841), *L. regnyi* (Simon, 1898), *L. bracniki* (Taczanowski, 1874), *L. henryi* Mello-Leitão, 1940, and *L. gemminipunctata* Chamberlin & Ivie, 1936 were included as outgroups (see map in Fig. 1). These species were selected based on preliminary results of an ongoing study on the higher level phylogenetic structure of *Leucauge* that will be published elsewhere. Most of these outgroups are represented by more than one specimen and therefore they serve as external validation points for the species delimitation analyses, under the assumption that these species are well defined.

To assess the effect of the presence of outgroups and missing data in the species delimitation analyses we assembled three different matrices. Matrix M0 includes eight nominal species (based on the current morphological-based circumscription) represented by 72 terminals, including ingroup and outgroups. The main property of M0 sampling is completeness (all terminals have both COI and ITS2 sequences). Matrix M1 is a subset of M0, where only the ingroup (*L. venusta* s. l.) and specimens of a single outgroup species (*L. argyra*) are present (63 terminals). Finally, matrix M2, is a superset of M0, including additional specimens for which COI sequences are present and those of ITS2 are missing, including specimens from publicly available databases (140 terminals).

2.2. DNA extraction and sequencing

Total DNA was extracted from two or three legs of each specimen using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. Two selected genes were amplified using the following primers, COI: LCO-1490 = 5′-GGT CAA CAA ATC ATA AAG ATA TTG G (Folmer et al., 1994), HCOout = 5′ CCA GGT AAA ATT AAA ATA TAA ACT TC (Carpenter and Wheeler, 1999) and ITS2: FITS = 5′ GGG ACG ATG AAG AAC GGA GC (Agnarsson, 2010, N.B.: This primer in the reference is attributed to White, 1990 – probably ITS3– but the sequence is different.), RITS = 5′-TCC TCC GCT TAT TGA TAT GC (White et al., 1990, originally referred as ITS4 in White op. cit., we use Agnarsson's naming for consistency). Polymerase chain reactions (PCR) were carried out in 25 μl total volume using 0.12 μl PROMEGA Go Taq G2 Flexi DNA polymerase, 5 μl colorless buffer (5 ×), 2 μl $MgCl_2$ (25 mM), 0.5 μl of dNTP mix (10 mM), 1 μl of each primer (10 mM) and 1 μl of DNA sample. PCR conditions for amplification of COI used the following protocol: initial denaturation step at 94 °C for 5 min followed by 35 amplification cycles (94 °C for 30 s, 40 °C for 30 s, 72 °C for 45 s) and a final extension cycle at 72 °C for 7 min. Amplification condition for ITS2 were the same as those for COI, except that 2 μl of template were used, initial denaturation step was only 3 min. and annealing temperature was set to 47 °C for 30 s. PCR products were visualized on 1% agarose gels stained with GelRed. PCR products were sent to MACROGEN, USA facilities for purification and sequencing. Raw reads were quality clipped and assembled into contigs and inspected visually using Geneious version 6.1.8 Kearse et al. (2012) or the Staden package version 2.0.0b11 Bonfield et al. (1995) and Staden (1996).

2.3. Sequence alignments

The COI fragments were aligned using the translation aware algorithm as implemented in MACSE version 1.2 (Ranwez et al., 2011). The multiple sequence alignment of COI sequences is usually trivial due to the absence of internal gaps, however the use of MACSE's translation aware algorithm facilitates the preservation and identification of the reading frame. Alignment of the ITS2 fragments was performed using 10 iterations of the PASTA routine (Mirarab et al., 2014), using MAFFT (Katoh and Standley, 2013, LINSI), OPAL (Wheeler and Kececioglu, 2007) and RAxML (Stamatakis, 2014, GTRGAMMA) as aligner, merger and tree estimator, respectively. Alignments were inspected and edited by eye in Aliview (Larsson, 2014). In the case of the ITS2 alignment, sites composed of more than 75% of gaps were removed using trimAl v1.2 (Capella-Gutiérrez et al., 2009). Final alignments for the COI and ITS2 partitions included 774 and 421 sites respectively.

2.4. Model selection

Models of sequence evolution and data partitioning were assessed with PartitionFinder v1.1.1 (Lanfear et al., 2012) using the Bayesian Information Criterion (BIC), testing codon based partitioning for COI and the subset of nucleotide models available for BEAST2 (Bouckaert et al., 2014) with unlinked branch lengths. The resulting best partition scheme combined all three codons position of COI in a single partition with a GTR + I + GAMMA substitution model. The ITS2 partition was assigned the TN93 + GAMMA.

Preliminary BEAST2 runs using a relaxed clock model (Drummond et al., 2006, uncorrelated log normal), rejected the use of a strict clock based on the coefficient of rate variation, showing mean estimates greater than 1.0 for both gene partitions. All matrices used a relaxed clock model with rates drawn from a lognormal distribution using a pure birth process tree model (Yule).

2.5. Species delimitation and phylogeny

Species delimitation was primarily evaluated using a multi-species

coalescent approach as implemented in the BEAST2 add-on package STACEY (Jones, 2016). This method was favored over alternative species discovery methods because it does not require *a priori* species trees nor group membership assignments. Additionally, this approach simultaneously co-estimates individual gene trees and species trees along with the species delimitation. Note that the term "species tree" in this context, refers to the distinction between the individual gene trees and the reconciled "tree", bearing in mind that terminals can refer to samples (the case in most analyses) or groups of terminals *a priori* grouped and deemed to represent species. Although not required, individual samples in STACEY can be *a priori* associated to species or populations, representing "minimal species cluster", i.e., groups of terminals that can be merged but not split. The main runs for the species delimitation ignored this species association feature, allowing for free grouping of the samples. The collapse weight parameter (ω), which affects the number of clusters, was given a flat prior bound from 0 to 1.0 and thus any possible grouping scheme received the same initial probability. Results from the STACEY runs were summarized with the species delimitation analysis tool provided with along with the STACEY package (speciesDA.jar) using collapse height = 0.001 and sim cutoff = 1.0 (no cluster similarity binning).

The results of the delimitation are shown both as the grouping of leaves into species groups, and pairwise group probability matrices, indicating the probability of any pair of leaves to belong to the same cluster (species). These matrices were computed with an R script, modified from the supplementary material in Jones et al. (2015). The influence of prior selection was inspected by comparisons with an additional analysis of matrix M0 sampling from the prior only. Additionally, delimitation by the Generalized Mixed Yule Coalescent Model (Pons et al., 2006; Fujisawa and Barraclough, 2013) was explored using the bayesian implementation in the package 'bGMYC' (Reid and Carstens, 2012) for the R environment (R Core Team, 2016). These analyses used a random sample of 100 trees from the species trees collected during the STACEY runs with default prior parameters.

Estimation of trees and species delimitations were performed under the multi-species coalescent paradigm as implemented in BEAST2 (Bouckaert et al., 2014). Each analysis was conducted using two independent runs of the Markov Chain Monte-Carlo (MCMC) for 200×10^6 generations, sampling every 5,000 states. Convergence of individual and combined runs was assessed with Tracer v 1.6 (Drummond and Rambaut, 2007). For each pair of runs, the tree and the state log files sampled during the MCMC were combined using Logcombiner v2.4.2 (Drummond and Rambaut, 2007) removing 10% of each run as burnin and resampling every 100 states.

With the aim to provide a temporal framework, we analyzed the matrix M0 with StarBEAST2 (Ogilvie and Drummond, 2016) with same priors and parameters as for the species delimitation analyses but using a mean clock rate for COI fixed at 0.0115 substitutions/my based on estimates of empirical rates observed in three previous studies: Brower (1994) for butterflies, and Bidegaray-Batista and Arnedo (2011) and Peres et al. (2015), for spiders. For the StarBeast2 analysis, individual samples were associated to "species" following the results from the previous species delimitation.

