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Evolutionary insights into the Andean genus *Nicandra* (Solanoideae, Solanaceae)

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Abstract

Nicandra (Solanaceae) is a small Andean genus, historically considered monospecific, with *N. physalodes* as its only species. This species is native to the South American Andes but naturalized in many countries worldwide. Recently, *N. john-tyleriana* and *N. yacheriana* were described using morphological evidence, which are endemic to Peru. Sequence data from four DNA markers, the plastidial intergenic spacers *trn*L-*trn*F and *ndh*F-*rpl*32, and the nuclear ITS region and GBSSI (*waxy*) gene, were used to test the identity of the nominal species using genomic evidence, to reconstruct the genus phylogenetic history through Maximum Likelihood and Bayesian Inference, and to estimate divergence times within the genus. The three *Nicandra* species formed distinct partitions and monophyletic lineages. *Nicandra physalodes*, originated in the late Miocene (ca. 8 My), was resolved as the first splitting branch, sister to the siblings *N. john-tyleriana* and *N. yacheriana*, which were originated at the end of the Miocene (ca. 5 My). The crown ages for the three species were estimated from the early Pliocene (*N. physalodes*) to the mid-Pleistocene (*N. john-tyleriana* and *N. yacheriana*), which is consistent with hypotheses of Neotropical species diversification. Open questions remain about the biogeography of the genus.

Keywords: Andes, shoo-fly plant, species delimitation

Introduction

Solanaceae, the nightshades family, encompasses a large species diversity (ca. 96 genera and ca. 2400 species; Barboza *et al.* 2016) distributed in all continents, except for Antarctica, although South America would be its center of diversity and ancestral range (Dupin *et al.* 2017). Specifically, the highest diversity of Solanaceae is found along the South American Andes and the Pacific coast up to Central America (D'Arcy 1991). A revised system for Solanaceae was recently presented by Barboza *et al.* (2016), which summarizes previous proposals (cf. Hunziker 2001, Olmstead *et al.* 2008, Särkinen *et al.* 2013) in a phylogenetic framework. Even though this work presents a comprehensive systematic scheme of the diversity of genera in Solanaceae, a number of them could not be assigned to any of the recognized subfamilies and/or tribes (i.e. *incertae sedis*). One of such genera is *Nicandra* Adanson (1763: 219), formed by three species and native to the South American Andes, which has been assigned to the Solanoideae subfamily but without any strongly defined closest affinity (Olmstead *et al.* 2008, Särkinen *et al.* 2013, Barboza *et al.* 2016). The genus is not only intriguing at the evolutionary level in the family, but it is also of biogeographical interest (cf. Dillon 2005) and has an added value due to the medicinal properties of its species. *Nicandra* was for a long time considered a monospecific genus, encompassing only *Nicandra physalodes* (Linnaeus 1753: 181) Gaertner (1791: 237), a species native to northwestern Argentina up to Ecuador (MacBride 1962, Brako & Zarucchi 1993, Dillon 2005, Carrizo García 2013, Lleellish *et al.* 2015, pers. obs.), although it has dispersed into tropical and subtropical areas worldwide (Dillon 2005). The species is well-known beyond its native range, recognized and cultivated as an ornamental (e.g. MacBride

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1962, Horton 1979, Pinto Carrasco 2019, pers. obs.), as well as for medicinal (Chen & Zhang 2019, Zhang *et al.* 2018) and nutritional uses (Thapa *et al.* 2014, Kshirsagar & Bhogaonkar 2015). The species also adapts easily to disturbed environments, such as road edges and modified fields, and therefore has also been considered a weed and treated with actions of control (Hawton 1976, Rahman 1985, Chivinge 1988). Two other species have been lately described for the genus: *N. john-tyleriana* S.Leiva & E.Pereyra (2007: 46; Leiva González & Pereyra Villanueva 2007) and *N. yacheriana* S.Leiva (2010: 26; Leiva González 2010), both endemic to Peru. There are only a few collections available for these newer species, which are respectively restricted to the Peruvian Departments La Libertad, on the northwest coast (Leiva González & Pereyra Villanueva 2007), and Ancash and Arequipa, on the northwest and south coasts (Leiva González 2010, voucher Barboza GE 4843).

