Phylogeny and Biogeography of the Amazonian Pachyptera (Bignonieae, Bignoniaceae)

Jessica Nayara Carvalho Francisco and Lúcia G. Lohmann

Universidade de São Paulo, Instituto de Biociências, Departamento de Botânica, Rua do Matão, 277, 05508-090, São Paulo, SP, Brazil

Authors for correspondence (jn_c_francisco@yahoo.com.br; llohmann@usp.br)

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Abstract—The Amazon houses a large proportion of the overall biodiversity currently found on Earth. Despite that, our knowledge of Amazonian biodiversity is still limited. In this study, we reconstruct the phylogeny of Pachyptera (Bignonieae), a genus of neotropical lianas that is centered in the Amazon. We then use this phylogenetic framework to re-evaluate species limits and study the biogeographic history of the genus. We sampled three molecular markers (i.e. ndhF, rp32-trnL, and PstC) and 51 individuals representing the breadth of morphological variation and geographic distribution of all species recognized in the genus. We used this information to reconstruct phylogenetic relationships among individuals of Pachyptera using Bayesian and maximum likelihood approaches. The resulting molecular phylogeny was used as a basis to test species limits within the P. kerere species complex using a cpDNA coalescent approach (GMYC). GMYC identified five potential species within the P. kerere species complex that were subsequently evaluated in the light of morphology. Morphological data supported the recognition of four of the five potential species suggested by GMYC, all of which were also supported by a multispecies coalescent model in a Bayesian framework. The phylogeny of Pachyptera was time-calibrated and used to reconstruct the biogeographical history of the genus. We identified historically important migration pathways using our comprehensive cpDNA dataset and a Bayesian stochastic search variable selection (BSSVS) framework. Our results indicate that the genus originated in lowland Amazonia during the Middle Eocene, and subsequently occupied Central America and the Andes. Most of the diversification of Pachyptera occurred in the Miocene, a period of intense perturbations in South America.

Keywords—Ancestral area reconstructions, coalescent approaches, haplotype networks, neotropics, species delimitation.

The Amazon houses a large proportion of the overall biodiversity available on Earth (Da Silva et al. 2005). Despite that, Amazonian biodiversity is relatively little studied, leaving a major gap in our understanding of global biodiversity patterns (Wesselingh and Salo 2006; Hopkins 2007; Cardoso et al. 2017). Amazonian biodiversity has a complex history and no single factor can explain the patterns observed (Hoorn et al. 2010; Wesselingh et al. 2010; Rull 2011). Detailed species-level studies, based on a comprehensive sampling of taxa, are critical for the understanding of the main processes shaping the Amazonian biota.

Pachyptera DC. (Bignonieae, Bignoniaceae) is a clade of lianas centered in the wet Amazonian forest (Francisco and Lohmann 2018), representing an interesting model for diversification studies in the Amazon. The genus belongs to tribe Bignonieae, which includes 21 genera and ca. 393 species (Lohmann and Taylor 2014), representing the largest clade within the Bignonieae (Gentry 1989; Lohmann 2006; Olmstead et al. 2009). Pachyptera has a complicated taxonomic history and underwent various taxonomic changes during the last 150 yr (see review in Francisco and Lohmann 2018). For example, the Pachyptera kerere (Aubl.) Sandwith species complex traditionally included three varieties, i.e. P. kerere var. kerere, P. kerere var. incarnata (Aubl.) A.H.Gentry (Gentry 1973), and P. kerere var. erythraea Dugand (Dugand 1955), distinguished by subtle differences in reproductive traits (Sprague and Sandwith 1932).

The first molecular phylogenetic study to sample Pachyptera (Lohmann 2006) focused on deciphering generic limits within the tribe Bignonieae as a whole and sampling within the genus was limited. Based on these phylogenetic findings and additional morphological studies, Lohmann and Taylor (2014) resurrected Pachyptera from Mansoa and recognized four species within the genus: P. aromatica (Barb. Rodr.) L.G.Lohmann, P. erythraea (Dugand) A.H.Gentry, P. kerere (Aubl.) Sandwith, and P. ventricosa (A.H.Gentry) L.G.Lohmann. However, P. erythraea and P. ventricosa were not sampled in the study of Lohmann (2006), and their phylogenetic placement remained uncertain.

A second phylogenetic study of Pachyptera (Francisco and Lohmann 2017) sampled all four species recognized by Lohmann and Taylor (2014). This phylogeny indicated that P. ventricosa is more closely related to species of Mansoa than to other species of Pachyptera, leading to the resurrection of Mansoa ventricosa A.H.Gentry and the recognition of three species of Pachyptera, i.e. P. aromatica, P. kerere, and P. erythraea (Francisco and Lohmann 2017). Despite the advances in our understanding of phylogenetic relationships within the genus, all studies to date only sampled a few individuals per species and did not encompass the breadth of morphological variation included in the group. Species of Pachyptera show overlapping distribution patterns and are quite variable morphologically, complicating species delimitation. A comprehensive phylogeny of the genus is thus needed so species limits can be revised, and the biogeographic history of the genus can be understood.

In this study, we: 1) reconstruct phylogenetic relationships within Pachyptera, 2) clarify species limits within the genus, and 3) assess patterns of temporal and spatial variation in the group. For that, we infer phylogenetic relationships among species of the genus using chloroplast (cpDNA) and nuclear (nDNA) markers and a broad sampling of individuals representing the breadth of morphological variation and geographical distribution of taxa. We then use this framework as a basis to coalescent approaches to re-evaluate species limits and conduct biogeographic analyses to understand the diversification history of this Amazonian centered group.

