An Acad Bras Cienc (2021) 93(4): e20191446 DOI 10.1590/0001-3765202120191446
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ECOSYSTEM

Rethinking the pollination syndromes in *Hymenaea* (Leguminosae): the role of anthesis in the diversification

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Abstract: Floral traits associated with functional groups of pollinators have been largely employed to understand mechanisms of floral diversification. *Hymenaea* is a monophyletic legume genus widely recognized to being bat-pollinated, with nocturnal anthesis and copious nectar. The most of species has short-paniculate inflorescences, white and robust flowers, congruent with a bat-pollination syndrome. However, other *Hymenaea* species show a different floral pattern (e.g., long-paniculate inflorescences and smaller flowers) which we report here as being bird pollinated. We examined the floral traits and visitors of *Hymenaea oblongifolia* var. *latifolia* and identified evolutionary shifts in floral traits associated with potential pollinators of *Hymenaea* species. Floral traits of *H. oblongifolia* var. *latifolia* differ from those expected for bat-pollinated flowers in species of sect. *Hymenaea*, and we observed hummingbirds collecting nectar legitimately. Our phylogenetic analysis did not support the monophyly of the taxonomic sections and suggests that bat pollination is ancestral in *Hymenaea*, with bird pollination evolving later. The transition coupling with shifts in the timing of anthesis and other floral traits. Pollinator-mediated evolutionary divergence hypothesis partially explains the *Hymenaea* diversification in the Neotropics. It is congruent with those species shifting from traits linked traditionally to bat pollination to hummingbird pollination.

Key words: Bat pollination, ethological isolation, floral traits, hummingbirds, legume.

INTRODUCTION

Pollination syndrome concept is based on the notion that suites of floral traits reflect adaptations to a specific pollinator or group of most effective pollinators (Stebbins 1970, Faegri & van der Pijl 1979, Grant 1994). It is a proxy for understanding floral trait diversification and evolution towards specialized pollination systems (Fenster et al. 2004, Armbruster et al. 2014, Rosas-Guerrero et al. 2014). This specialized interaction of floral traits and pollinators is a key component that can drive convergent floral evolution in distinct lineages (Johnson 2010, Papadopulos et al. 2013, Newman et al. 2014, Ferreira et al. 2015). On the other hand, multiple functional groups of pollinators can drive ancestral polymorphic populations to different floral traits (i.e., divergent evolution processes) in an idiosyncratic geographic-ecological context (Shemske & Bradshaw 1999, Eaton et al. 2012, Armbruster 2014). In other possible scenario, the conservatism of floral traits is intuitively understood as a direct result of the evolutionary history in the context of sharing of the common ancestry without selective pressures exerted by pollinators (i.e., phylogenetic inertia; Sakazono et al. 2012, Papadopoulos et al. 2013).

Hymenaea L., a Neotropical monophyletic genus of subfamily Detarioideae in the

Leguminosae, is a good biological model to investigate the mechanisms that drive the floral evolution in plants, mainly due to its great variation in floral and inflorescence attributes. In the first revision of the genus, two sections were proposed in accordance to morphological traits (i.e., sect. *Hymenaea* and sect. *Trachylobium*; Lee & Langenheim 1975). *Hymenaea* has long been considered entirely bat-pollinated, because most species have floral traits related to chiropterophily (e.g., nocturnal anthesis, white and robust petals, and copious nectar production; Langenheim et al. 1973, Lee & Langenheim 1975, Arroyo 1981, Gibbs et al. 1999, Dunphy et al. 2004, Paiva & Machado 2008, Fleming et al. 2009, Domingos-Melo et al. 2019). This generalization is supported by empirical evidence in natural populations of *H. stigonocarpa* Mart. ex Hayne, *H. courbaril* L, and *H. cangaceira* R.B. Pinto, Mansano & A.M.G. Azevedo, which have been observed to be pollinated by Phyllostomid bats (sect. *Hymenaea*; Arroyo 1981, Gibbs et al. 1999, Dunphy et al. 2004, Lacerda et al. 2008, Moraes & Sebbenn 2011, Domingos-Melo et al. 2019). All of these species are members of sect. *Hymenaea*, but no data are available in the literature about the pollination mode in sect. *Trachylobium*.

Phyllostomid bat diversity is particularly important in tropical rainforests surrounding the equator, which suggests that this diversity could be explained by environmental triggers such as warm temperatures, low seasonality and wet conditions (Stevens 2011, 2013, Alroy 2019). Furthermore, a rapid expansion of potential geographical ranges after evolving has been proposed for phyllostomid lineages (Weber et al. 2014), but with geographic gaps due to fluctuations in fruit and flower resources. These limitations, driven by rainfall and temperature seasonality, in specialists frugivorous and nectarivorous bats (Stevens 2013), are similar with nectarivorous birds (e.g., hummingbirds; McGuire et al. 2014), which share some floral attributes with nectarivorous bats (e.g., dilute nectar, large pollen grains). Nectarivorous birds and bats, have a high diversity associated with Neotropical rainforests, but they are ethologically separated (i.e., diurnal vs. nocturnal foragers). The high diversity associated to Neotropical rainforests is congruent with the geographic distribution and time of diversification of *Hymenaea* (Lee & Langemheim 1975, Mackinder 2005). The genus has its center of distribution associated to Amazonian hylaea, in which Neotropical *Trachylobium* species are restricted to the Amazonian domain, except by the disjunct distribution of *H. oblongifolia* Huber, which presents a variety restricted to the Atlantic Rainforest (Lee & Langenheim 1975). Unfortunately, the Atlantic Rainforest – a hotspot of diversity and physiographic domain of Neotropical realm – is one of the most fragmented and endangered biomes of Brazil due to intense deforestation (SOS Mata Atlântica and INPE 2016). The habitat fragmentation might affect the fitness of plant populations and pollinator guilds services (Elmqvist et al. 2003).