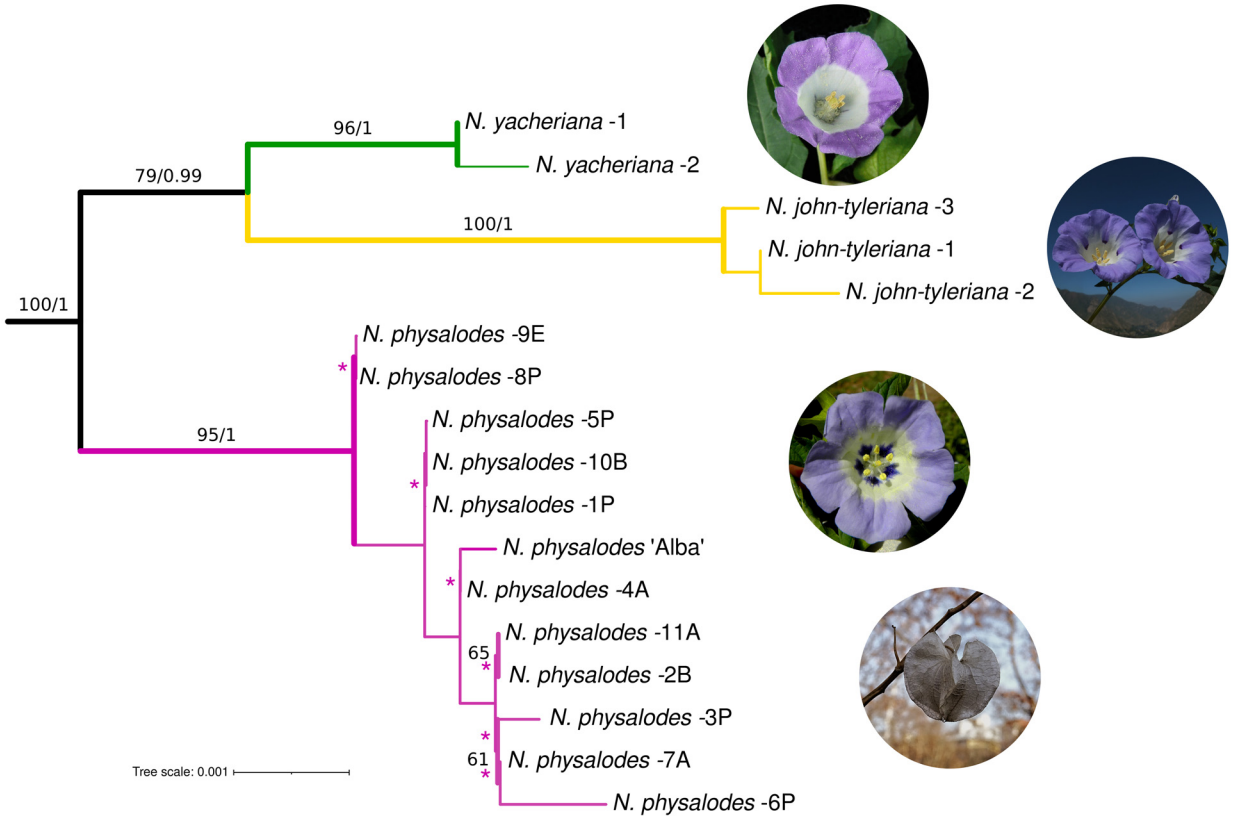

FIGURE 1. Phylogenetic reconstruction of *Nicandra*. Best-scoring maximum likelihood tree obtained from a sequence dataset of four DNA markers: *trn*L-*trn*F, *ndh*F-*rpl*32, ITS and *waxy*. Width of branches proportional to the support: widest – strong, thinnest – low, intermediate – moderate. Asterisks indicate very short branches. Branch supports above 50/0.8 (bootstrap after ML/posterior probabilities after BI) specified by branches. The outgroup was excluded (cf. Suppl. Figure 1). Samples identified as in the Suppl. Table 1. Tree scale: expected changes per site. Images illustrate flowers of *N. yacheriana* (upper), *N. john-tyleriana* (second from above) and *N. physalodes* (center), and a mature fruit of *N. physalodes* (bottom) showing the inflated accrescent (drying) calyx. Photos by S. Leiva González (*N. yacheriana* and *N. john-tyleriana*) and C. Carrizo García (*N. physalodes*).

Nicandra species are vigorous annual herbs with showy flowers, characterized by campanulate corollas, distally pale violet and white in the throat, with various patterns of spots (Figure 1). The three species develop accrescent inflated fruiting calyxes with auriculated segments, that at maturity resemble those observed in the (phylogenetically distant) genus *Physalis* Linnaeus (1753: 182) (Figure 1). The species are mainly (but not only) distinguished by differences in the pigmentation patterns inside the corolla, in the ovary and nectary colors, as well as in the calyx shape (Leiva González & Pereyra Villanueva 2007, Leiva González 2010). *Nicandra* has been repeatedly studied from a chemical perspective because its species produce withanolids with exclusive arrangements, a type of secondary metabolites with anti-inflammatory, anti-tumoral and anti-bacterial properties, among several others (Misico *et al.* 2011). A range of withanolids have been isolated from different plant organs, mainly from *N. physalodes* (e.g. Carrero *et al.* 2018, Wang *et al.* 2018, Zhang *et al.* 2018), but also from *N. john-tyleriana* (Gutiérrez Nicolás *et al.* 2015). In fact, *N. physalodes* is registered in the Catalog of Peruvian Medicinal Plants (Santiváñez Acosta *et al.* 2013). *Nicandra physalodes* is also