2.6. Isolation by distance

We evaluated the potential relationship of geographic and genetic distances using Mantel tests (Mantel, 1967). The test was conducted with the package "vegan" (Oksanen et al., 2016) using 999 replicates. Geographic distances (in kilometers) were calculated from the geographic coordinates with the aid of the package 'geosphere' (Hijmans, 2016) and genetic distances of COI were estimated using the Kimura two-parameters model (K80, Kimura, 1980) as implemented in the package "ape" (Paradis et al., 2004), for the ITS2 partition distances were estimated with the "indel" metric; more complex models, such as GTR are not implemented in R for genetic distances. The samples for these pairwise analyses were partitioned as follow: (1) *L. venusta* s. l., all localities, (2) *L. venusta* s.s. all localities, (3) *L. argyrobapta* s.l. all localities and (4) *L. venusta* s. l. USA localities.

In addition, the multivariate effect of the climate and geography was evaluated with a distance based redundancy analysis (RDA) as implemented in the rda function of "vegan". The analyses were performed only on the COI partition because gaps prevented calculation of the principal coordinates. Sequence based distances were computed using "K80" metric and principal coordinates estimated from the resulting genetic distance matrix. These principal coordinates were then used as the response variable in RDA analyses using the geographic coordinates and the climate values interpolated from the set of uncorrelated environmental variables as explanatory variables (see ENM section). Conditional RDA were performed to dissect the contribution to the variance attributed to pure geographic, climatic and combined variables. Significance was established with the permutation test included with vegan.

2.7. Ecological Niche modeling

Potential distribution models for *L. venusta* s. s. and *L. argyrobapta* were produced using the 19 bioclimatic layers from WordClim version 1.7 (Hijmans et al., 2005) and three additional layers form the beta version of Wordlclim 2.0 (solar radiance, wind speed, and water vapor pressure, http://worldclim.org/version2 on Aug.15. 2016). All layers used 2.5 arc resolution and were clipped and edited using GDAL (GDAL Development Team, 2016) and GRASS 6.4.4 (GRASS Development Team, 2016).

To account for the effect of undersampling of background cells (pseudoabsence), models were computed from two geographic regions, spanning from North to South America bound at 49°N and 30°S (continental scale), and a second restricted to the better-sampled Eastern USA region. Correlation coefficients of the environmental variables were calculated for all available climatic rasters (supplementary Tables 5 and 6). Highly correlated variables ($|r| \geqslant 0.9$), were removed with the exception of one representative layer, selected for its ease of interpretation. For the continental scale analyses, 13 layers were retained: Bio1 = Annual mean temperature, Bio2 = Mean diurnal range (Mean of monthly (max temp − min temp)), Bio5 = Max. temperature of warmest month, Bio7 = Temperature annual range (Bio5–Bio6), Bio8 = Mean temperature of wettest quarter, Bio9 = Mean temperature of driest quarter, Bio12 = Annual precipitation, Bio14 = Precipitation of driest month, Bio15 = Precipitation seasonality (coefficient of variation), Bio18 = Precipitation of warmest quarter, Bio19 = Precipitation of coldest quarter, Solar radiation (kJ m^{-2} day^{-1}) and wind speed (m s^{-1}). For the Eastern USA region 12 layers were retained, the same as in the large scale but excluding Bio19, and Solar radiation and including Bio16 = Precipitation of wettest quarter.

Ecological niche models (ENM) were produced using MAXENT v 3.3.3 k (Phillips et al., 2006, 2004; Phillips and Dudík, 2008). Each model was averaged from 20 replicates using 20% of the data points from each dataset for testing. Niche comparisons, as implemented in the R package "phyloclim" (Heibl and Calenge, 2013), were calculated using the niche equivalency test (Warren et al., 2008) reporting both Schoner's *D* and *I* statistics from 99 replicates. Both indices range from 0 to 1, and show similar properties; however these metrics could show differences in their significance (Warren et al., 2008). Additional ENMs of two emblematic tropical orb-weaving species (*Gastercantha cancriformis* (Linnaeus, 1758) and *Nephila clavipes* (Linnaeus, 1767), both in the family Araneidae) were computed from GBIF records (GBIF, http://doi.org/10.15468/dl.gn2fjk http://doi.org/10.15468/dlfayv3p. Downloaded 09/05/2016). The ENM for these species were produced to be used as comparison for the niche equivalence test. These two araneid species were selected for being abundant, common orb-weavers with a broad distribution in the neotropics, somewhat similar to that of *L. venusta* s. l., extending from USA to Brazil. Although *L. venusta* has an

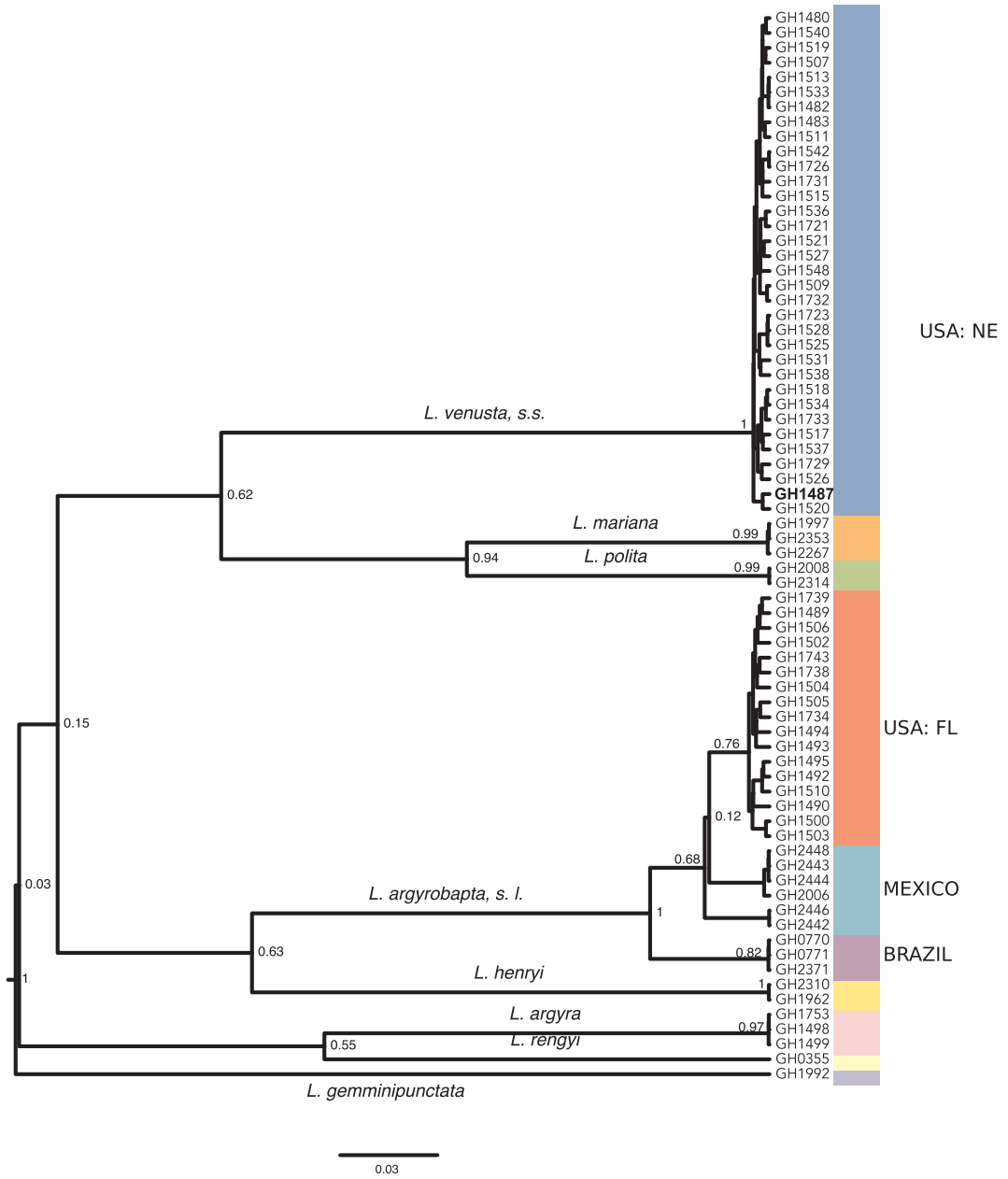

Fig. 2. Summary species trees obtained as part of the species delimitation. Colored bars mark the clusters identified as different species by STACEY. Sample GH1987, highlighted in bold was collected around the type locality of L. venusta. Posterior probability values are indicated for each node. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

entry in the GBIF portal, this dataset was not included because the records could not be unequivocally associated to the species recognized in the delimitation analyses, (see Results).