Materials and Methods

Taxon Sampling—We sampled 38 accessions of all three species recognized in Pachyptera by Francisco and Lohmann (2017), i.e. P. aromatica, P. kerere, and P. erythraea. We also sampled ten individuals of a previously recognized variety of P. kerere, i.e. Pachyptera kerere var. incarnata (Aubl.) A.H.Gentry [= Pachyptera incarnata (Aubl.) Francisco & L.G.Lohmann; Francisco and Lohmann 2018], and three individuals of a newly described species, i.e. Pachyptera linearis Francisco & L.G.Lohmann (Francisco and Lohmann 2018).

This led to a final sampling scheme that included 51 accessions representing five taxa. We also included sequences of four outgroups, selected
based on Lohmann (2006): Tanacetum biilatum (Sprague) L.G.Lohmann, Fridericia speciosa Mart., Lundia spruceana Bureau, and Siziphylum riparium (Kunth) Sandwith. All sequences were newly generated for this study, except for outgroup sequences, sequences from three individuals of Pachyptera that were retrieved from Lohmann (2006), and sequences of three other individuals of Pachyptera retrieved from Francisco and Lohmann (2017). All vouchers and GenBank accessions are shown in Appendix 1, while details about each dataset are in Table S1 available at the Dried Digital Repository (Francisco and Lohmann 2020).

Samples were selected to represent the breadth of morphological variation and geographic distribution of each taxon. For that, we divided Amazonia into four main biogeographic areas, defined based on the distribution patterns of the various taxa, paleogeological data, and geographical barriers (i.e. major rivers and geological history). The following four biogeographical areas were considered: 1) NE = North Eastern Amazonia, including the Guiana Shield region; 2) NW = North Western Amazonia; 3) SE = South Eastern Amazonia; and 4) SW = South Western Amazonia (Fig. 1).

The biogeographical areas were defined based on a digitized version (Löwenberg-Neto 2014) of the regionalization scheme proposed by Mora (2014), with slight modifications. For example, the division between South Western and South Eastern Amazonia following the Madeira river was also incorporated because this division can prevent gene flow (Simões et al. 2008). The North Western region is broadly defined here as to include the Venezuela province and Sabana (Morrone 2014). In addition, Central America and the Andes were treated as a fifth area (CA & A).

We tried to sample at least two individuals of each species per area. Our final sampling included five individuals of P. aromatic (occurring in NW, NE, and SE), two individuals of P. erythraea (restricted to NE), 31 individuals of the broadly distributed P. kerere (occurring in the five biogeographical areas), three individuals of P. linearis (restricted to NW), and 10 individuals of P. incarnata (occurring in NE and SE).

DNA Extraction, Amplification, and Sequencing—We extracted genomic DNA from silica-dried leaflets collected during fieldwork and herbarium specimens using the DNeasy plant mini kit (Qiagen, Düsseldorf, Germany) or the Invisorb Plant Mini Kit (Invitek, Berlin, Germany) following the manufacturer’s instructions. For old herbarium samples, we added 1.5 μL of β-mercaptoethanol to each reaction. We selected two chloroplast DNA markers, i.e. the rulF and rpl32-trnl dataset, 50 individuals; 2) rpl32-trnl dataset, 49 individuals; 3) PepC dataset, 16 individuals; 4) cpDNA dataset (rulF and rpl32-trnl), 51 individuals; and 5) combined dataset (rulF, rpl32-trnl, and PepC), 51 individuals. Given the wide distribution of taxa, we were able to collect at least one field sample throughout the entire species ranges. Hence, more than half of the samples were obtained from herbarium specimens collected between 1950-1990, which yielded a high failure rate in the amplification of sequences, especially for the nuclear gene. Although the nDNA dataset is smaller, it was sufficient to test major relationships recovered from the cpDNA dataset.

Phylogeny Reconstruction—We analyzed our molecular dataset using Bayesian inference (BI) and maximum likelihood (ML). For BI and ML analyses, we selected the best-fitting model of DNA substitution for each data partition using the Akaike information criterion implemented in JModelTest v. 2.1.4 (Darriba et al. 2012). We performed BI analyses in MrBayes 3.2.2 (Ronquist et al. 2012) using the GTR + G model for the rulF and rpl32-trnl datasets, and the GTR + I model for the PepC dataset. We conducted four Markov chain Monte Carlo (MCMC) runs using four chains each for a total of 10^6 generations. Trees were saved every 1000 generations to minimize autocorrelation among samples. Stationarity was determined by visually monitoring likelihood values in Tracer v. 1.5 (Rambaut and Drummond 2009). Trees were summarized into a consensus tree after discarding the burn-in (25%). We conducted ML searches in RaxML (Stamatakis 2006) using the graphical user interface RAxMLGUI v. 1.3 (Silvestro and Michalak 2012) with 1000 rapid bootstrap replicates. We performed the analysis using the same models of DNA substitution mentioned above, except for the analyses of the combined dataset, for which we used the GTR model. Nodes with posterior probabilities (PP) ≥ 95 and bootstrap (BS) ≥ 70 were considered well supported.

Species Delimitation—We used a combination of approaches and coalescent theory as a basis to investigate species limits within the P. kerere species complex. The number of species recognized within the P. kerere species complex ranged from one to three in the past, but species limits remained confusing during the last 150 yr (see Francisco and Lohmann 2018 for further details). Coalescent theory offers an opportunity for the accurate exploration and understanding of evolutionary units as it allows us to infer species limits based on a genealogical and population genetic perspective (Sites and Marshall 2003; Zhang et al. 2011; Fujita et al. 2012). As genetic data can contain the signal of historical processes involved in lineage divergence (Nielsen and Wakeley 2001), it provides primary data for lineage diagnosis, especially when associated with morphological data (Sites and Marshall 2003; Pons et al. 2006; Knowles and Carstens 2007; Fujita et al. 2012). More specifically, morphological data informs phenotypic divergences among species (Edwards and Knowles 2014) representing a secondary line of evidence to assess the separation of lineages (De Queiroz 1998, 2007).