reputed to have insect-repellent properties, leading to its English vernacular name "shoo-fly plant" (Pinto Carrasco 2019). By contrast, given the low number of collections available for *N. yacheriana* and *N. john-tyleriana*, and the scarcity of field data in general for *Nicandra*, it has been difficult to achieve a comprehensive knowledge of the genus diversity regarding the species biology and evolution. Using DNA sequence data, we undertook the first exploration into the genus *Nicandra* diversity and evolutionary history, and also sought to provide additional evidence on the species delimitation to obtain a more integrative perspective, beyond their morphological diagnosis. To this end, the following questions were addressed: 1. Can the nominal species currently recognized for *Nicandra* be delimited using DNA sequence data? 2. How did the species diversification history developed within the genus? and 3. Which is the timeline of diversification events within *Nicandra*? It is hypothesized that the three currently recognized *Nicandra* species are distinguishable from an integrative point of view that incorporates their evolutionary history. The unified species concept proposed by De Queiroz (2007) will be used as framework, and the analyses planned will be done under the criteria of (reciprocal) monophyly as guiding line of evidence (De Queiroz 2007) to perform species-level phylogenetic inferences. This approach allows to take taxonomy beyond the naming of species, enabling to understand the processes that have shaped them (Schlick-Steiner *et al*. 2010).

Materials & methods

Sampling and data collection

Leaf material of the three *Nicandra* species was collected from wild individuals along their native ranges (Suppl. Table 1). A sample of *N. physalodes* cv. 'Alba' (which displays pure white corollas) was obtained from a specimen grown in Córdoba (Argentina) from seeds acquired in an ornamental plant market. *Lycium ciliatum* Schlechtendal (1832: 69), *L. cestroides* Schlechtendal (1832: 70) (tribe Lycieae), and *Brugmansia sanguinea* (Ruiz & Pavón 1799: 15) D.Don (1835: 272) (tribe Datureae) were included as outgroup. The outgroup species were selected based on phylogenetic reconstructions published for the family (Olmstead *et al.* 2008, Särkinen *et al.* 2013). Fresh leaves from the outgroup species *L. ciliatum* and *B. sanguinea* were also collected in the field (Suppl. Table 1). In all cases, the leaves were dried and preserved in silica-gel until further treatment. Herbarium vouchers were prepared for most samples and deposited in CORD and HAO (Suppl. Table 1).