3. Results

3.1. Species delimitation analyses

All species delimitation analyses, across all three matrices, consistently divided *L. venusta* s. l. into more than one species (Fig. 2). Two highly supported and deeply divergent monophyletic groups are easily recognized: a northern clade containing most of samples from the NE-USA and Canada and a southern clade that includes specimens from Florida, Mexico and Brazil. Because the northern species clade includes a sample collected in the area of the probable type locality of *Epeira venusta* (GH1487, in Georgia), this northern species is hereafter referred as *L. venusta* sensu stricto (s.s.). The southern clade by virtue of its association with specimens from the type locality of *L. argyrobapta*, are referred collectively as *L. argyrobapta* sensu lato (s. l.). All of the species delimitation analyses consistently divided *L. argyrobapta* in three groups that correspond to the country of origin of the specimens, nevertheless this result must be interpreted with caution due to the low posterior probability of each of these nodes on the species trees ($PP < .95$) and the sparse sample of specimens from Central and South America. In all analyses the individuals of the outgroup species were placed either as conspecific (for each of the seven outgroup species analyzed), or as highly supported sister clusters, providing an external validation for the delimitation analyses.

The group membership probability heatmap resulting from STACEY delimitation analysis using matrix M0 is shown in Fig. 3. Individual bGMYC and STACEY heatmaps for matrices M0, M1 and M3 are depicted in supplemental Fig. 9. For the matrix M0 the number of species

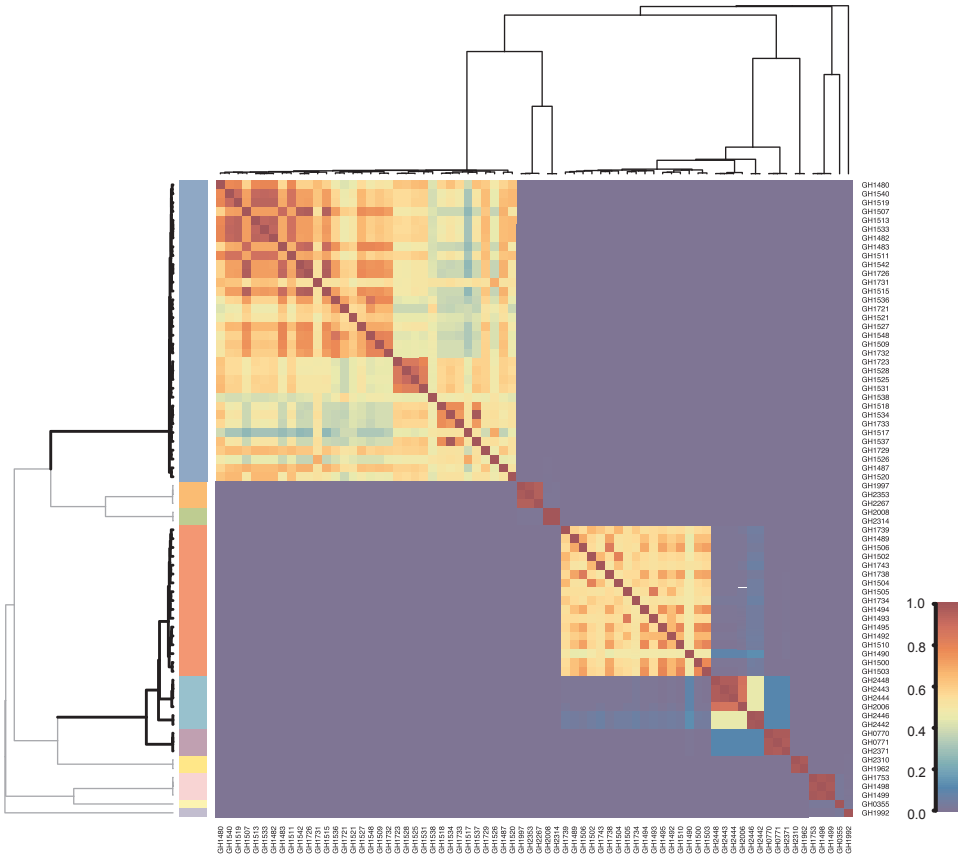

Fig. 3. Group membership probability matrix from STACEY delimitation on the M0 dataset. Each cell in the matrix show the posterior probability for any pair of specimens to belong to the same cluster. The tree on the left and top margin is the same shown in Fig. 2. Branches leading to specimens identified as *L. venusta* s. l. are shown with thicker lines.

in the 95% highest posterior density (HPD) ranged from 10 to 16 with a median of 13 clusters. The single highest posterior probability (HPP) clustering found by STACEY recognized 10 clusters (PP = .13%). This clustering is in agreement with the morphological delimitation except in dividing *Leucage venusta* s. l. into four clusters, separating the Florida, Mexico and Brazilian populations in clusters independent from the rest of the North American specimens (see membership probability heatmap in Fig. 3). A rerun of STACEY delimitation on matrix M0, where individuals were associated to minimal clusters based in this 10 species scheme recovered the same 10 clusters (no merging) grouping with a PP = 99.85%. The results from bGMYC analyses of matrix M0 (Log coalescent rate/Yule rate, Log(Coal/Yule) = 3.814, 95%, HPD = 3.623–4.016) are congruent with those obtained by STACEY. The mean number of clusters across the sampled trees was 13 species but using a probability threshold greater than 0.5 as a point estimate resulted in 10 species clusters, the same groupings that were found in the STACEY analyses (Fig. 3).

In the matrix where *L. argyra* is the only outgroup (M1), the single posterior probability clustering divided the southern populations into three clusters of *L. venusta* s. l. corresponding to the countries of origin: USA (Florida), Mexico and Brazil (*PP* = .32%). In bGMYC the point estimate of the species delimitation from the posterior (cut off 0.5), grouped them in three species: *L. argyra* (outgroup), *L. venusta* (NE-USA) and *L. venusta* (Florida + Mexico + Brazil). In bGMYC M1 the mean number of species in the posterior was 8 (Log (Coal./Yule) rate = 3.595, 95% HPD = 3.297–3.891). The summarized point estimate recognizes 6 species. The partitioning of the ingroup is identical to that of STACEY, except that it further divides the Mexican population of *L. argyrobapta* in two clusters. For the extended matrix (M2), the number of clusters ranged from 12 to 20 with a median of 16 clusters. The highest PP clustering resulted in 12 clusters (0.18%). This HPP clustering divides *L. venusta* s. s. into two groups, which are polyphyletic in the maximum credibility tree. The rest of the members of *L. venusta* were additionally divided in three clusters, in this case not grouped by geographic provenance. Each of the seven outgroup species were recognized as independent clusters except for *L. argyra*, where the specimen from Brazil (GH1967) was classified in an independent cluster, and the samples of *L. branicki* and *L. regnyi* were lumped in the same cluster. The bGMYC delimitation, with Log (coalescent/Yule) rate = 4.174 [4.009–4.1750] conflicted with STACEY's, the mean number of species in the dataset was also 16 species, but the point estimate with $P > .5$ recognized 13 species. Unlike STACEY's result, *L. venusta* s.s. was not split, *L. argyrobapta* was divided by countries, as in M0 and M1, but the cluster from Mexico was further divided into two species. The delimitation in the outgroups also separated *L. argyra* in two clusters but *L. branicki* and *L. regnyi* were recognized as independent clusters.