We adopted a three-stage approach, as follows: 1) a species discovery approach used to assign putative species based on molecular characters, 2) a morphological approach used to evaluate the putative species suggested based on molecular data, and 3) a validation approach used to estimate the support of the individual evolutionary lineages identified as distinct. These approaches aimed at reducing investigator-driven biases and alleviated substantial shortcomings of each method. Given our limited sampling of individuals for the nuclear marker (16 individuals vs. 51 individuals sampled in the cpDNA dataset), we only used the chloroplast dataset for our species delimitation analyses. Single-locus data derived from a small sample of individuals and species has been shown to provide sufficient information for species delimitation methods (Fujita et al. 2012; Fujisawa and Barraclough 2013).

First, we used the generalized mixed Yule coalescent (GMYC) method to identify putative species (Pons et al. 2006). GMYC overcomes quantitative species delimitation methods by establishing confidence intervals while evaluating the uncertainty associated with species delimitation hypothesis populations a priori, thus avoiding problematic population delimitation (Pons et al. 2006). As such, this method does not require previous information about species, allowing a careful evaluation
of groups with uncertain taxonomy. For this analysis, we generated an ultrametric time-tree based on the Bayesian analysis of the combined cpDNA dataset using BEAST v. 2.4.3 (Bouckaert et al. 2014).

The analysis of GMYC was performed in the R package ‘splitS’ (Species Limits by Threshold Statistics project; Ezard et al. 2009) fitted for the single-

threshold algorithm. We also ran Bayesian Poisson tree processes (bPTP; Zhang et al. 2013) to detect possible evolutionary lineages associated with the time cali-

bration procedures required by GMYC. This analysis verifies whether the uncertainty associated with the time-calibrated branch lengths are affecting the ultrametric phylogeny. We used the same Bayesian combined cpDNA tree generated with BEAST to infer the phylogenetic tree. We ran the analyses on the bPTP web server (http://species.h-isms.org/bptp/) using the following parameters: MCMC = 500,000 generations; thinning = 1000; burn-in = 0.25; seed = 1234.

Because the GMYC is used to estimate infraspecific variation and to identify the highest possible number of species in the case of broadly distributed taxa (Bergsten et al. 2012; Talavera et al. 2013), we re-evaluated the results based on “morphological distinctiveness” (Table 1) as an ad-

ditional criterion to assign species limits. For this, we conducted com-

parative morphological studies following the same methodology described in Francisco and Lohmann (2018). In sum, we examined 378 specimens collected in the field or deposited in the following herbaria: A, B, COL, ESA, F, G, HB, HERBAM, HRCB, HUA, INA, INPA, K, LINN, MBM, MG, MICH, MO, NY, NX, P, R, RB, RBR, S, SPF, SPF SPSF, UEC, UC, UFACPZ, US, VEN and WU (acronyms following Thiers 2015). All analyses followed the terminology of Lohmann and Taylor (2014), with additional terms from Nogueira et al. (2013) for trichomes, and Gentry and Tomb (1979) and Hesse et al. (2009) for pollen.

We searched for congruence among the putative species suggested by GMYC and key morphological characters. We then validated our species hypotheses based on estimated support, using a multispecies coalescent model in a Bayesian framework, implemented in BEAST (Bouckaert et al. 2014). While GMYC does not require a priori species hypotheses allowing an unbiased evaluation of groups with uncertain taxonomy (Pons et al. 2006), BEAST requires the definition of a priori species and is usually used to validate hypotheses pre-defined based on other methods. This approach allowed us to evaluate whether the species hypothesized by GMYC and subsequently corroborated by morphology are indeed likely to represent reproductively isolated lineages. BEAST provides a species tree estimation that is assumed to not exchange genes and to form independently distinct lineages. Posterior probabilities ≥ 95 were considered as sufficiently in-

formative for the assignment of species status. Due to the lower sampling of our nuclear dataset, we ran BEAST using the combined chloroplast dataset exclusively. Since BEAST uses a clock model to estimate the roots of in-

dividual gene trees through a multispecies coalescent approach (Heled and Drummond 2009), no outliers were included in this analysis. The follow-

ing parameters were used: linked trees, unlink substitution models, strict clock model parameters, and Yule process. For more details see Table S1 (Francisco and Lohmann 2020).

**Divergence Time Estimation**—Divergence time estimation was carried out in BEAST. For this analysis, we used our combined molecular dataset (i.e. ndhF, rpl32-trnL, and PepC), including a single accession per species recognized in species delimitation analyses. We defined three unlinked partitions and clock models, corresponding to the different genes with linked trees. We used a relaxed clock log normal approach with a Yule tree prior speciation model.

We used divergence time constraints, applying a normal distribution to the prior probabilities of the dates based on the genus age (40.0 Mya [95% HPD: 43.2–37.0 MyA] estimated by Lohmann et al. (2013). We assigned a mean 40, sigma 1.6, and offset 0.5 to the root of the clade. We performed the analysis with four runs using four MCMC chains each for 10^6 generations, sampling every 1000 generations. Results were verified in Tracer to ensure the convergence of runs and if the effective sample sizes (ESS) were > 200. We used LogCombiner in BEAST to combine trees derived from the four runs. We built a maximum clade credibility tree with median height nodes in TreeAnnotator in BEAST, after excluding 25% of samples as burn-in.