DNA extraction, sequencing and alignment

Total genomic DNA was extracted from dried leaves using the DNeasy® Plant Mini Kit (Qiagen, California, EEUU), following the manufacturer's instructions, or employing the CTAB protocol (Doyle 1987). Four spacer regions from the chloroplast genome, *trn*L-*trn*F, *ndh*F-*rpl*32, *psb*A-*trn*H and *rpl*32-*trn*$L_{(UAG)}$, and two nuclear markers, the ITS region and the gene GBSSI (*waxy*), were PCR-amplified and sequenced. Published protocols, using the specified primers, were followed in most cases: Taberlet *et al.* (1991) for *trn*L-*trn*F, Sang *et al.* (1997) for *psb*A-*trn*H, Shaw *et al.* (2007) for *ndh*F-*rpl*32 and *rpl*32-*trn*$L_{(UAG)}$, and White *et al.* (1990) for ITS. Commercial PCR master mixes were used, and the appropriate quantities for each reagent were adjusted to comply with the manufacturer's instructions (Suppl. Data 1). The gene *waxy*, from exons 1 to 8, was amplified using the primers forward 5' and reverse 3' developed by Peralta & Spooner (2001) for tomatoes. The PCR conditions had been adjusted for the target species, which were variable across *Nicandra* (Suppl. Data 1). PCR amplicons were cleaned with enzymes, following Werle *et al.* (1994), and sequenced at Macrogen Inc. (Seoul, South Korea) or at the University of Vienna (Vienna, Austria). The plastid spacers *psb*A-*trn*H and *rpl*32-*trn*$L_{(UAG)}$ were not variable and were therefore discarded for further analyses. Sequences of the other four markers were aligned using Muscle in MEGA7 (Kumar *et al.* 2016). The final concatenated dataset consisted of 20 accessions. New sequences were obtained for all the markers used for most samples, including from the outgroup (Suppl. Table 1); only a few sequences for the outgroup species were retrieved from GenBank (Suppl. Table 1).

Species delimitation tests

The delimitation of the nominal species of *Nicandra* was tested through two different methods that use DNA barcoding datasets, both categorized as exploratory approaches that propose *de novo* species partitions (Puillandre *et al.* 2020): ASAP (Assemble Species by Automatic Partitioning; Puillandre *et al.* 2020) and GMYC (General Mixed Yule-Coalescent model; Pons *et al.* 2006). ASAP is based on pairwise genetic distances. It calculates scores for each defined partition, resulting in the lowest score being favored (Puillandre *et al.* 2020). The K80 model (Kimura, 1980) was used, as it is the standard in DNA barcoding (Ratnasingham & Hebert 2007). The samples with incomplete or missing

waxy sequences were excluded (*N. john-tyleriana* 2, *N. physalodes* 10B, 3P and 7A). The analysis was run in the web version of this tool (https://bioinfo.mnhn.fr/abi/public/asap/). GMYC uses an ultrametric phylogenetic tree as input to infer the transition point between branching events caused by speciation and allele coalescence, i.e. the transition from interspecific to intraspecific branching patterns (Bryson *et al.* 2013, Puillandre *et al.* 2020). GMYC resolves a species partition by maximizing the likelihood of that transition. The GMYC analysis was performed with the gmyc function of the splits package (Ezard *et al.* 2009) for R (R Core Team 2020; http://www.rstudio.com), setting the multi-threshold method (i.e. it relaxes the premise of a single threshold time for the transition from interspecific to intraspecific branching events). The input tree used was the maximum likelihood tree obtained in this study (see below), which was made ultrametric with the function chronos of the ape package (Paradis & Schliep 2019) in R. Given that the input for the GMYC analysis is a tree, instead of the sequence data used to build it, no samples were excluded in this case.