3.2. Isolation by distance

The results of the Mantel test over all data partitions are summarized in Table 1. The partition of *L. argyrobapta* was the only one showing strong and significant correlation between the geographic distances and genetic distances ($r = 0.89$). Significant values were observed for the partitions of *L. venusta* s. l. at both geographic scales, indicating that the genetic and geographic distances are not independent, however the correlations of these two matrices is relatively weak ($r < 0.5$). The genetic response was stronger for the COI datasets than for the ITS2. Bivariate plots of the geographic vs. genetic distances partitions tested in with the Mantel test are shown as supplementary Figs. 14–21.

The results for the RDA are summarized in Table 2. The biplot of the unconditioned RDA is shown in Fig. 4. Notice that the RD1 axis, which explains 93% of the observed variance, segregates the samples of *L. venusta* s. s. (blue dots) from *L. argyrobapta* (red dots). The RD2 axis, explaining only 2.6 % of the variance, only affect samples in the *L.*

Table 1
Summary statistics of the Mantel test by gene over different partitions of *L. venusta* specimens showing correlation (r) and probability values (p). Significant values are highlighted in bold type. When all samples are lumped together as one species, the test shows weak correlation between geographic and genetic distances. Grouping the samples into the delimited species, only *L. argyrobapta* shows strong and significant correlation in the mitochondrial locus.

	COI		ITS2	
	r	p	r	p
L. venusta s.l.	0.3799	**.001**	0.3139	**.001**
L. venusta s.l. (USA)	0.3269	**.001**	0.2721	**.001**
L. venusta s.s.	0.01559	.408	0.03866	.158
L. argyrobapta	**0.8959**	**.001**	0.1947	.069

Table 2
Summary statistics of the complete and partial redundancy analyses.

Type	p-value	Variance (inertia)	RD1 (%)	RD2 (%)
Complete	**.001**	0.0024922	93.101	2.6
Climate alone	**.001**	0.0011159	99.7	4.8
Geography alone	.163	6.386×10^{-6}	8.33	1.67

argyrobapta. The vectors of explanatory variables show the magnitude of correlation of each variable with the redundancy axes, sharper angles and larger arrows indicate greater correlation. Several purely climatic variables strongly correlate with the RD1 axis while pure geographic variables (lat, long) show relatively higher correlations with RD2. The partial redundancy analysis, which removes the effects of a subset of the explanatory variables, shows that the set of pure climatic variables explains 44% of the observed variance with bio2 (diurnal temperature range) and bio12 (mean annual precipitation) being the variables that contribute the most to RD1. Conversely the geographic coordinates alone, when the climatic variation is factored out, explain only a small fraction of the genetic variation observed (2%).

3.3. Phylogenetic structure and dated phylogeny

In all species trees resulting from the STACEY analyses, *Leucage venusta* s.s. and *L. argyrobapta* s. l. were reciprocally monophyletic, with $PP > .95$ (Fig. 2 and supplemental Figs. 10 and 11). The same pattern is obtained for the individual gene trees (supplemental Figs. 12 and 13). In the analyses of the M0 and M2 matrices, all the outgroup species were found monophyletic with $PP > .95$, except for *L. argyra* ($PP = .63$) and *L. branicki* ($PP = .63$). Although interspecific relationships are, for the most part, poorly supported, none of the analyses grouped *L. venusta* and *L. argyrobapta* as sister species.

Multi-species coalescence methods accommodate discordance between individual gene tree and the species tree and therefore benefit from the combined signal from several loci. The use of the multispecies coalescent paradigm however, allows for discordance of these two genes, avoiding the possibility of producing trees dominated by the signal of one locus over the other. In this study, reciprocal monophyly of *L. venusta* s. s. and *L. argyrobapta* in gene and species trees, increases our confidence in the distinction of these two species.

The dated phylogeny based on the M0 matrix and using the assignment of samples to the 10 populations (species) found in both delimitation analyses places the divergence of *L. venusta* s.s. and *L. argyrobapta* s.l. around 27 million years ago, in the Paleogene (Fig. 5). Although the confidence interval of the node age estimates is considerably broad, the results suggest that the clades representing these two species have diverged for at least 10 my (95% HPD 10.4–49.9). The clock rate for the ITS2 partition was estimated at 0.0123 ± 0.013, very similar to the fixed rate of COI. This similarity in the clock rate was also observed on the free clock rate analyses in STACEY with a mean clock rate of in M0 0.667 for COI and 0.566 for ITS2.

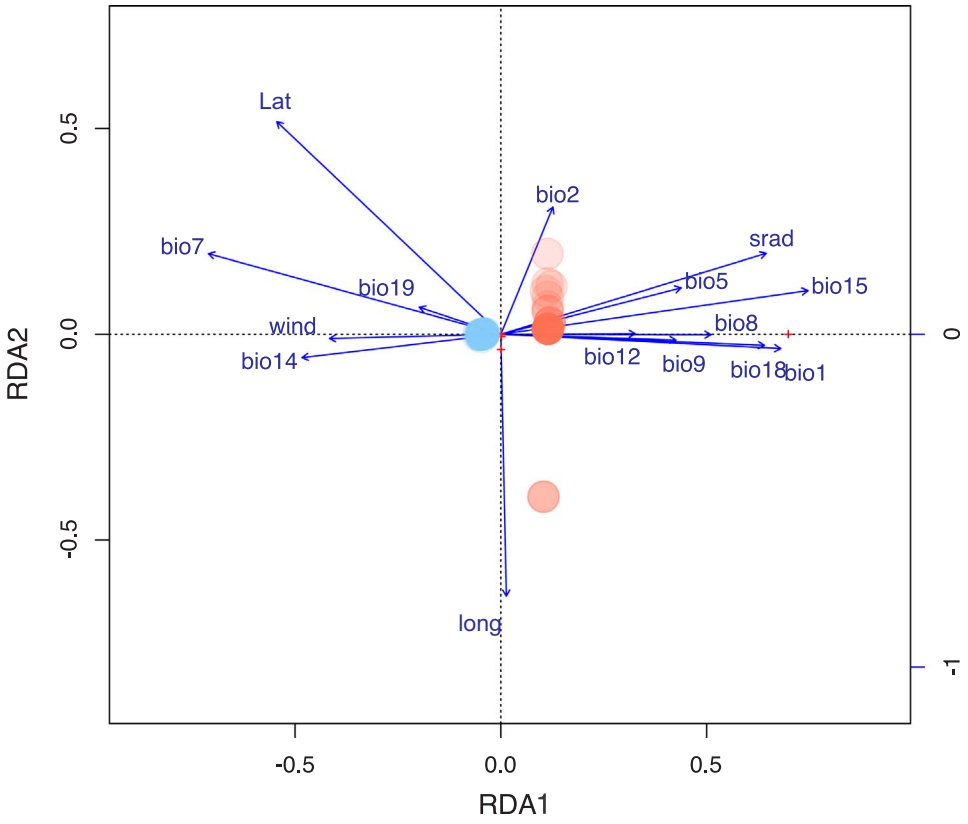

Fig. 4. Biplot of the unconstrained redundancy analysis (RDA). This graph summarizes the variation in the genetic differentiation principal coordinates as explained by a set of explanatory variables, climate and geography in this case. Blue points identify the *L. venusta* samples and *L. argyrobapta* points in red. Explanatory variable arrows as blue colored vectors. See text for details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