**Biogeographical Analyses**—We conducted ancestral area reconstruc-

tions using the same five biogeographical regions defined for our sampling (Fig. 1). We performed the dispersal extinction cladogenesis model (DEC, Ree et al. 2008) implemented in RASP v. 3.1 (Yu et al. 2015) to reconstruct the biogeographic history of Pachyptera across the Amazon and Central America.

For this analysis, we used the time-calibrated tree estimated for Pachyptera using the combined dataset (i.e. ndhF, rpl32-trnL, and PepC) and a single individual per species. Species movements across areas were unconstrained, i.e. dispersal rate was modeled equal among areas. This analysis reconstructed uncertainty in the geographic origin of Pachyptera, which is likely due to the broad distribution of P. kerere.

In order to identify the ancestral area of Pachyptera, we constructed a haplotype network of P. kerere, our broadly distributed taxon, using the cpDNA dataset. For this analysis, we assumed that higher levels of genetic diversity and central positions in the haplotype network should be located at more ancient areas than newly colonized areas located at the tips of the network (Kingman 1982; Posada and Crandall 2002). We constructed a haplotype network using the cpDNA dataset of P. kerere (n = 31) and statistical parsimony implemented in TCS v. 1.21 (Clement et al. 2000). For this analysis, we used a connection limit of 95% and treated gaps as a fifth character state. We visualized the network with tcsBU (Dos Santos et al. 2015). The network of chloroplast haplotypes recovered 19 haplotypes with the ndhF dataset and 31 haplotypes with the rpl32-trnL dataset. The net-

work derived from the analysis of the ndhF dataset indicated that North Western Amazon and Central America and Andes were the most likely ancestral areas of P. kerere (Fig. S4, Francisco and Lohmann, 2020). On the other hand, the network derived from the analysis of the rpl32-trnL dataset indicated that South Eastern Amazon might be the ancestral area. How-

ever, the network constructed with the rpl32-trnL dataset included loops suggesting recombination. As such, we used North Western Amazon and Central America and Andes as putative areas for the distribution of P. kerere in our final ancestral area reconstruction with the DEC model (Ree et al. 2008).

We also explored whether model choice influenced biogeographical reconstruction. For that, we applied the statistical dispersal-vicariance model (S-DIVA, Yu et al. 2010) implemented in RASP. We performed the S-DIVA analysis with the default setting. The ancestral areas recon-

structed with DEC and S-DIVA models were similar for most lineages (Table S2, Francisco and Lohmann 2020). A best likelihood was recovered for DEC instead of S-DIVA and DEC was selected as the most suitable model for our dataset.

We also conducted a BSSVS (Lemey et al. 2009) analysis of discrete states using a diffusion model in BEAST. This approach incorporates a pure dispersal model allowing us to infer the spatial history of Pachyptera across the landscape over time, and to identify historically important migration pathways within the genus. This method accommodates uncertainty in

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**Table 1. Morphological traits of Pachyptera species.**

| Clade              | Flower color | Corolla shape              | Androecium | Ovary                  | Fruit                      | Seed                                      | Seed ornamentation                           |
|--------------------|--------------|-----------------------------|------------|------------------------|----------------------------|-------------------------------------------|----------------------------------------------|
| P. aromatica       | White        | Hypocrateriform             | Included   | Pubescent              | Linear and flattened       | Oboblong, thin, characeous, winged        | Striated, secondary sculpture smooth         |
| P. erythraea        | Orange to red| Tubular, campanulate        | Sub-exserted| Lepidote               | Linear and flattened       | Oboblong, thin, characeous to winged      | Striated, secondary sculpture not seen       |
| P. incarnata        | Light pink to pale purple | Infundibuliform              | Included   | Lepidote               | Linear and flattened       | Oboblong, thin, characeous to sub-characeous, winged | Striated, secondary sculpture with randomly distributed micro pores |
| P. kerere           | White to cream| Infundibuliform             | Included   | Pubescent              | Fusiform and inflated      | Irregularly circular and obcordate, thick, corky, wingless | Striated, secondary sculpture with two pairs of medium micro pores on each striation |
| P. linearis         | White        | Infundibuliform             | Included   | Pubescent              | Linear and flattened       | Oboblong, thin, characeous to winged      | Striated, secondary sculpture regularly interrupted by lateral rays |
dispersal and in the unknown phylogeny, preventing overstated conclusions. For this analysis, we used the comprehensive tree derived from the combined chloroplast dataset (cpDNA) and partitioned the samples into geographic regions linked to the trait “location.” We used a symmetric substitution model for the discrete traits in the location partition with the social network using the BSSVS procedure. We also used a relaxed clock log-normal with a coalescent constant population tree prior. For a temporal frame, we applied divergence time constraints (see Table S1, Francisco and Lohmann 2020). Furthermore, a strict clock model was employed for the “location” trait, exponential prior for the discrete location state rate (locations.clock.rate), keeping the default settings for the remaining parameters. We assessed the convergence of model parameters using Tracer and discarded the first 25% of sampled generations as burn-in. A chronogram with ancestral areas with the maximum sum of clade credibility (MCC) was obtained with TreeAnnotator and visualized in FigTree v. 1.4.0 (Rambaut and Drummond 2012). We assessed statistical significance for dispersal events using the Bayes factor (BF) test in SPREAD v. 1.0.6 (Bielesj et al. 2011).