Based on the morphology of floral traits used to delimit the sections recognized by Lee & Langenheim (1975), the chiropterophily widely attributed to *Hymenaea* does not explain the suites of floral traits observed in the genus. Here, we predict that the bat pollinated generalization reflects a misclassification for sect. *Trachylobium*. This hypothesis is grounded in the proposition of pollination syndromes (sensu Faegri & van der Pijl), excluding *a priori* the distinct floral patterns being a result of stochastic processes (e.g., drift, environment filters). In the present study, we examined the floral traits and potential pollinators of *Hymenaea oblongifolia* var. *latifolia* Lee & Langenh. (sect. *Trachylobium*; sensu Lee & Langenheim 1975) – an endemic Atlantic Rainforest species, with records only to

Bahia and Pernambuco States, Northeastern, Brazil (Flora do Brasil 2020), in which the floral traits are distinct from the traditionally chiropterophilous species. In addition, we investigated the evolution of the pollination syndromes in the genus based on a phylogenetic framework of *Hymenaea* and related genera of the subfamily Detarioideae (LPWG 2017). In addition, we review the morphology of other *Hymenaea* species and outgroups (based on Fougère-Danezan et al. 2007, 2010, Bruneau et al. 2008) to build a matrix of floral traits used for identifying pollination syndromes. In this sense, we addressed the following questions: (1) Are the morphological sections (Lee & Langenheim 1975) phylogenetically supported? (2) What is the pollination syndrome of *Hymenaea oblongifolia* var. *latifolia* Y.T. Lee & Langenh. based on morphological and field observations? (3) Is the divergence of floral traits associated to distinct potential pollinators in the genus? (4) Are the pollination syndromes a consequence of divergent evolution driven by distinct pollinator guilds or are they a result of phylogenetic inertia?

MATERIALS AND METHODS

Hymenaea L. and focal species

The diversity of *Hymenaea* L. is typically centered in the tropical rainforest, except for *H. verrucosa*, which is distributed in Sub-Saharan Africa (Mackinder 2005, Fougère-Danezan et al. 2007, Bruneau et al. 2008, LPWG 2017). *Hymenaea* comprises 20 recognized species, occurring in several biomes, like tropical rainforests, seasonally dry tropical forests and woodlands (SDTFW), and savannas (Mackinder 2005, Fougère-Danezan et al. 2007, Bruneau et al. 2008, Souza et al. 2014, Lima & Pinto 2015, Ribeiro et al. 2015, LPWG 2017, Aguilar et al. 2018). In the first revision of the genus, *Hymenaea* was traditionally divided into two sections based on morphological criteria: (i) sect. *Hymenaea*, with short-paniculate inflorescences, large flowers, and additional mass of nectariferous tissue; and (ii) sect. *Trachylobium* with long-paniculate inflorescences, smaller flowers, without additional mass of nectariferous tissue (Lee & Langenheim 1975).

We investigated the floral biology and potential pollinators of *Hymenaea oblongifolia* var. *latifolia*, one of the only three species of the sect. *Trachylobium*, with a disjunct distribution in the Amazon and Atlantic Rainforest (Lee & Langenheim 1975), and for which no information (in the genus) over on the floral biology and pollinators is available yet. Despite the wide distribution through the ombrophilous forests in the Brazil, *H. oblongifolia* is divided into four taxonomic varieties (i.e., *H. oblongifolia* var. *oblongifolia*; *H. oblongifolia* var. *davisii* [Sandwith] Y.T. Lee & Langenh.; *H. oblongifolia* var. *latifolia*; and *H. oblongifolia* var. *palustris* [Ducke] Y.T. Lee & Langenh.), with the variety under study is restricted to the Atlantic Rainforest where it naturally occurs as a very large canopy tree (Lee & Langenheim 1975, Lima & Pinto 2015) at low population densities (0.29 ind/ha; Thomas et al. 2009). The species is 15–40 m tall with paniculate inflorescences (14–22 cm long), flowers 14–16 mm long, with a campanulate hypanthium and a stalk-like base 1–2 mm long; the four sepals are brownish, fleshy, and widely ovate; the five petals are white and spatulate, with 5–7 mm long claws; the ten stamens are 14–23 mm long, with anthers c. 3 mm long; the ovary is oblong, pubescent throughout and hirsute at the base, with a c. 2 mm long stipe at the base (Souza et al. 2016).

We studied a population located in the municipality of Ibirapitanga, Bahia State, Brazil (13°58’S, 39°28’W, at 342 m a.s.l.), within the Atlantic Forest phytogeographic domain. The southern coastal sub-region of Bahia State consists of a fragmented agricultural landscape containing cacao plantations (cabruca) and primary and secondary rainforest fragments (Landau et al. 2008) subject to a tropical climate (type Af; Alvares et al. 2013) without a dry season, with intense rainfall throughout the year and mean monthly temperatures varying between 21.6 (July) and 25.9 °C (February).

Floral biology of the focal species

Field observations of floral attributes were performed from the canopy of one tree (ca. 40 m tall, including the canopy), during three days (January 19–21, 2015), totaling 41 hours of diurnal and nocturnal observations. We conducted observations using climbing techniques (Fig. 1), to identify the exact moment of flower opening and availability of floral resources, comprising all the floral activity range. Only one plant of *H. oblongifolia* var. *latifolia* was observed due: (i) the tree size and the necessity of climbing techniques; (ii) the low population density (Thomas et al. 2009); and (iii) the asynchrony among five individuals in the studied population (i.e., only one individual with flower production during the focal observations). In addition, the aggregate floral production of *H. oblongifolia* var. *latifolia* (December to January, Souza & Funch 2016) restricted the sampling

Figure 1. ***Hymenaea oblongifolia* var. *latifolia* Lee & Langenh. in a fragmente of ombrophilous forest, Ibirapitanga, Bahia, Brazil. (a) Individual focal plant in its habitat; (b and c) Hummingbird (Hylocharis sapphrina) making contact with the anthers of *H. oblongifolia* var. *latifolia* during its visits (red arrow); (d, e and f) Sequence of anthesis of *H. oblongifolia* var. *latifolia* (floral bud at the beginning of anthesis, flower at the middle of the anthesis, and flower at end of anthesis, respectively). Red bar = 1 cm.**

effort, once January we performed the first focal observations for the species, and February the population did not present flowers anymore.