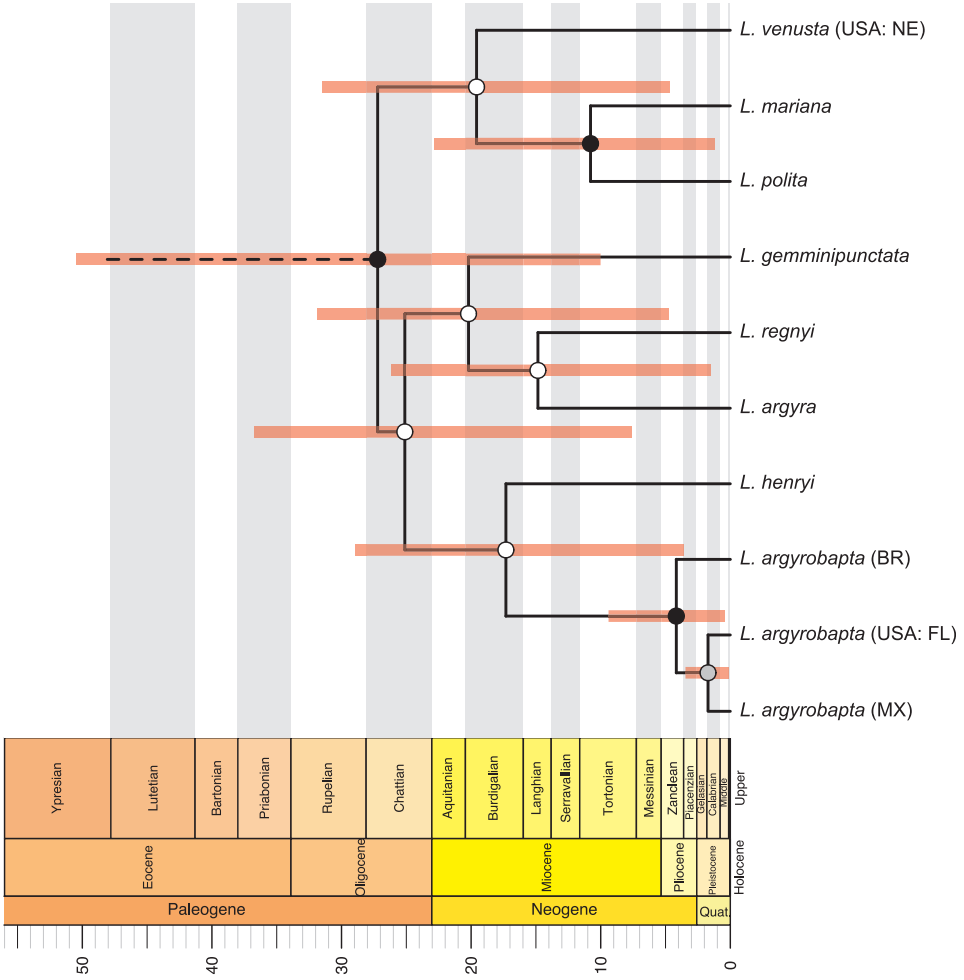

Fig. 5. Time calibrated species tree generated with starBeast2 analyses and using a fixed median substitution rate. Red bars show 95% HPD age interval for each node. Black circles indicate nodes with $PP > 95\%$, gray circles $75\% < PP < 95\%$ and white circles $PP < 75\%$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Ecological niche models and niche equivalence

The resulting ENM's of *L. venusta* and *L. argyrobapta* are shown in Fig. 6. Models for *Gasteracantha cancriformis* and *Nephila clavipes* are provided as supplemental Fig. 22. The habitat suitability of *L. venusta* s. s. (AUC = 0.980) is restricted in North America to the eastern half of USA and Canada and it clearly excludes the Florida peninsula. The distribution model of *L. argyrobapta* (AUC = 0.993) extends in the north from coastal plains of North Carolina, USA prominently in Florida continuing along the Atlantic coast to Mexico and Central America with disjunct patches of suitable habitat in the northwest parts of Mexico, West Indies, Venezuela, the Atlantic Forest of Brazil extending to the Chaco region in the south. For the most part the ENM's of these two species are mutually exclusive, except for a narrow area of contact (Fig. 6) where it is probable to find both species in sympatry.

The ENM of these two species corresponds with a parapatric pattern of distribution. Notably, neither model predicted the presence of this species in California, from which specimens *L. venusta* have been

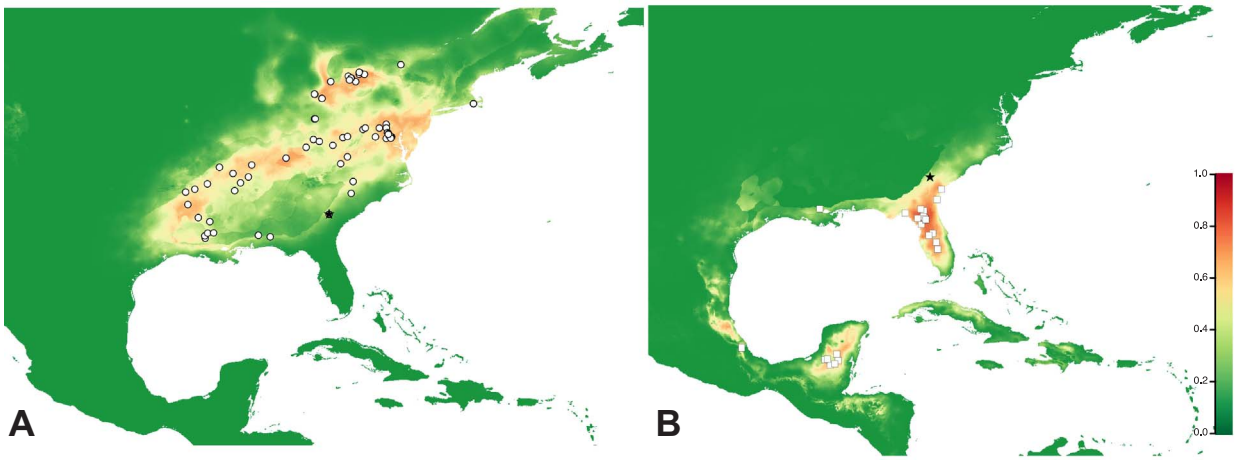

Fig. 6. Detail of the ecological niche models of *L. venusta* s.s (A). and *L. argyrobapta* (B) in North America. The locality closest to the probable type locality for of *L. venusta* is indicated with a black star.

Table 3
Results of the Ecological Niche Equivalence Tests.

	Metric	*L. argyrobapta*		*N. clavipes*		*G. cancriformis*	
		SE-USA	Americas	SE-USA	Americas	SE-USA	Americas
L. venusta	D, p	0.073, 7.4e−99	0.086, 6.63e−93	0.093, 8.9e−266	0.129, 9.9e−196	0.115, 1.8e−194	0.140, 1.5e−146
	I, p	0.2, 0	0.217, 0	0.192, 0	0.260, 0	0.276, 0	0.321, 0
L. argyrobapta	D, p	–	–	0.408, 2.8e−31	0.416, 1.68e−18	0.385, 1.26e−16	0.389, 4.38e−17
	I, p	–	–	0.701, 7.3e−44	0.704, 8.11e−24	0.689, 6.47e−20	0.693, 1.8e−18

reported before (Levi, 1980). The model of *L. argyrobapta* shows the most geographically close patch of suitable habitat to the cited localities in California but the identity of these is pending until specimens from this region are available.

Precipitation seasonality (Bio15) and temperature annual range (Bio7) were the environmental factors that contributed the most to the *Leucauge venusta* s. s. model in both the continental and Eastern USA regions. For the model of *L. argyrobapta*, the most important variables were Bio18 (Precipitation of Warmest Quarter) and Bio8 (Mean Temperature of Wettest Quarter).

The results from the niche equivalence pairwise comparisons are summarized in the Table 3. In all cases the niche equivalence hypothesis (null) was rejected ($p \ll 0.05$). The values of niche similarity indexes (D, I) with the tropical species (*Nephila clavipes* and *Gasteracantha cancriformis*) were higher when compared with *L. argyrobapta*, than those for *L. venusta* s. s. The geographic scales had no influence on the results and the actual values of D and I were very similar.

3.5. Taxonomic changes

All species sequence delimitation analyses that we have performed clearly separate Northern from Southern specimens as different species of *Leucauge*. This division is consistently observed in the individual gene trees (see supplementary Figs. 12 and 13), and species trees across different matrices. The clades sustaining these groupings show high posterior probabilities across all analyses ($PP > 0.95$, see Fig. 2, and supplementary Figs. 10 and 11); moreover, based on the Mantel test and RDA results the genetic differences cannot be explained by the geographic structure alone. In addition to the genetic partitioning, the ecological niche model analyses show that members of these two clades occupy different environmental envelopes. All these result favor a separation of the specimens currently classified under the epithet of *L. venusta* in at least two distinct species.