RESULTS

Phylogeny of Pachyptera—The analyses of the ndhF and rpl32-trnL datasets recovered congruent topologies (Figs. S1–S2, respectively; Francisco and Lohmann 2020). In both analyses Pachyptera emerged as monophyletic and divided into three main clades, as follows: 1) a clade that includes all specimens of P. aromatica; 2) a clade that includes a monophyletic P. erythraea sister to a monophyletic P. incarnata; and 3) a clade that includes all individuals of P. kerere sister to a clade composed of P. linearis. The topology derived from the analysis of the combined cpDNA dataset recovered a similar topology but with higher resolution and support overall (Fig. S2, Francisco and Lohmann 2020). The topology derived from the analysis of the PepC dataset also recovered a monophyletic Pachyptera and the same three main clades (Fig. S3, Francisco and Lohmann 2020).

Topologies derived from the Bayesian and maximum likelihood analyses of the cpDNA and nDNA datasets were congruent (Fig. 2). The phylogeny derived from the analysis of the combined ndhF, rpl32-trnL, and PepC dataset also recovered a monophyletic Pachyptera (PP = 100%, BS = 100%). Pachyptera aromatica is strongly supported as monophyletic (PP = 94%, BS = 84%) and recovered as sister to the remaining species of the genus, all of which are included in the P. kerere species complex. This species complex includes two major sub-clades: 1) a highly supported clade (PP = 100%, BS = 100%) composed of P. erythraea (PP = 100%, BS = 100%) and P. incarnata (PP = 100%, BS = 99%), and 2) a moderately supported clade (PP = 76%, BS = 84%) composed of P. kerere (PP = 65%, BS = 76%) and P. linearis (PP = 99%, BS = 86%).

Species Delimitation—The GMYC analysis using the single threshold approach recovered six putative species within the genus (Fig. 3) with confidence intervals that ranged from five to 20 (logL_null = 31.77327; logL_GMYC = 35.6921; LR = 7.836675; p = 0.01987411), and threshold time (transition in branching rate occurring) of ca. 6.5 MYA. The likelihood ratio test was significant, indicating that the null model (i.e. a single population) could be rejected. The bPTP resulted in five to 29 estimated species that were highly similar to those suggested by GMYC, indicating that the species boundaries suggested by GMYC did not vary with branch length.

The six initial putative species recovered within the genus were evaluated using morphological data (Table 1; Figs. 2–3). This data only provided support for the recognition of five out of the six possible species identified with GMYC. More specifically, morphological data provided further support for the recognition of P. aromatica, P. erythraea, P. incarnata, P. kerere, and P. linearis, but did not support the recognition of the earliest diverging clade within P. kerere as a separate species. All the morphological data are summarized in Table 1.

Within this framework, P. aromatica is unquestionably an independent species. In contrast, members of the Pachyptera kerere species complex share a higher number of morphological features. Pachyptera kerere and P. linearis share white infundibuliform corollas, included androecia, and a pubescent ovary, but differ in fruit traits. Namely, members of the P. linearis differ from P. kerere by the linear and flattened fruits, with oblong, thin, coriaceous to woody and winged seeds (vs. the fusiform and inflated fruits with irregularly circular and obcordate, thick, corky and wingless seeds of P. kerere). Fruits of P. linearis are similar to those of P. incarnata and P. erythraea. Namely, P. linearis has a pubescent ovary, while P. incarnata and P. erythraea share a lepidote ovary. Furthermore, while P. incarnata has light pink to pale purple infundibuliform corollas, and included androecium, P. erythraea has orange to red tubular-campanulate corollas, and a sub-exserted androecium. All of these morphological features provide support for the recognition of four species within the Pachyptera kerere species complex.

The five species identified within the genus based on GMYC and recognized with morphology were further tested with BEAST, which provided very strong support (PP > 0.98%) for the recognition of five taxa (Fig. 3).

Divergence Time Estimation—Divergence time estimation suggested that the most recent common ancestor (MRCA) of the genus originated at ca. 40.3 million years ago (MYA), in the Middle Eocene [95% highest posterior density (HPD): 43.5–37.2 MYA]. These analyses further suggest that the genus diversified during the Miocene, at ca. 23.5 MYA [95% HPD: 38.5–10.5 MYA]. The species diversity belonging to P. kerere species complex dates back to the Late Miocene, with the split between P. erythraea and P. incarnata occurring at approximately 15.4 MYA [95% HPD: 34.9–3.4 MYA], and the divergence between P. kerere and the P. linearis at ca. 9.8 MYA [95% HPD: 23.8–1.0 MYA] (Table S2, Francisco and Lohmann 2020).

Biogeography of Pachyptera—Biogeographical analyses inferred by the DEC model and coding the distribution of P. kerere using the two ancestral areas identified by the haplotype networks (i.e. North Western Amazon, and Central America and Andes, Fig. S4, Francisco and Lohmann 2020), ambiguously recuperated the ancestral area of the genus and the crown node of the species-complex (Fig. 4; Table S2, Francisco and Lohmann 2020). In this analysis, a disjunct area composed of Central America and Andes plus Eastern Amazon was recovered as the most likely ancestral area for the MRCA of the P. erythraea + P. incarnata clade (RP = 0.50), with vicariance presenting an important role in the history of clade. On the other hand, two likely ancestral areas for the MRCA of the P. kerere + P. linearis clade were recovered almost equally for the North Western Amazon and/or Central America and Andes plus North Western Amazon origin (RP = 0.53 and 0.47, respectively), implying dispersal.