The timing and duration of anthesis and pollen availability were analyzed by observing 20 floral buds isolated in voile bags, and 20 non-isolated floral buds. Pollen grains were collected directly from the anthers of 40 flowers every two hours from 06:00 to 12:00 h. Laboratory analyses of pollen viability were performed using lactophenol (Willis 1999). Receptivity of the stigma was determined by dipping the stigmas of 40 flowers in hydrogen peroxide every two hours (from 06:00 to 12:00 h) to detect esterase activity. Nectar sugar concentrations were sampled in the field every hour, throughout the floral cycle, between 06:00 and 12:00 h, using a hand-held refractometer. The presence of osmophores (i.e., odoriferous glands – usually present in chiropterophilous flowers and absent in ornithophilous ones) was verified by immersing the flowers in 1% neutral-red solution (10 min) with subsequent washing in a 5% solution of glacial acetic acid (Dafni et al. 2005). Finally, we investigated the pigments presence with absorption ranges within the ultra-violet spectrum by maintaining the flowers in an atmosphere of ammonium hydroxide for five minutes (Scogin et al. 1977).

Flower visitors and potential pollinators of the focal species

We performed surveys of floral visitors during 3 days (from 05:00 to 22:00 on the first day, and from 04:00 to 16:00 in the second and third days) in a tree canopy by observing the harvesting of floral resources and foraging behaviors during visits. The observed visitors were ranked as: (i) potential pollinators, who performed legitimate visits; or (ii) pollen or nectar thieves, who performed illegitimate visits but collected resources without damaging the flower (Inouye 1980).

DNA extraction, amplification and sequencing

The taxonomic sampling included 13 species of *Hymenaea* (i.e., 10 species of sect. *Hymenaea* and three species of sect. *Trachylobium*) and six outgroups belonging to *Guibourtia* Benn., *Peltogyne* Vogel, and *Goniorrhachis* Taub. based on previous phylogenetic studies of Deatrioideae (Fougère-Danezan et al. 2007, 2010, Bruneau et al. 2008, de la Estrella et al. 2018).

We obtained sequences previously published in studies from the plastid *trnL* intron, *matK* gene and the nuclear ribosomal internal transcribed spacer (ITS) (Table I; Bruneau et al. 2001, 2008, Fougère-Danezan et al. 2003, 2007, de la Estrella et al. 2018) available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank). We selected sequences that could be validated by voucher specimens in herbarium. In addition, samples of a few species were obtained from specimens collected in the field (preserved in silica gel) and from herbarium material (Table I). Vouchers were deposited in the HUEFS – Herbário da Universidade Estadual de Feira de Santana.

We extracted total genomic DNA from fresh leaves (preserved in silica gel) and herbarium samples using, respectively, modified CTAB protocol of Doyle & Doyle (1987) and DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). PCR reactions were performed using the TopTaq Master Mix Kit (QIAGEN GmbH, Hilden, Germany; according to the manufacturer's protocol) for a final volume of 10 µL. For herbarium samples, PCR reactions also included 2 µL of TBT-PAR [trealose, bovine serum albumin (BSA), polysorbate-20 (Tween-20)] (Samarakoon et al. 2013), and for ITS they also included 0.4 µL of DMSO 99.5% (dimethyl sulfoxide). Amplifications were purified using PEG 11% (Paithankar & Prasad

Table I. List of taxa (*Hymenaea*, *Guibourtia* and *Peltogyne*) used in the phylogenetic inference, markers, vouchers and GenBank accesses.

	Taxa	**Phytogeographial domain**	**Habitat**	**Vouchers and GenBank acc. №**		
				matK	***trnL***	***ITS***
Ingroup	*Hymenaea altissima* Ducke	Atlantic Rainforest	wet		I.M. Souza 380	I.M. Souza 380
	Hymenaea aurea Y.T. Lee & Langenh.	Atlantic Rainforest	wet	KY046051		
	Hymenaea cangaceira R.B. Pinto, Mansano & A.M.G. Azevedo	Caatinga	dry		FJ009872	FJ009817
	Hymenaea courbaril L.	Amazon, Caatinga, Savannah, Atlantic Rainforest, Pantanal	dry/wet	EU361972	I.M. Souza 205	AY955800
	Hymenaea eriogyne Benth.	Caatinga, Savannah	dry	KX162185	AY958474	KY306587
	Hymenaea fariana R.D. Ribeiro, D.B.O.S. Cardoso & H.C. Lima	Atlantic Rainforest	wet	KT724877		
	Hymenaea martiana Hayne	Caatinga, Savannah, Atlantic Rainforest	dry/wet	KT724869	I.M. Souza 223	
	Hymenaea oblongifolia Huber	Amazon, Atlantic Rainforest	wet	EU361973	AF365161	
	Hymenaea parvifolia Huber	Amazon	wet	KY046054	AY958476	
	Hymenaea reticulata Ducke	Amazon	wet	KY046055		
	Hymenaea stigonocarpa Mart. ex Hayne	Amazon, Caatinga, Savannah, Pantanal	dry/wet	KX162186	AY958475	KY306588
	Hymenaea travassii Kuhlmann ex Paes	Chaco	dry	KT724879		
	Hymenaea verrucosa Gaertn.	-	wet	EU361974	AF365162	
Outgroup	*Goniorrhachis marginata* Taub.	Caatinga	dry	EU361959	AF365185	AY955776
	Guibourtia chodatiana (Moric.) J. Léonard	Savannah	dry	KY118208	I.M. Souza 232	AY955802
	Peltogyne confertiflora (Mart. ex Hayne) Benth.	Caatinga, Savannah, Atlantic Rainforest	dry/wet	EU362021	AF365163	AY955798
	Peltogyne floribunda (Kunth) Pittier	Amazon	wet	EU362022	AY958483	KY306637
	Peltogyne pauciflora Benth.	Caatinga	dry	KX162256	G. Costa 429	AY955799
	Peltogyne paniculata Benth.	Amazon	wet/dry	KX162255	AY958482	AY955797

1991), and then sequenced bidirectionally using the Big Dye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's protocol and using the same primers as for the amplification (Appendix 1). Sequencing products were cleaned using isopropanol 80% and ethanol 70%, and analyzed on a 3130xl Genetic Analyzer (Applied Biosystems/HITACHI, Tokyo, Japan) at the Laboratório de Sistemática Molecular de Plantas of the Universidade Estadual de Feira de Santana.