Phylogenetic analysis

Maximum Likelihood (ML) analyses were done in IQ-Tree v1.6.12 (Nguyen *et al.* 2015). The dataset was partitioned in four according to the individual markers (Chernomor *et al.* 2016), and the substitution model for each one was determined using ModelFinder (Kalyaanamoorthy *et al.* 2017). The best-fitting model according to the BIC values were different for each marker = partition (Table 1). Standard bootstrap analyses (BS) with 1000 replicates were performed to calculate branch supports. Bayesian Inference (BI) analyses were computed in Mr. Bayes v. 3.2.6 (Ronquist *et al.* 2012). This software allows fewer substitution models than IQ-Tree and therefore equivalents were used (Table 1). Two Markov Chain Monte Carlo (MCMC) were run, with 10 million generations (initial 25% discarded as burn-in), and sampling every 1000 generations. Effective Sampling Sizes (ESS) were evaluated using Tracer v1.7.1 (Rambaut *et al.* 2018), with a 10% burn-in; ESS > 10000 were recovered for all parameters. A consensus tree along with branches posterior probabilities (PP) was produced. The trees generated by ML and BI were visualized and annotated in FigTree v1.3.1 (Rambaut 2006–2018; http://github.com/rambaut/figtree/) and iTOL v5.7 (Letunic & Bork 2019). Branch supports are presented as BS and PP values for ML and BI trees, respectively, hereafter specified in that order.

TABLE 1. Features of the DNA markers used. Unique sites = Number of unique site patterns; informative sites = Number of parsimony-informative sites; invariable sites= Number of invariant sites.

DNA marker	Max. length (bp)	Unique sites	Informative sites	Invariable sites	Substitution model (ML)	Substitution model (BI)
*trn*L-*trn*F	819	59	25	762	TPM3+F	GTR
*ndh*F-*rpl*32	866	32	11	836	K3Pu+F	GTR
ITS	603	78	47	523	TN+F+G4	HKY
waxy	1139	141	89	1002	HKY+F	HKY

Divergence time estimation

Divergence times were estimated using BEAST2 v2.6.3 (Bouckaert *et al.* 2019). The .xml input file was compiled with BEAUti v2.6.3. The GTR substitution model, a relaxed lognormal clock and a Yule tree prior were selected. No fossil record exists for *Nicandra*, therefore two secondary calibration points were used, as calculated by Särkinen *et al.* (2013), which were set as stem ages: the most recent common ancestor (MRCA) between *N. physalodes* and *Lycium* L. (20.98 million years ago –My), and the MRCA between *N. physalodes* and the tribe Datureae (15.9 My). Four MCMC were run, with 15 million generations and sampling every 1000 generations. ESS were evaluated with Tracer v1.7.1 (25% burn-in); ESS>200 were recovered for all parameters and the four chains converged. The output tree files were combined using LogCombiner v2.6.3 (Drummond & Rambaut 2007), with the initial 25% discarded as burn-in. The combined trees were annotated using TreeAnnotator v2.6.3 (Drummond & Rambaut 2007), with 25% burn-in, and a Maximum Clade Credibility (MCC) tree was calculated. The MCC tree was visualized and edited in FigTree v1.3.1; median node ages along with the 95% highest posterior density (HPD) are presented.

Results

Sequence data

Good quality sequences ranged from ca. 600 to 1139 bp among the four DNA markers used, being the nuclear markers the shortest (ITS) and longer (*waxy*) ones (Table 1). The highest proportions of informative sites were observed in both

nuclear markers, while the plastid marker *ndh*F-*rpl*32 had the lowest (Table 1). The concatenated dataset had a total length of 3427 bp.