The ancestral area reconstructions within Pachyptera using the BSSVS are summarized in Fig. 5, and Table S2, Francisco and Lohmann (2020). These reconstructions show uncertainty in the geographic origin of Pachyptera and its species, recovering multiple possible ancestral areas with low support (PP < 31%). BSSVS recovered three equally likely (PP = 21%) biogeographical areas for the MRCA of all extant Pachyptera
species, i.e. North Western Amazon, North Eastern Amazon, and South Eastern Amazon. Subsequent splitting occurred in Central America and the Andes (PP = 24%) on the crown node of the *Pachyptera* species complex. Two areas, Central America and Andes and North Eastern Amazon, were recovered as equally likely (PP = 23%) for the MRCA of the *P. erythraea* and *P. incarnata* clade. Central America and Andes was recovered as the most likely ancestral area for the MRCA of the *P. kerere* and *P. linearis* clade (PP = 31%). The Bayes factor test for dispersal frequencies revealed two significant (BF > 3) routes for dispersal between: 1) North Western Amazon to South Western Amazon (BF = 131); and 2) North Western Amazon to South Eastern Amazon (BF = 21). Furthermore, one moderately significant route (BF > 2.9) was recovered between North Eastern Amazon to South Eastern Amazon (BF = 2.9). Two additional routes were not significant and only supported by BF < 1.4: 1) North Eastern Amazon to Central America and Andes (BF = 1.4), and 2) North Eastern Amazon to South Eastern Amazon (BF = 1.4).
South Eastern Amazon to Central America and Andes (BF = 1.3).

**Discussion**

In this study, we used molecular data (i.e. \textit{ndhF}, \textit{rpl32-trnL}, and \textit{PepC}) to reconstruct relationships among species of \textit{Pachyptera}, a small genus of Neotropical lianas. Our analysis included a broad sampling of taxa (i.e. 51 individuals from all species recognized), and was used as basis to evaluate species boundaries. A polyphyletic \textit{P. kerere} species complex was recovered, indicating the need for taxonomic adjustments, all of which were recently proposed by Francisco and Lohmann (2018). The phylogenetic framework was used as basis to evaluate species limits within the \textit{P. kerere} species complex, estimate divergence times, and reconstruct the biogeographic history of the genus. The genus likely originated in lowland Amazonia during Middle Eocene and diversified during the Miocene, a period of intense geological, marine, hydrological and climatic changes. Below, we summarize our major findings and discuss their implications for the systematics and biogeography of \textit{Pachyptera}.

**Phylogeny of Pachyptera**—The phylogeny constructed here provides further support for the monophyly of \textit{Pachyptera} recovered in earlier studies (Lohmann 2006; Francisco and Lohmann 2017). \textit{Pachyptera aromatica} is strongly supported as sister to the rest of the genus, which includes two main clades: 1) a clade composed of \textit{P. erythraea} and \textit{P. incarnata}, and 2) a clade composed of \textit{P. kerere} and \textit{P. linearis} (Fig. 2). This new phylogenetic framework provides additional support for the position of \textit{P. aromatica} within \textit{Pachyptera}. These findings are further supported by morphological synapomorphies such as the papery peeling bark, patelliform glands arranged in lines on the upper portions of the calyx and corolla tube (Lohmann 2006; Lohmann and Taylor 2014), and prophylls of the axillary buds organized in a series of three, all of which are unique within Bignonieae (Lohmann and Taylor 2014; Francisco and Lohmann 2018). The phylogenetic framework reconstructed here also clarifies species limits within the \textit{P. kerere} species complex. Namely, \textit{P. kerere} s. l. (sensu Gentry 1973 and Lohmann and Taylor 2014; also including \textit{P. kerere} var. \textit{incarnata}) is polyphyletic as circumscribed previously. Furthermore, a lineage with mixed flower and fruit features between \textit{P. kerere} and \textit{P. incarnata}, emerged as a strongly supported clade that is sister to \textit{P. kerere}, providing further support for the recently recognized \textit{P. linearis} (Francisco and Lohmann 2018).

**Species Delimitation and Taxonomic Implications**—Species delimitation is a controversial topic in biology, which is partly due to the fact that speciation is a continuous process and organisms may be at different stages of differentiation. Multiple species concepts have been proposed (see De Queiroz 1998, 2007 for a review). For this study, we treat independently evolving meta-populations as species (De Queiroz 1998, 2007) and integrate morphological and molecular data while defining species limits in a reproducible and falsifiable framework (Padial et al. 2010; Fujita et al. 2012).

Our findings provide important new insights for the delimitation of species within \textit{Pachyptera}, especially in what concerns the taxonomically complicated \textit{P. kerere} species complex. As previously circumscribed, the \textit{P. kerere} species complex is polyphyletic (Lohmann and Taylor 2014). This species complex is treated as four separate taxa in a recent monograph of the group (Francisco and Lohmann 2018). The pink flowered \textit{P. incarnata} is sister to the red flowered \textit{P. erythraea}, instead of a variety more closely related to the white flowered \textit{P. kerere} as previously thought (Gentry 1973; Lohmann and Taylor 2014). On the other hand, \textit{P. kerere} is sister to \textit{P. linearis}, another white flowered species. Despite the similarities in flower morphology, these taxa differ in fruit and seed morphology. More specifically, the seeds of \textit{P. kerere} are corky, wingless, and water dispersed, while those of \textit{P. linearis} are thin, winged, and wind dispersed. The cryptic \textit{P. linearis}
described by Francisco and Lohmann (2018) was never noticed before, likely due to the restricted distribution, small population sizes, and overlapping patterns of morphological variation with *P. kerere* and *P. incarnata*. The recognition of these four clades as separate species within the *P. kerere* species complex is strongly supported by the molecular phylogeny of the genus (Fig. 2). Furthermore, the GMYC species delimitation analyses, morphological studies, and the final BEAST validation (Fig. 3) provide additional support for the recognition of four separate taxa within the *P. kerere* species complex. Cryptic species are indistinguishable morphologically and difficult to diagnose (Bickford et al. 2007). The initial GMYC analysis (Fig. 3) suggested the recognition of six species, only five of which were supported by morphological data. The lineage not recognized as a separate taxon is restricted to Madre de Dios (Peru) and would benefit from additional studies. A lack of gene flow between this lineage and the remaining *P. kerere* is indicative of incipient speciation (Avise 2000). However, given the lack of morphological differentiation of this lineage, we prefer to adopt a more conservative approach and keep this putative cryptic species within *P. kerere* until more comprehensive population level studies can be conducted.