Alignment, phylogenetic reconstruction and divergence time estimation

We aligned sequences in MUSCLE using the default settings (Edgar 2004) and with manual adjustments for visual improvement in program MEGA version 10.0.4 (Kumar et al. 2018). We coded gaps as a fifth character state, and performed Bayesian analyses using MrBayes v.3.2.5 (Ronquist et al. 2012) on the concatenated data matrix (all regions). We selected the best-fit substitution models for each plastid datasets and ITS partitions based on Maximum Likelihood Criterion (MrModeltest v.2.3; Nylander 2004). The best models identified were: (i) GTR for the *matK* gene, ITS1 and ITS2; (ii) F81+G for the *trnL*-intron; and (iii) K80 for the 5.8S of the nuclear ribosomal locus.

Divergence time estimates were inferred using the concatenated data matrix for the 13 *Hymenaea* taxa and outgroups used in the phylogenetic reconstruction. To estimate divergence time we used BEAST v.1.8.2 (Drummond et al. 2012) with a GTR substitution model, an uncorrelated lognormal relaxed clock model, a tree prior with a Yule speciation model, and a random starting tree. For the analyses, two nodes were constrained. The stem node representing the Most Recent Common Ancestor (MRCA) of the clade, that includes the genera *Peltogyne*, *Guibourtia* and *Hymenaea*, was set at 46.1 Mya (normal distribution; mean = 46.0; stdev = 2.65) based on the divergence time estimated by Bruneau et al. (2008). The MRCA of the genus *Hymenaea* was calibrated at 24 Mya (lognormal prior; offset = 23.0; mean = 0; stdev = 1) based on a fossil flower preserved in amber from the Dominican Republic (Hueber & Langenheim 1986). Because this flower has morphological features of sect. *Hymenaea* and this section was reconstructed as paraphyletic (see Results), we set the crown node of the MRCA of the genus *Hymenaea* because it matches the age estimated for the same node by Bruneau et al. (2008: mean = 24.7; stdev = 1.369). Four independents Metropolis-coupled Markov Chain Monte Carlo (MCMC) were run for 10 million generations, and parameters and trees were sampled every 5000 generations. Convergence and stationarity were checked in Tracer v1.6 (Rambaut & Drummond 2013). All parameters had ESS (effective sample size) values > 200. Trees sampled after exclusion of 25% burn-in of each run were combined with LogCombiner (Rambaut & Drummond 2010a). The maximum clade credibility (MCC) tree was estimated using mean ages and 95% highest posterior density (HPD) intervals of node ages using TreeAnnotator (Rambaut & Drummond 2010b). The MCC tree was visualized and edited with FigTree version 1.4.2 (Rambaut 2014).

Evolution of pollination systems

Pollination systems (i.e., bee, bird and bat) were scored for the sampled species of *Hymenaea*, *Guibourtia*, *Peltogyne* (the closest outgroups) and *Goniorrhachis*, based on the pollination syndromes inference (morphological criteria according to Faegri & van der Pijl 1979), and confirmed by field observations for *Peltogyne pauciflora* Benth. and *Goniorrhachis marginata* Taub. (I.M. Souza, unpublished data). Despite the recognition of varieties for some species of *Hymenaea*, such as the focal species in the present study (i.e., *H. oblongifolia* var. *oblongifolia*; *H. oblongifolia* var. *davisi*.; *H. oblongifolia* var. *latifolia*; *H. oblongifolia* var. *palustris*), here we assume that missing lineages (i.e., infraspecific categories) will not affect further character reconstructions mainly due to the conserved floral pattern at species level (Lee & Langenheim 1975).

The match between the morpho-functional interpretation of pollinator mode (i.e., flower morphology, anthesis and odor) and empirical field observations of pollination mode is supported by earliest studies with *H. courbaril* and *H. stigonocarpa* (Arroyo 1981, Gibbs et al. 1999, Dunphy et al. 2004) and by our own field observations of *H. eriogyne*, *H. martiana* and *H. velutina*. We treated pollination syndromes as categorical states (standard data; Table III) and modeled using the *mk* model of Lewis (2001) as implemented by Ronquist et al. (2012), optimized onto 1,000 trees sampled at stationarity to account for phylogenetic uncertainty, and presented at the MCC tree in Mesquite v.3.40 (Maddison & Maddison 2018).

RESULTS

Floral biology of *H. oblongifolia var. latifolia*

The species has flexible pedicels and curved flowers, slightly zygomorphic. At the end of anthesis, the base of the petal remains erect, extending the hypanthium cavity, and the reproductive organs are projected forward (Fig. 1). Anthesis is diurnal, occurring from 04:00 to 07:00 h, and flowers lasted for circa 12 hours. Some buds that had not reached anthesis until c. 07:00 h remained partially opened, finishing that process at the next sunrise. The anthers became dehiscent shortly before 06:00 h and pollen grains are available until c. 11:00 h. The stigma became receptive from 06:00 h. The species showed high pollen viability (100%) during the floral cycle. No scent was perceived, and osmophores were absent. Pigments reflecting UV were observed on large sections of the sepals and petals, and at the base of the stamens and pistil. Nectar production was observed between 08:00 and 12:00 h, and the sugar concentration varied from 8% to 23% (absolute values) in the same time interval.

Floral visitors and potential pollinators of *H. oblongifolia* var. *latifolia*

The flowers were visited by hummingbirds, perching birds, bees, butterflies, and beetles (Table II). The hummingbirds *Discosura longicaudus*, *Eupetomena macroura*, and *Hylocharis sapphirina* were first observed foraging in the canopy near 06:00 h, feeding on nectar (Fig. 1), and apparently disputing territories. Their visits were principally in the morning, until around 11:00 h, and they came into direct contact with the reproductive organs (stigma and stamens) during times of pollen grain availability and stigma receptivity (between 06:00 and 07:00 h). Pollen grains were deposited on the throats of the hummingbirds due to the exserted stamens, floral opening in relation to axis of the inflorescence, and flexible pedicel. In addition, the stigmatic surface is spatially distant from the set of anthers (i.e., positive hercogamy). Several flowers were visited when the hummingbird entered in the feeding territory, and perching on a branch or leaving the tree canopy. In the afternoon period, hummingbirds were only observed making short visits to occasional flowers.