In spite of uncertainty of the exact type locality of *Epeira venusta*, the specimen collected closest to the probable source of the specimen (which bears the voucher label "GH1487") is clearly nested in the Northern clade. We therefore restrict the name *L. venusta* to the northern species and thus *L. argyrobapta* White, is removed from the synonymy proposed by Dimitrov and Hormiga (2010). All our species delimitation analyses suggest that the *L. argyrobapta* populations from USA, Mexico and Brazil represent different species. However, given the limited geographic sampling, which was designed to establish the species limit of the North American *Leucauge*, low branch support and the lack of diagnostic morphological characters, we refrain from interpreting this partitioning as conclusive. As with *L. venusta*, testing if these populations represent independent lineages or a geographic continuum, demands a more thorough sampling across the whole distribution range.

It must be noted that the ENMs predict the potential occurrence of both *L. venusta* and *L. argyrobapta* around the putative type locality of *Epeira venusta* and therefore it is possible that the specimen illustrated by Abbot was conspecific with *L. argyrobapta*. In the absence of an actual specimen, such a hypothesis cannot be tested. The decision of interpreting Abbot's specimens as a member of the northern clade is based on the phylogenetic placement of the specimen GH1487 and on promoting nomenclatural stability, as recommended by the ICZN (ICZN 1999).

L. venusta and *L. argyrobapta* are very similar morphologically and previous authors have reported variation on size and coloration that would require more rigorous study Levi (1980), Henaut et al. (2006), and Dimitrov and Hormiga (2010). A thorough investigation of the morphological variation of these two species is beyond the scope of our study. Such an effort would require the detailed study and comparison of many more specimens than the ones included in the present study, including the large amounts of specimens in museums preserved in 70–80% ethanol.

In most cases, geographic distribution can be used as a proxy for distinguishing these species as our results demonstrate that the geographic distributions of these species show little overlap. The morphological features described below as diagnostic should be interpreted as preliminary guidelines and further observations of specimens from an broad geographic range are required to validate its usefulness. In our samples the subtle differences observed in the male pedipalps seem constant enough to allow morphological diagnosis of these two species. Adult males are however rarer in our samples and in the case of females, for now, the differences are more subtle and thus female specimens remain virtually indistinguishable on morphological basis, as the observed variation is confounded with intraspecific variation. Note that although Levi (1980) examined specimens of both species for his description of *L. venusta*, he illustrated and described specimens from Florida (his Figs. 44–59), which correspond to *L. argyrobapta* (e.g., compare the apical conductor process in Fig. 7 and in Levi (1980, Fig. 58)). In his "Variation" section Levi reported differences in the ventral silver patches between the southern specimens and those of the northern states. In addition, Levi (1980) also reported variation in the degree of epigynal sclerotization and the relative length of the male palpal tibia. It seems that Archer (1951) may have correctly interpreted these two species when he described *L. mabelae* Archer 1951 (synonymized by Levi (1980) under *L. venusta*). The specimens of the type series of *L. mabelae* were collected in Florida, within the distribution area of *L. argyrobabta*, while the specimens of *L. venusta* that Archer illustrated and used for the diagnosis came from Tuscaloosa (Alabama), within the range of *L. venusta* s.s. If confirmed by examination of the Archer type, this is of potential nomenclatural relevance if future work determines that the specimens from Rio de Janeiro and Florida, interpreted by us as one species (*L. argyrobapta*), represent two species (the name *L. mabelae* would be available for the Florida species).

Untangling the species affinity of the taxonomic references is particularly difficult for cryptic and very similar species. The species assignments provided in the section below are mostly based on the geographic origin of the specimen(s) and the images provided therein. The use of this geographic pattern assumes that the current distribution of these two species is applicable to historical records (1841–present). In some cases, particularly in the southern states of the US where the two species are likely to coexist, the designations to one or the other species is dubious and further evidence will be required for sorting these cases.

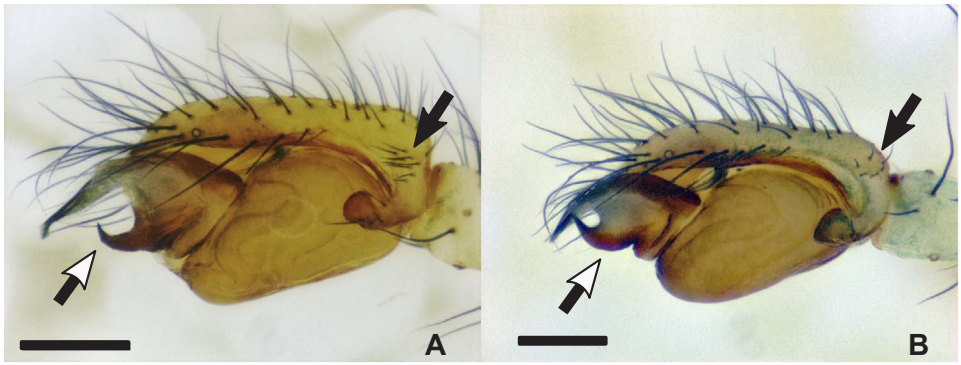

Fig. 7. Detail of the male palps in ectal view showing the differences observed in (A) *L. venusta* (Virginia, GH1480) and (B) *L. argyrobapta* (Florida, GH1777). Notice the difference in the curvature of the conductor apohyses (white tip arrows) and the density of setae at the base of the paracymbium (black tip arrow). Although these two specimens vary in the density of paracymbial setae, this feature is highly variable within species. Scale bar = 0.2 mm.

3.6. Taxonomy

Family Tetragnathidae Menge, 1866

***Leucauge* White, 1841**

Type species: *Linyphia (Leucauge) argyrobapta* White, 1841.

***Leucauge argyrobapta* (White, 1841) stat. rev.** contra Dimitrov & Hormiga (2010).

Neotype, male from Brazil, Rio de Janeiro, Botanical garden of the Museu Nacional do Rio de Janeiro, lat. −22.90842, long. −43.223547 21 VIII 2007, leg. Abel Pérez-Gonzlez, Adriano B. Kury, Thiago S. Moreira, Dimitar Dimitrov and Gustavo Hormiga (deposited in MNRJ). (examined).

Linyphia (L.) argyrobapta White, 1841.

Argyroepeira venusta (Walckenaer): McCook, 1893, p. 242, P. 20, f. 1–6, specimens from the Mississipi Valley and Texas and Central America likely are of dubious affinity.

Leucauge hortorum (Hentz): (Banks, 1909, p. 163), no descriptions or drawings provided but the distribution in Cuba excludes the known range of *L. venusta*.

Leucauge argyrobapta (White): Petrunkevitch (1911, p. 355), recorded from Rio de Janeiro, probably listing only Walckenaer's specimens.

Leucauge mabelae Archer, 1951.

Lecuauge venusta (Walkenaer): Pickard-Cambridge (1903), in part, records from Mexico, Guatemala, Costa Rica, Panama Colombia and Antilles; Petrunkevitch (1930, p. 266, f. 121–122), in part, only records from Bermuda Bahamas, Mexico, West Indies, Guatemala, Costa Rica, Panama; Colombia; Levi (1980, p25, f. 44–59), record from Florida, Mexico, Panama, specimens illustrated from Florida; Coddington (1990, p. 17. f. 55), figure based on Levi (1980); Hormiga et al. (1995, p. 324, f. 6H-I, 13H), No locality data provided but shape of conductor similar to *L. argyrobapta*; Kuntner et al. (2008, p. 177, f. 19A-C), not locality data provided but shape of conductor more similar to *L. argyrobapta*; Dimitrov and Hormiga (2010, 24, f. 1A-G, 2A-F, 3A-B, 4A-D, 5A-G, 6A-G, 7A-D, 8A-G, 9A-F, 10A-g, 11A-G), neotype specimen illustrated from Rio de Janeiro, Brazil; Barrantes et al. (2013, p. 59, f 9A-D), female specimen illustrated from Baton Rouge, Louisiana, most likely *L. argyrobapyta*.