In our study, morphology and molecular data acted in concert, allowing a clear definition of species boundaries. Thus, although the practice of species delimitation focused exclusively on genetic data has been advocated by some (e.g. Cook et al. 2010; Renner 2016), we highlight the paramount and inextricable link between morphology and genetic data for accurate species recognition (Sites and Marshall 2003; Pons et al. 2006; Knowles and Carstens 2007; Padial et al. 2010). Species boundaries can be semi-permeable, reflecting limited gene flow among taxa due to a set of intrinsic barriers, e.g. phenotypic differences (Harrison and Larson 2014). Differences in floral structure and phenology have been hypothesized to represent a key speciation driver within this tribe (Gentry 1974, 1990; Alcantara and Lohmann 2010). Specifically in *Pachyptera*, the white flowered *P. aromatica* is hawk-moth pollinated (Barbosa Rodrigues 1891; Gentry 1974), while the red flowered *P. erythraea* is hummingbird pollinated (Gentry 1974).
and the white flowered *P. kerere* is likely pollinated by large to medium-sized bees (Gentry 1974). The pink flowered *P. incarnata* and the *P. linearis* are likely pollinated by bees, too. The great diversity in pollination systems found in such a small genus of tropical lianas may be associated with the diversification of this group. The ecological consistency among the evolutionary units recognized as separate species provides further support for the recognition of four separate taxa within the *P. kerere* species complex. A new taxonomic revision of *Pachyptera* proposed all required taxonomic changes (Francisco and Lohmann 2018).

**Biogeography of *Pachyptera*—**Our results indicate that the MRCA of *Pachyptera* diverged ca. 40.3 MYA (95% HPD, 43.5–37.2 MYA), a period when the Amazon rainforest covered an area much larger than today (the pan-Amazonia; Hoorn et al. 2010). This time coincides with high global temperatures (Jaramillo et al. 2006) and increases in species numbers for several plant families in the Neotropics (Jaramillo et al. 2010), leading to higher rainforest diversity that was perhaps even greater than that found in modern Amazonian forests (Jaramillo et al. 2006). Our reconstruction suggests that *Pachyptera* originated in lowland Amazonia during the Middle Eocene (Fig. 6I), corroborating earlier findings (Lohmann et al. 2013). These data provide additional support for the hypothesis that Amazonian forests were highly diverse in the Eocene (Jaramillo et al. 2006; Wing et al. 2009), as well as for the hypothesis that the diversity currently found in the Amazon resulted largely from in situ diversification (Gentry 1982; Antonelli et al. 2018). Although our analysis could not establish the complete diversification history of the genus with certainty, it suggests historically important dispersal routes from North to South within the Amazon (BF > 3). This pattern has also been documented for other Amazonian organisms such as birds (Aleixo and Rossetti 2007), ants (Solomon et al. 2008), butterflies (Condamine et al. 2012), palms (Roncal et al. 2012), and glassfrogs (Castroviejo-Fisher et al. 2014).

*Pachyptera aromatica* was the first lineage to diverge from the remaining species in the genus (Fig. 6I). The diversification of this taxon likely began in the Guiana Shield and was followed by the occupation of South Eastern Amazon and North Western Amazon, at approximately 2 MYA (Fig. 5). Early diverging evolutionary units are often associated with more stable and geologically older terrains that may have acted as “species-pumps” (Aleixo and Rossetti 2007). The Guiana Shield remained stable since the Late Cretaceous (Rossetti et al. 2005) and represents the center of diversity of *Pachyptera* (Francisco and Lohmann 2018). Below we discuss the potential impact of three key Neotropical geological events for the diversification history of *Pachyptera*.

(i) **Closure of the Isthmus of Panama**—The rise of the Isthmus of Panama represented one of the major geological changes in the Americas (Gentry 1982; Antonelli and Sanmartín 2011). Recent geological studies associated with biological evidence have suggested a more complex history for the closure of the Isthmus of Panama, with a stepwise formation and biotic interchange between North and South
America since the Oligocene–Miocene (Cody et al. 2010; Bacon et al. 2012, 2015; Montes et al. 2012, 2015; O’Dea et al. 2016; Jaramillo 2018). Despite that, the most important faunal wave of migration (e.g. the “Great American Biotic Interchange,” GABI) seems to have only occurred in the Late Pliocene (Stehtli and Webb 1985).