The other floral visitors did not make contact with the sexual organs of the flowers and were considered illegitimate. Bananaquit birds (*Coereba flaveola*) made intense visits during the morning hours but came into contact with the flowers only laterally (i.e., piercing the flower). *Apis mellifera* bees were observed

Table II. **Floral visitors of *Hymenaea oblongifolia* var. *latifolia* Y.T. Lee & Langenh. in a rain forest area, Ibirapitanga, Bahia State, Brazil. Flower visitor classification follows Inouye (1980) terminology.**

Groups	Order/Family/Species	Flower visitors classification
Birds	APODIFORMES	
	Trochilidae	
	Discosura longicaudus (Gmelin, 1788)	legitimate/potential pollinator
	Eupetomena macroura (Gmelin, 1788)	legitimate/potential pollinator
	Hylocharis sapphirina (Gmelin, 1788)	legitimate/potential pollinator
	PASSERIFORMES	
	Thraupidae	
	Coereba flaveola (Linnaeus, 1758)	illegitimate/nectar thieving
Insects	COLEOPTERA	
	Indet.	
	spp. indet.	illegitimate/pollen thieving
	DIPTERA	
	Indet.	
	spp. indet.	illegitimate/pollen thieving
	HYMENOPTERA	
	Apidae	
	Apis mellifera (Linnaeus, 1758)	illegitimate/pollen thieving
	Xylocopa sp.	illegitimate/nectar thieving
	LEPIDOPTERA	
	Nymphalidae	
	spp. indet.	illegitimate/nectar thieving

foraging on the flowers at around 05:00 h and during the entire day, but they only made contact with the anthers and collected pollen grains (they did not contact de stigma). Carpenter bees (*Xylocopa* sp.) made occasional visits between 10:00 and 11:00 h and were observed feeding on nectar laterally in the flowers. Butterflies were observed only in the early morning (around 07:15 h), landing on the petals and introducing their proboscis into the hypanthium cavity to feed on the nectar. Flies and small beetles collected pollen grains but they did not contact the stigma of the flower.

Floral traits and pollination syndrome evolution

The Bayesian analyses of the concatenated dataset did not support the monophyly of morphological sections (Fig. 2) and recovered two clades: (i) one major clade associated with distinct ecological preferences (Fig. 2; Table III), with chiropterophilous species from distinct habitats (i.e., dry and wet forests); and (ii) a second clade aggregating only species from wet forests, but which have distinct morphological traits and, consequently, distinct pollination syndromes (Fig. 2). The age of the most recent common ancestor of the genus (MRCA) was estimated at between 32.97 Mya (mean stem age) and 23.95 Mya (mean crown age) (Fig. 2).

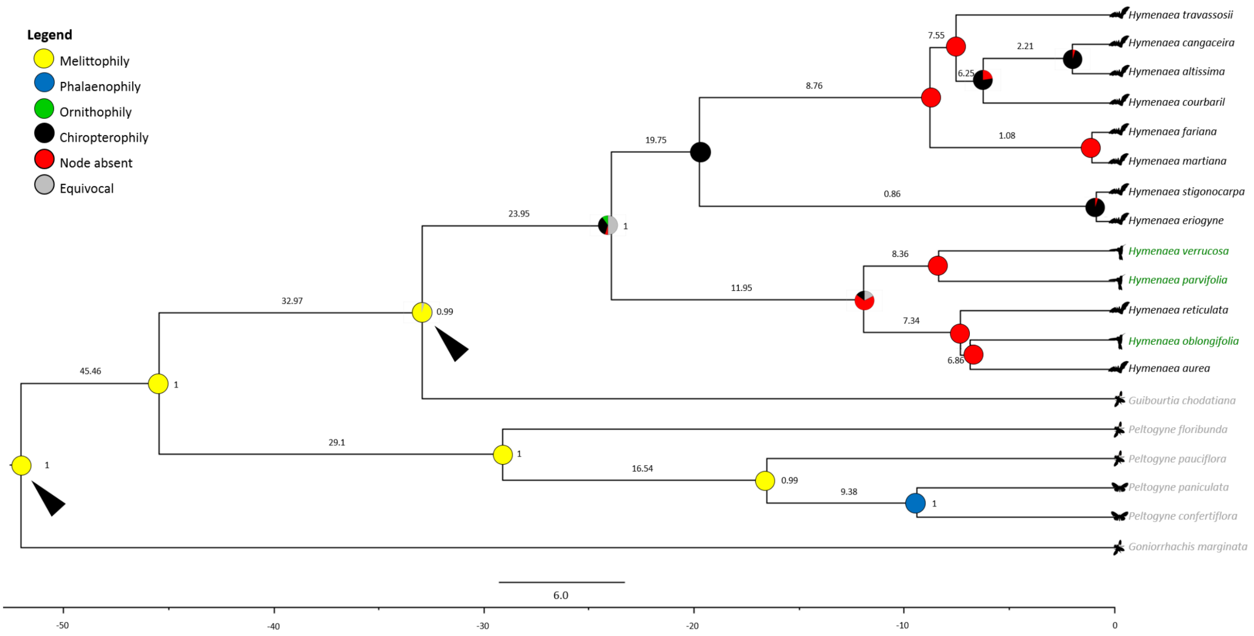

Figure 2. Phylogenetic analysis of *Hymenaea* clade: outgroups are colored in gray, *Hymenaea* sect. *Hymenaea* (sensu Lee & Langenheim 1975) is in black, and *Hymenaea* sect. *Trachylobium* (sensu Lee & Langenheim 1975) is in green. Summary of the Bayesian majority-rule consensus tree sampled, with optimization of pollination syndromes, and Maximum clade credibility tree of *Hymenaea* L. obtained from BEAST analyses. Numbers above branches are divergence time estimates. Black arrow refesr to the calibration point for the genus *Hymenaea* (Hueber & Langenheim 1986). Absent node mean an equivocal reconstruction node. Numbers on the nodes refer to the Bayesian posterior probabilities (only values > 95%).