Diagnosis: *Leucauge argyrobapta* males differ from those of *L. venusta* in the shape of the conductor (Fig. 7.) In prolateral view the conductor ectal apophysis of *L. venusta* is straight for most of its length with a sharp turn apically, the ectal apophysis runs parallel to the mesal one at its base, before curving sharply towards the ectal apophysis. In *L. argyrobapta* both apophyses are smooth and continuously curved with no sharp angles or turns. These diagnostic features are shown in previous illustrations of *L. venusta* in Archer (1951, Figs. 3 and 4) or Álvarez-Padilla and Hormiga (2011, Fig. 45A-D) and *L. argyrobapta* Archer (1951, Figs. 1 and 2) and Dimitrov and Hormiga (2010, Fig. 2A-F). Females of both species are very similar in both external and internal genitalic anatomy (see Dimitrov and Hormiga, 2010; Álvarez-Padilla and Hormiga, 2011, figs. 3A and 45D, respectively) and no consistent differences have been discerned in their epigyna. Some specimens of *L. argyrobapta* have epigynal plate wider than longer, where in most specimens of *L. venusta* the plate is as long or longer than wide. Variation in this character may be difficult to assess because the shape and degree of sclerotization of the epigynal plate is highly variable, even among specimens of the same locality (as mentioned by Levi, 1980). In fresh and 95% ethanol specimens of *L. argyrobapta*, the paturon base and the endites are black or very dark, whereas in *L. venusta* the distal part of the chelicerae is dark colored and the rest is uniformly buff colored.

***Leucauge venusta* (Walckenaer, 1841)**

Type: Abbot illustration Plate 13 No. 113 In: "The Insects and spiders of Georgia" (ca. 1798, unpublished). Photocopy of the plate from H. Levi collections at the MCZ (examined).

Epeira venusta Walckenaer, 1841.

Argyroepeira hortorum (Hentz): Emerton (1884, p. 332, P. 37, f. 29–32) Illustration and records from New Haven Connecticut and Milton, Massachusetts; Emerton (1902, p. 192, f. 446–447).

Argyroepeira venusta (Walckenaer): McCook (1893, p. 242, P. 20, f. 1–6), in part. specimens from Philadelphia and the Atlantic Coast.

Not *Leucauge horturum* (Hentz): (Franganillo, 1936, p.85, f. 40), although the figure does not allow proper identifications, the palp illustrated is not similar to that of *L. venusta*; the outline resembles more closely to member of the genus *Chrysometa* Simon.

Leucauge venusta (Walckenaer): Pickard-Cambridge (1903), in part. record from Baltimore; Petrunkevitch (1911) in part., only records from NE; Petrunkevitch (1930, p. 266, f. 121–122), in part, specimens illustrated from New Jersey and NE USA records; Kaston (1948, p. 265, f. 836–837, 843–846), Archer (1951, p. 6, f. 3–4), specimens from Tuscaloosa, Alabama; Wiehle (1967, p. 193, f. 40, 42–43), although the origin of the specimens illustrated is not stated Fig. 42 shows straight conductor of *L. venusta*; Levi (1980, p. 25), in part., specimen illustrated from Florida, most likely *L. argyrobapta*, most records in North America, including USA (except FL) and Canada are likely *L. venusta*; Dondale et al. (2003, p. 51, f. 44–51), records and illustrations from Levi (1980) and ÁlvarezPadilla (2007, p. 291, f. 3F,4F), specimen from Fairfax, Virginia; Álvarez-Padilla et al. (2009, p. 540, f. 5), Paquin et al. (2008, p. 31, f.25-29), Álvarez-Padilla and Benjamin (2011, p. 58, f. 4C-D, 5D), and Álvarez-Padilla and Hormiga (2011, p. 764, f. 41A-F, 42A-H, 43A-F, 44A-F, 45A-D). Not *Leucauge venusta* (Walckenaer):Saito (1933, p. 48, f. 24), specimen from Taiwan, cf. *L. tesselata* (Thorell, 1887); (Dierkens, 2010, p. 38. f. 16–18), photographs of specimen from the island of Martinique in the lesser Antilles does not correspond with *L. venusta*, cf. *L. taczanowskii* (Marx, 1893). Not *Leucauge venusta* Saito, 1933 (missidentification); cf. *L. tesselata* (Thorell, 1887). Diagnosis: See diagnosis of *L. argyrobapta*.

Nomina dubia:

Epeira hortorum Hentz 1847.

Synonymized with *Epeira venusta* Walckenaer by McCook (1893, p. 242).

In the original description Hentz (1847, p. 477) listed "All the

United States" as the habitat of for this species. Although this author collected and worked in the southern states (particularly in Alabama) he had access to specimens from other parts of the US, thus obscuring provenance of the specimen(s) upon which the description is based. The collection of Hentz along with the type specimen is reported as "destroyed" (Hentz et al., 1875; Levi, 1980). This name is a junior synonym of either *L. venusta* or *L. argyrobapta* but in the lack of a specimen or precise locality its identity is dubious.

Tetragnatha 5-lineata Keyserling, 1864.

Synonimized with *E. horturum* Hentz by Keyserling (1893). The original description and drawing does not allow proper identification. Keyserling mentions "several" specimens from St. Fe de Bogota (N. Granada) in its collection. Several specimens from the Keyserling collection were acquired by the British Museum in 1890 (Pocock, 1906) including several types of species pulished between 1880–1894 but no reference is made of previous specimens. *Tetragnatha 5-lineata* is listed as a synonym of *E. hortorum* Hentz in Keyserling (1893), published postmortem and edited by George Marx, thus it is not clear if the species assignment was made by Keyserling or Marx. The description included in (1893) is the same (verbatim) to the original from 1864 but includes additional drawings. The diversity of epygina depicted in these drawings (Keyserling, 1893, Plate XVII, F. 246b, c, d, f) suggest several species lumped under the same name. Levi (1980), does not includes *T. 5-lineata* in his list of synonyms, and records of this species specimens are missing from his survey of types of American *Leucauge* (Levi, 2008).

4. Discussion

4.1. Species delimitation

In recent years there has been a proliferation of computational methods and criteria to identify and circumscribe independently evolving lineages (e. g. Pons et al., 2006; Reid and Carstens, 2012; Puillandre et al., 2012; Zhang et al., 2013; Grummer et al., 2014; Jones et al., 2015; Jones, 2016; Yang and Rannala, 2014; Leaché et al., 2014). In this study we explored and compared two Bayesian delimitation approaches. The default and more immediate summary of the STACEY delimitation method (Jones, 2016) is the posterior probability of a specific grouping, i.e., the most frequent grouping found in the sampled trees. Results across STACEY delimitation analyses have in common a strikingly low posterior probability for the single highest posterior probability groupings in each matrix, all below 1%. The explanation of these results lies in the complexity of the classification problem when the number of elements, in this case individuals, is relatively large. The number of all possible non-empty subsets of n elements, known as the Bell number (B_n) increases dramatically with the number of elements to classify. For 72 terminals (M0), the number of possible groupings $B_{72} = 9.314528 \times 10^{75}$ and for the samples in M2 $B_{140} = 7.516119 \times 10^{176}$. A run of STACEY on matrix M0, where the individual samples were grouped into populations (minimal clusters in STACEY which can me merged but not split) based on the highest posterior probability clustering from the association free run, resulted in a dramatically much higher posterior probability value for the grouping 10 clusters ($PP = 99.85\%$). Potentially longer runs of the MCMC would increase the posterior probability of the "correct" clustering, because convergence is evaluated on number of clusters identified, not the specific clusterings themselves. Both STACEY and bGMYC summarize their results in the form of probability matrices in which each entry represents the probability of a pair of individuals (i,j) to belong to the same species. However, the approach to point probability estimate is different for both programs. In STACEY, the default estimate is the posterior probability of a clustering scheme, whereas in bGMYC the point estimate is the probability of each clusters above a threshold. Using bGYMC point estimate estrategy on STACEY's probability matrices, produces clusters identical to those from the bGYMC, except on M0, where samples of *L. argyrobapta* from Mexico are divided in two clusters.