The split event that gave rise to the crown node of the \textit{P. kerere} species complex occurred in the Early Miocene (Fig. 6II), ca. 23.5 Mya (95% HPD: 38.5–10.5 Mya). This age predates the full closure of the Isthmus of Panama (Montes et al. 2012, 2015; Leigh et al. 2014; Bacon et al. 2015, 2016; O’Dea et al. 2016) suggesting early dispersal of \textit{Pachyptera} from Amazonia to Central America and Andes (Fig. 6II) through a stepping-stone mechanism, a pattern also documented for other plant groups (e.g. Croat and Busey 1975; Gentry 1982; Antonelli et al. 2009; Cody et al. 2010; Lohmann et al. 2013; Bacon et al. 2015; Thode et al. 2019). This early dispersal might have been facilitated by an emerging land bridge connecting Central and South America (~20 MYA; Cody et al. 2010; Bacon et al. 2012, Montes et al. 2012, 2015; O’Dea et al. 2016). The temporary connection through GAARlandia (i.e. Greater Antilles and the Aves Ridge land bridge, 33–35 MYA; Iturralde-Vinent and MacPhee 1999; Pennington and Dick 2004) could represent an alternative route of dispersal. However, extant species are absent in the Antilles and the time frame recovered here is more congruent with an emerging land bridge connection through the Panama Isthmus.

(ii) Andean Uplift and the Formation of the Pebas Lake System—The Andean orogeny led to a series of geological, climatic, and hydrological changes that strongly impacted the Amazonian landscape. These changes are thought to have represented triggers for Neotropical diversification (Hoorn 1993; Hoorn et al. 1995, 2010; see more in Antonelli and Sanmartín 2011). The uplift of the Andes began in the Paleogene (65–34 MYA) with the subduction of the oceanic Nazca plate beneath South America and a series of plate movements from the south to the north and from the west to the east, along the western coast of South America. Because of plate tectonic readjustments, the Central and Northern Andes rapidly uplifted during the last 20–10 MYA (Gregory-Wodzicki 2000; Hoorn et al. 2010). As mountain building progressed and reached a critical elevation (~2000 m), rainfall increased along the eastern flank leading to erosion, water and sediment inflows to lowland Amazon. Meanwhile, ongoing uplift of the Eastern Cordillera in the Central Andes promoted geological submergence of western Amazon creating a huge...
Our biogeographical reconstructions suggest two alternative ancestral areas for the *P. erythraea* + *P. incarnata* clade: 1) a disjunct ancestral area composed of Central America and the Andes and Eastern Amazon (inferred by DEC, Fig. 4; Table S2, Francisco and Lohmann 2020), or 2) a smaller disjunct ancestral area composed of Central America and the Andes and North Eastern Amazon (inferred by BSSVS, Fig. 5; Table S2, Francisco and Lohmann 2020). While the former scenario invokes vicariance, the latter invokes dispersal. Comparisons between the divergence times of clades and geographic barriers help to identify the most likely scenarios. The age of the MRCA of the *P. erythraea* + *P. incarnata* dates back to 15.4 MYA (95% HPD: 34.9–3.4 MYA), which corresponds to the age of formation of the Pebas system that separated western and eastern South America (Hoorn 1993; Hoorn et al. 1995, 2010; Wesselingh et al. 2002; Wesselingh and Salo 2006; Antonelli and Sanmartín 2011). This coincidence in timing suggests that allopatric speciation between the northern Andes and Eastern Amazonia, sometime between 17 to 11 Mya (Hoorn 1993; Hoorn et al. 1995, 2010; Wesselingh et al. 2002; Wesselingh and Salo 2006; Antonelli et al. 2009) may have played a role in the diversification history of this clade (Fig. 6III). *Pachyptera erythraea* is endemic to the Magdalena Valley, which separated the northeastern Cordillera into Eastern and Western Cordilleras at ca. 11.8 MYA (Hoorn et al. 1995), indicating the parallel role of emergent mountains in genetic isolation (Antonelli and Sanmartín 2011). On the other hand, *P. incarnata* is restricted to Eastern Amazon, suggesting an ecological constraint as the geographical barrier no longer exists today.

## Conclusion
We reconstructed a robust phylogeny of *Pachyptera*, which provided a framework for species delimitation, establishing a strong basis for a species-level account for the whole genus (Francisco and Lohmann 2018). Overall, our findings corroborate the recognition of five species within *Pachyptera*, namely the previously recognized *P. aromatica* and *P. erythraea*, the newly circumscribed *P. kerere* and *P. incarnata*, and the newly described *P. linearis*. Coalescence analyses indicated that the studied populations of each of these taxa are genetically and historically isolated from each other, providing additional support for the recognition of four species within the *P. kerere* species complex. These molecular findings are further supported by morphological traits and differences in pollination syndrome.

Our study provided the first biogeographic hypothesis for *Pachyptera*. Paleogeographical events and a dynamic environment during the Neogene seem to have played a significant impact on the history of the genus. A similar pattern has been documented for other angiosperm groups (Antonelli et al. 2009; Hoorn et al. 2010; Antonelli and Sanmartin 2011; Rull
markers for patterns recovered here. Recently developed microsatellite markers for Pachyptera (Francisco et al. 2016) offer a great opportunity for population genetic and phylogeographic studies within this group.

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Author Contributions

JNCF and LGL designed the research; JNCF and LGL collected samples; JNCF conducted molecular laboratory work and performed data analyses; LGL contributed with reagents/materials/analytical tools; JNCF and LGL wrote the paper.

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Outgroup: Fridericia speciosa Mart., BRAZIL, Minas Gerais, PE do Rio Doce, Lombardi 2521 (MO): DQ222584.1*, KC914604*, DQ222730*. Lundia spruceana Bureau, PERU, Madre Dios, Manu National Park, Lohmann 610 (MO): DQ222593*, KP775734.1*, DQ222745*. Stizephyllum perforatum (Cham.) Miers, BRAZIL, Paraná, Londrina, Fonseca 105 (SPF): KP691457.1*, KP697987.1*, KP697965*. Tanacium bilabiatum (Sprague) L.G.Lohmann, BRAZIL, Amazonas, Rio Solimões, Lohmann 92 (SPF): DQ222540*, KP775333*, DQ222667*.  

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