Reconstruction of the pollination syndrome indicated that the MRCA of the genus *Hymenaea* was probably pollinated by bats (bat-pollination = 37.5%, bird-pollination = 0.4%, equivocal reconstruction = 62.1%) (Fig. 2). Species with hummingbird pollination diverged later (ca. 11.95 Mya; Fig. 2), but internal resolution within the clade is too low to determine if this type of pollination evolved once or more times. Bird-pollinated species of *Hymenaea* differs from bat-pollinated ancestors by a shift in inflorescence arrangement, in shape and size of the flower, arrangement of the stamens and pistil, degree of asymmetry, time of anthesis and loss of odor (Table III).

DISCUSSION

Hymenaea has been associated to bat pollination due to the nocturnal flowers of the majority of species studied so far (e.g., Arroyo 1981, Gibbs et al. 1999, Dunphy et al. 2004). However, we report, for the first time, diurnal anthesis and legitimate visits by hummingbirds to the flowers of one species, *Hymenaea oblongifolia* Huber, which occurs in the Atlantic Rainforest. *Hymenaea oblongifolia* is restricted to wet habitats and exhibits a disjunct distribution, occurring in the Amazonian and Atlantic Rainforests (Lee & Langenheim 1975, Lima & Pinto 2015). The species includes four taxonomic varieties, of which *Hymenaea oblongifolia* var. *latifolia* (the focal species) is endemic to the Atlantic Rainforest (sect. *Trachylobium*; Lee & Langenheim 1975). In addition, we present a preliminary analysis of the evolution of pollination syndromes in the genus *Hymenaea*, and reveal that the morphological sections proposed by Lee & Langenheim (1975) are not supported phylogenetically. Here, the "morphological sections" were reinterpreted as 'floral morphs or groups'. We suggest that divergent evolution, driven by distinct pollinator guilds, might explain the distinct floral patterns observed in the genus, therefore contradicting the phylogenetic inertia hypothesis.

Bird pollination

The floral attributes of *H. oblongifolia* var. *latifolia* do not match the typical chiropterophily syndrome, as reported for *H. courbaril*, *H. stigonocarpa* and *H. cangaceira*, all of them with robust and strongly scented flowers, with a landing platform, rigid pedicels, and nocturnal anthesis (Arroyo 1981, Gibbs et al. 1999, Domingos-Melo et al. 2019). On the other hand, *H. oblongifolia* var. *latifolia* shows several morphofunctional floral traits that match those expected for bird-pollinated species (Muchhala

Table III. Inflorescence and floral traits of *Hymenaea* from herbarium, field data and literature, used to infer about potential pollinators in the reconstruction of character state. Legend: Inflor = inflorescence; ds = dish-shaped; cs = cup-shaped; int = intermediate (10 < int < 20 mm long); lar = large (lar ≥ 20 mm long); Sym = symmetry; act = actinomorphy; szyg = slightly zygomorphy; zyg = zygomorphy; Noc = nocturnal; Diu = diurnal; pres = present; abs = absent; Pol = pollination syndrome; chi = chiropterophily; orn = ornithophily; Hab = habitat; wet = wet forest; dry = dry forest.

Species	Inflor	Pedicel	Flower shape	Flower size	Arrangement of stamens	Sym	Anthesis	Odor	Nectar	Pol	Hab
Hymenaea altissima Ducke	dense	robust	ds	lar	non-grouped	act	-	-	-	chi[1]	wet
H. aurea Y.T. Lee & Langenh.	dense	robust	ds	lar	non-grouped	act	-	-	-	chi[1]	wet
H. cangaceira R.B. Pinto, Mansano & A.M.G. Azevedo	dense	robust	ds	lar	non-grouped	act	-	-	-	chi[1]	dry
H. courbaril L.	dense	robust	ds	lar	non-grouped	act	noc	pres	diluted	chi[1,2,3]	dry
H. eriogyne Benth.	dense	robust	ds	lar	non-grouped	act	noc	pres	diluted	chi[1,3]	dry
H. fariana R.D. Ribeiro, D.B.O.S. Cardoso & H.C. Lima	dense	robust	ds	lar	non-grouped	act	-	-	-	chi[1]	wet
H. martiana Hayne	dense	robust	ds	lar	non-grouped	act	noc	pres	diluted	chi[1,3]	dry
H. oblongifolia Huber	laxa	delicate	cs	int	grouped	zyg	diu	abs	diluted	orn[1,3]	wet
H. parvifolia Huber	laxa	delicate	cs	int	grouped	zyg	-	-	-	orn[1,3]	wet
H. reticulata Ducke	dense	robust	ds	lar	non-grouped	act	-	-	-	chi[1]	wet
H. stigonocarpa Mart. ex Hyne	dense	robust	ds	lar	non-grouped	act	noc	pres	diluted	chi[1,2,3]	dry
H. travassii Kuhlm. ex L.E. Paes	dense	robust	ds	lar	non-grouped	act	-	-	-	chi[1]	dry
H. verrucosa Gaertn.	laxa	delicate	cs	int	grouped	zyg	-	-	-	orn[1]	wet

[1] Pollination syndromes based on morphological attributes (Faegri & van der Pijl 1979); [2] Pollination syndromes cited in the literature (Langenheim et al. 1973, Lee & Langenheim 1975, Arroyo 1981, Gibbs et al. 1999, Dunphy et al. 2004, Paiva & Machado 2008, Fleming et al. 2009); [3] Pollination syndromes attested by field observations of I.M. Souza (unpublished data).

2006), such as delicate and odorless flowers, without landing platform, a narrow tube aperture (composed of the hypanthium and corolla base), stamens and pistil clustered and projected beyond the perianth, flexible pedicels and diurnal anthesis.

The floral structure and architecture of *H. oblongifolia* var. *latifolia* enables different animals to illegitimately access floral rewards (i.e., without contacting simultaneously the reproductive organs). The stamens and pistil are clustered and projected beyond the perianth/corolla/petals so that insects (collecting pollen or nectar) generally do not come into contact with the anthers and stigma during their visits because of their small body sizes and foraging behaviors. This exposure of the reproductive organs (exserted pistil and stamens) requires a precise approach to the flower and for nectar collection to effect pollination, as exhibited here only by hummingbirds. The correspondence between the floral display and the attributes of the animal (e.g., behavior and body size) contributes to the plant pollination success and as has already been well documented for different plant species. For example, *Tillandsia macropetala* Wawra is a bromeliad species with bat pollinated flowers for which have been recorded o be visited by different insects, which despite accessing the floral reward, because of their small body size they did not make contact with the anthers and stigma during their visits (Aguilar-Rodríguez et al. 2014); and *Burmeistera* (Campanulaceae) has many species with bat and hummingbird pollinated flowers, and a floral architecture that limits and ensures the pollinator fidelity, but illegitimate visits by several moths have nonetheless been recorded (Muchhala 2006).