The bGMYC allows point calculations that sum the probabilities of individual clusters across the sampled groupings, reporting maximum inclusive groups where the probability of association of the member is larger that an arbitrary threshold value (in our case 0.5), lower values would tend to lump clusters and higher values would tend to split, and therefore this strategy transfers the problem of species delimitation to selecting this critical threshold. STACEY's approach of finding the highest posterior probability grouping is free from this user-defined threshold. However, in the cases where the number of elements to classify is large, the computing effort required to produce effective sample sizes of any particular clustering may result prohibitive. The complexity of the problem can be greatly reduced by using reasonable assumptions about the minimal number of species (predefined clusters). For complex scenarios such initial clusters can be defined using explicit criteria, e.g. single point GMYC delimitation, and these clusters analyzed under the Bayesian framework.

4.2. Isolation by distance

Our Mantel test results show signs of dependence between genetic and geographic distances. For most of the data partitions the correlation between geographic distance and genetic differentiation was weak ($r < 0.5$). For the combined data partition that includes the mixed samples of *L. venusta* and *L. argyrobapta*, the Mantel test suggests that geography and genetic differentiation are not independent ($p < 0.05$), although the correlation between these matrices is mild (0.4). In the context of species delimitation, geographic-genetic independence is generally interpreted as supporting evidence for the species differentiation; however the opposite does not constitute evidence for rejecting cryptic species scenarios. Meirmans (2012) shows that Mantel test is unable to differentiate continuous from abrupt changes in the genetic structure, caused for example by a geographical barrier and some models of speciation are expected to produce linear response of genetic difference to the geographic scenario (Ribera et al., 2011). Additionally, measurements of geographic distances are not universally adequate, where the effective spatial separation differs from "as the crow flies" distances, for example in the presence of barriers to dispersal, e. g. Isolation by Resistance (McRae, 2006).

To address the limitations of the Mantel test mentioned above we explored the use of the redundancy analysis (RDA) following the recommendations in Legendre et al. (2015). First, we transformed the genetic variation into its principal coordinates and these were used as the response variable matrix in RDA and partitioned RDA. It is difficult to asses the general properties of this approach but the use of RDA provided a more complete insight into the relationships of the genetic distances and a variety of variables than show variable degrees of autocorrelation. The main purpose of testing for IBD in species delimitation analysis is to rule out the effects of geography in producing genetic structure, for example when samples are drawn from extremes of a continuous spectrum of the variation. The results of the RDA and the partial RDA in particular show that the geographic component has little explanatory power for the genetic differences in the samples. Simultaneously, the analyses reveal a strong relation of climate and the genetic differences, specially of variables associated with humidity and temperature. This finding is in agreement with the results from the ENM and support the hypothesis of these two species having different habitat preferences.

The inspection of the bivariate plots proved illustrative in the interpretation of the results. Plots containing mixed samples of *L. venusta* and *L. argyrobapta* show two discrete clouds of points, one representing intraspecific distances which tend to vary little in spite of large geographic gaps and a cloud of interspecific comparisons which show high genetic differentiation even with geographic proximity.

The use of fixed mutation rates for tree calibration is controversial and therefore the age derived from the analyses shown in Fig. 5 should be interpreted with caution, especially as it involves the root node

(Drummond et al., 2006; Bidegaray-Batista and Arnedo, 2011; Lukoschek et al., 2012; Duchêne et al., 2011; Duchêne et al., 2014). Unfortunately, there are no known fossils of *Leucauge* and empirical studies have demonstrated that confidence in age estimates increases when many calibration points are used, especially when combined with tip dating techniques where the placement of the calibration points is explicitly tested (Lukoschek et al., 2012; Sauquet et al., 2012). In the absence of reliable calibration points, the use of rates of molecular substitution is the only alternative to provide a temporal framework for certain evolutionary events. Alternatively, some authors have resorted to a two-step approach in which the rates on the branches of interest are estimated from previous analyses using these as secondary calibration points, with an extended taxon sample to include clades with reliable calibration points (Hipsley and Müller, 2014; Sauquet et al., 2012). This second approach is prohibited by the required scale to incorporate such calibrations points (c. f., Dimitrov et al., 2016).

4.3. Phylogenetic structure

One of the most surprising outcomes of this study is the hypothesis that *L. venusta* and *L. argyrobapta* are not sister species. Moreover, the estimated age of divergence implies that the morphologies in this two species, have remained seemingly invariable for at least 10 million years. This result is more surprising when one considers that secondary sexual characters have high rates of evolution in spiders (Hormiga, 2002; Hormiga et al., 2003; Eberhard, 2004). This paradigm in the evolution of spider genitalia is the foundation for recognizing and describing most of the spider species using characters from external and internal genitalia (e.g Levi, 1965; Eberhard and Huber, 2006; Peretti and Eberhard, 2010; Barrantes et al., 2013). The morphology of the genitalia of both sexes of *L. venusta* s. l. (including specimens from localities in USA and Brazil) has been studied and illustrated in detail in recent taxonomic literature, including dissections and scanning electron microscopy (Dimitrov and Hormiga, 2010; Álvarez-Padilla and Hormiga, 2011). The subtle variations observed (if any) have been attributed to geographic or intraspecific variation Levi (1980).

In our study, most of the nodes relevant to interspecific relationships have low support. The most likely reason for this low support lies in the level of phylogenetic informativeness sensu Townsend (2007), of the genetic markers used in this study. Both ITS2 and the COI loci are known to have higher rates of mutation compared to the alternative genetic markers used in spider evolutionary biology. This property allows them to show differentiation between individuals and populations but the signal to inform deeper relationships is obscured due to saturation. A broader sampling of species and loci is required to test interspecific relationships (in prep.) and thus we limit our conclusion from these analyses to the implications on species delimitation.

4.4. Ecological models and niche equivalence

The results from the niche equivalence test suggest that *L. venusta* and *L. argyrobapta* occupy different ecological niches. This result conforms to previously reported parapatric patterns for the distribution of cryptic species Vodă et al. (2015), which agrees with the hypothesis that sister species tend to exist in allopatry. Such a biogeographic pattern could be explained as means to avoid competition although cryptic species have been reported to occur in sympatry (Kurushima et al., 2016). Although not equivalent, the habitat preferences of *L. argyrobapta* are more similar to other tropical orb-weavers, such as the araneids *Gasteracantha cancriformis* and *Nephila clavipes*.

5. Conclusions

Based on the species delimitation analyses, *Leucauge venusta* s.l. includes at least two species in North America. The name *Leucauge venusta* needs to be restricted to the North Eastern populations of orchard spiders while *Leucauge argyrobapta*, is removed from junior synonymy with *L. venusta* and used to collectively denote the populations in the southern clade. Based on the results of the Mantel test and RDA, the observed genetic differences cannot be explained by geographic distance alone, showing a poor correlation in these dimensions. Methods to study the complex effects of the environment and geography on the genetic structure require further development followed by empirical tests. We followed the recommendations in the literature for the use of ordination methods to complement the results from the Mantel test. However, most of these methods, traditionally used in community ecology studies, lack proper implementation for its use with sequence based data and have not, to our knowledge, been explored in the context of species delimitation in simulated nor empirical data.

Ecological niche models suggest that these two species have different environmental preferences. Although not statistically equivalent, the niche model of *L. argyrobapta* showed more similarity with other tropical orb-weavers, supporting the hypothesis that *L. venusta* and *L. argyrobapta* occupy different climatic niches, restricting the occurrence of *L. venusta* to temperate climates. The species delimitation methods favored the partition of *L. argyrobapta* into three species. However, it is possible that this result is an artifact resulting from insufficient geographic sampling of *L. argyrobapta* in Central and South America. Future work should expand geographic sampling (Central America, South America, US West populations) and assess intra-inter morphological variation.

Competing interest

The authors have no competing interest to declare.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the

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