Bird-pollinated flowers do not always display all the traits typically associated with ornithophily, and hummingbirds often visit a wide spectrum of floral types while foraging for nectar (Temeles et al. 2002, Micheneau et al. 2006, Navarro et al. 2008, Las-Casas et al. 2012, Marques et al. 2015). Here, *Discosura longicaldus* (Gmelin, 1788) and *Hylocharis sapphirine* (Gmelin, 1788) (hummingbirds) were identified, for the first time, as pollinators of *H. oblongifolia* var. *latifolia* – a component of the canopy trees of a tropical rainforest. So far, only *D. longicaudus* has been reported as a floral visitor of *Parkia nitida* Miq. in the Amazonian rainforest (Hopkins 1984), but its contribution to the pollination of trees in the Atlantic Rainforest remains unexplored.

Floral adaptations related to hummingbird pollination are principally (Cronk & Ojeda 2008) supported by: (i) attraction mechanisms, often food rewards (such as the dilute nectar of *H. oblongifolia* var. *latifolia*), and (ii) specific pollination mechanisms, often related to the spatial and temporal presentation of the reproductive organs (e.g., exserted stamens and pistil). The timing of anthesis is another relevant trait for identifying potential pollinators, implying ethological isolation (Grant 1994). In *Hymenaea*, many species belonging to floral morph typical of the sect. *Hymenaea* (6 of the 17 total species) exhibit nocturnal anthesis (i.e., *H. stigonocarpa*, Gibbs et al. 1999; *H. courbaril*, Dunphy et al. 2004; *H. cangaceira*, Domingos-Melo et al. 2019; H. *eriogyne*, *H. martiana* and *H. velutina*, I.M. Souza, *personal observations*), restricting plant-pollinator interactions to nocturnal foraging animals, which when associated with other floral traits, indicate typical attributes of chiropterophily. Bat-pollination seems to be a predominant feature in the floral morph *Hymenaea*, and this has been generalized for the entire genus (Lee & Langenheim 1975, Arroyo 1981, Gibbs et al. 1999, Dunphy et al. 2004, Domingos-Melo et al. 2019).

Trachylobium group, composed only of three species (i.e., *H. oblongifolia*, *H. parvifolia* and *H. verrucose*), is morphofunctionally distinct from floral morph *Hymenaea* by presenting long-paniculate inflorescences with smaller, slightly zygomorphic and horizontally positioned flowers on flexuous pedicels, without an additional mass of nectariferous tissue (see section description proposed by Lee & Langenheim 1975). Given the strong morphological floral similarities among the species of this group, it is possible that bird pollination (reported here for *H. oblongifolia* var. *latifolia*) is a general feature of this floral morph, as well as is bat pollination for the morphological sect. *Hymenaea* (i.e., ecological signatures).

Finally, bat-pollination is typically encountered in tropical regions (Faegri & van der Pijl 1979, Cronk & Ojeda 2008), and there are many similarities between bat and bird-pollinated species (Sazima et al. 1999, Fleming et al. 2005). Nectars with low-sugar concentrations are found in both bat- and bird-pollinated species (Faegri & van der Pijl 1979, Gibbs et al. 1999). However, bird-pollinated species often exhibit the combination of diurnal anthesis and dilute nectar (with sugar concentrations varying between 20% and 26%) (Hainsworth & Wolf 1972, Baker 1975, Cruden et al. 1983, Ackermann & Weigend 2006, Cronk & Ojeda 2008) as was found here for *H. oblongifolia* var. *latifolia*.

Evolution of floral traits and pollination syndromes

Pollinators exert selective pressures on reproductive traits of plants (e.g., morphological and ecological floral attributes; Dudash et al. 2011, Temeles et al. 2013, Suzuki & Ohashi 2014, Ferreira et al. 2015), contributing indirectly to floral evolution (Shemske & Bradshaw 1999, Eaton et al. 2012, Armbruster 2014). In the present study, the MRCA of *Hymenaea* was probably bat-pollinated (i.e., approximately 24 Mya) and the bird pollination emerging later (i.e., approximately 11.95 Mya); The morphological sections (i.e., *Hymenaea* and *Trachylobium*) proposed by Lee & Langenheim (1975) were not phylogenetically supported; and the transitions in pollination syndromes were accompanied by shifts in specific floral traits (e.g., morphology of flowers and inflorescence, timing of anthesis, presence/absence of floral scent). These results reinforce the notion that distinct floral patterns observed in the genus might be a result of divergent evolution processes driven independently by distinct pollinator guilds (i.e., hummingbirds vs. nectar-feeding bats).

Plants pollinated by vertebrates share certain morphofunctional floral traits, such as dilute nectar and large pollen grains with striate-verrucate ornamentation (Banks & Rudall 2016). Different degrees of evolutionary specialization to vertebrate pollination by tropical and subtropical plants have been associated with taxonomic richness, body mass, and hovering ability as the result of the substantial adaptive radiation of tropical hummingbirds and nectar-feeding bats (Fleming & Muchhala 2008). New World specialized nectarivorous vertebrates are largely glossophagine bats (family Phyllostomidae, with c. 38 species) and hummingbirds (family Trochilidae, c. 338 species; Fleming et al. 2005, 2009, McGuire et al. 2014). Boundaries between bird and bat-pollination have been hypothesized to be related to floral morphology and reward accessibility (Muchhala 2003), or primarily to absolute nectar volumes and sugar concentrations (Ackermann & Weigend 2006). These are the results of divergent evolution processes, in which distinct pressures on floral traits were driven by distinct, ethologically isolated (i.e., diurnal hummingbirds vs. nocturnal nectar-feeding bats; Fleming et at. 2005) pollinators guilds. In addition, for the paraphyletic group composed of species considered to be bird pollinated, we might infer a process of convergent evolution driven by different birds in the geographic ranges (i.e., *H. oblongifolia* in Atlantic and Amazonian forests, *H. parvifolia* in Amazonian forests, and *H. verrucosa* in wet forests in Africa). These results are supported by a pollinator-mediated divergent evolution hypothesis.

Glossophaginae and hummingbirds radiated almost synchronously during the Early Miocene, approximately 22 Mya ago (Datzmann et al. 2010, McGuire et al. 2014) – which is highly congruent with age estimates for the appearance of bat pollination and the early diversification of *Hymenaea*. Different groups of plants (e.g., *Tiquilia* Pers., Boraginaceae, Moore & Jansen 2006, *Agave* L., Agavaceae, Good-Avila et al. 2006, *Heliotropium* sect. *Cochranea* (Miers) Kuntze, Heliotropiaceae, Luebert & Wen 2008, Cactaceae, Arakaki et al. 2011) diversified during drier and cooler global conditions after the Middle Miocene Climatic Transition that promoted the expansion of dry biomes throughout the tropics (Egan & Crandall 2008, Couvreur et al. 2011, Janssens et al. 2016).

Environmental factors may drive floral diversification among species (Koski & Ashman 2016), with selective forces acting on floral traits (e.g., color, timing of anthesis, ultraviolet pigmentation; see Schemske & Bierzychudek 2001, Warren & Mackenzie 2001, Coberly & Rausher 2003, Koski & Ashman 2016) favoring adaptation to a particular pollinator group. For example, the rainforests present low inter-annual rainfall fluctuations and a greater abundance of hummingbird species than dry habitats, implying a larger number of bird pollinated plant species (Fleming et al. 2005). Abiotic and biotic factors may also have been important in *Hymenaea*, where bat pollinated species have colonized both rainforest and seasonally dry vegetation, whereas the bird pollinated species seem to be restricted to rainforests, with its higher proportion of flower-visiting birds than seasonally dry habitats (Machado & Lopes 2004, Fleming et al. 2005, Las-Casas et al. 2012).

In conclusion, we assume that shifts in particular floral traits throughout the diversification of *Hymenaea* have implicated in the distinct floral patterns observed in genus: (i) inflorescence dense, with large and robust flowers, and nocturnal anthesis; and (ii) inflorescence laxa, with small and delicate flowers, and diurnal anthesis. These patterns are the result of divergent evolution processes, in which pollinators ethologically isolated have driven distinct pressures on floral traits. On the other hand, for the paraphyletic group composed by supposed bird pollinated species, we inferred a process of convergent evolution. These evidences are supported by pollinator-mediated flower divergent evolution hypothesis – common in different lineages of angiosperm (Valente et al. 2012, van der Niet & Johnson 2012, Schiestl & Johnson 2013, van der Niet et al. 2014). Finally, we reinforce the necessity of increasing the collection efforts on flower-pollinator interactions should be mobilized to assess the contribution of the divergent evolution in *Hymenaea* species diversity in the Neotropics.

Acknowledgments

This paper is part of the PhD Thesis of IMS in the Pos-Graduate Program in Botany of the Universidade Estadual de Feira de Santana. Constructive suggestions and questions by two anonymous referees greatly improved the final version of the manuscript. This work was sponsored by the Programa de Pesquisa em Biodiversidade do Semiárido (PPBIO), and the Sistema Nacional de Pesquisa em Biodiversidade (SISBIOTA, processes CNPq 563084/2010-3 and FAPESB PES0053/2011). This study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001: IMS was supported by a CAPES grant. FMH was supported by a CNPq-302381/2020-1 grant. LPQ was supported by a CNPq-Pq1A grant.

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Appendix 1. Primers used for PCR amplification and sequencing, and PCR conditions.

DNA region	Primer name	Primer Sequence 5'–3'	References	Pre-melting	Denaturation	PCR Conditions		
						Primer Annealing (II)	Primer Extension (III)	Cycles Final (I + II + III) Extension
ITS min)	17SE F	ACGAATTCATGGTCCGGTGAAGTGTTCG	Sun et al. (1994)	94 °C (4 min)	94 °C (1 min	53 °C (1 min)	72 °C (2 min 28 30 sec)	72 °C (7
trnL C min)	26SE R	TAGAATTCCCCGGTTCGCTCGCCGTTAC CGAAATCGGTAGACGCTACG	Sun et al. (1994) Taberlet et al. (1991)	94 °C (4 min)	92 °C (45 sec)	50 °C (45 sec)	72 °C (2 min) 35	72 °C (5
matK min)	F	ATI'TGAACTGGTGACACGAG	Taberlet et al. (1991)	94 °C (3 min)	94 °C (40 sec)	51 °C (45 sec)	72 °C (1 min) 36	72 °C (7
	trnk685F	GTATCGCACTATGTATCATTTGA	Wojciechowski et al. (2004)					
	matk4R	CATCTTTCACCCAGTAGCGAAG	Hu et al. (2000)					
	matk4La	CCTTCGATACTGGGTGAAAGAT	Wojciechowski et al. (2004)					
	matk1932R	CCAGACCGGCTTACTAATGGG	Wojciechowski et al. (2004)					
	matk1100L	TTCAGTGGTACGGAGTCAAATG	Wojciechowski et al. (2004)					
	trnK2R	CCCGGAACTAGTCGGATGG	Wojciechowski et al. (2004)					

How to cite

SOUZA IM, HUGHES FM, FUNCH LS & QUEIROZ LP. 2021. Rethinking the pollination syndromes in *Hymenaea* (Leguminosae): the role of anthesis in the diversification. An Acad Bras Cienc 93: e20191446. DOI 10.1590/0001-3765202120191446.

Manuscript received on November 25, 2019;
accepted for publication on August 31, 2020

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I.M.S. and L.P.Q. conceived of the presented idea; L.P.Q. and L.S.F. encouraged I.M.S. to investigate and supervised the findings of this work; I.M.S. developed the theory; I.M.S. and F.M.H. performed the molecular and evolution analyses. All authors discussed the results and contributed to the final manuscript.