Species delimitation

The three nominal *Nicandra* species were supported by the two approaches followed. Under the ASAP method, the lowest score (= 1) was assigned to a single partition with three species (P-value 5.93e-02), conforming to the three nominal *Nicandra* species, which was therefore the favored one (Suppl. Data 2). In the GMYC analysis, the likelihood of the GMYC model was significantly higher than the likelihood of the null model, which postulates that all individuals belong to a single species cluster. The GMYC model returned three entities = species and the samples associated to each one matched the nominal *Nicandra* species (Suppl. Data 3).

Phylogenetic analysis and divergence time estimation

The ML and BI tree topologies were fully congruent (Figure 1). *Nicandra* formed a strong monophyletic group (100/1; Suppl. Figure 1). Two main clades were resolved within the genus (Figure 1): one formed by all *N. physalodes* samples (95/1), and the second one by the sister species *N. yacheriana* and *N. john-tyleriana* (79/0.99). The two latter species formed strong monophyletic groups (96/1 and 100/1, respectively). *Nicandra physalodes* split at the root of the genus and is sister group to the remaining pair of species. The branching resolution was poor and weakly supported between most samples of *N. physalodes*, which were resolved in a number of extremely short branches (Figure 1).

The dated phylogeny obtained using BEAST was fully congruent with the results presented above (Figure 2). The crown age of *Nicandra* (i.e. intrageneric diversification) was estimated at 8.5 My (95% HPD 3.2–13.9; HPD: highest posterior density), in the late Miocene (Neogene), being *N. physalodes* the first diverging species. The split between *N. yacheriana* and *N. john-tyleriana* was 5.6 My (95% HPD 1.7–10.1), at the end of the Miocene (Neogene period). The crown ages for the three species (i.e. intraspecific diversification) were estimated at 4.5 My (95% HPD 0.7–9.2) for *N. physalodes*, in the early Pliocene (Neogene), and 2 My (95% HPD 0.15–4.9) for *N. john-tyleriana* and 1.4 My (95% HPD 0.02–4.2) for *N. yacheriana*, both in the early Pleistocene (Quaternary period).

Discussion

The South American Andes are one of the hotspots of Solanaceae diversity, which continue to be a source of novelties, as it has been for *Nicandra*. In this opportunity, the first exploration through the species diversity of the small Andean genus *Nicandra* was undertaken, which was recently enlarged with two additional species (Leiva González & Pereyra Villanueva 2007, Leiva González 2010). DNA sequence data of four markers, that have been successfully employed to reconstruct phylogenetic histories for several genera in the family in the past (e.g. Peralta & Spooner 2001, Moré *et al.* 2015, Carrizo García *et al.* 2018), were employed. In this study, they were used not only to explore the evolutionary history of *Nicandra*, but also to build DNA barcoding datasets to test the species identities, and they have proven to be useful in both cases following a multi-locus approach. In addition, the present results are the first report documenting the monophyly of *Nicandra.* The genus has been included in Solanaceae family-wide phylogenetic studies but represented so far only for the type species, *N. physalodes* (Olmstead *et al.* 2008, Särkinen *et al.* 2013).

The unified species concept proposed by De Queiroz (2007) recognizes species as lineages of metapopulations that evolve separately as their primary defining property. In addition, other properties that may add evidence of lineage separation are considered relevant to delimit species, while multiple lines of evidence would result in more robust hypotheses of lineage separation, i.e. the existence of different species (De Queiroz 2007). Taken together, the outcome of the species delimitation tests, in which the three nominal *Nicandra* species were recovered, and the phylogenetic analyses, in which the samples assigned to each species formed three well-resolved and strongly supported monophyletic groups, the current results support that *Nicandra* consists of three distinguishable species: *N. physalodes*, *N. john-tyleriana*, and *N. yacheriana*. Historically considered to be monospecific and in general not deeply studied, it can be wondered if the two recently described *Nicandra* species could have been merely overlooked in the field (and in the herbaria) and considered atypical *N. physalodes* samples. There is actually evidence of phenotypic variability in *N. physalodes* related to variable pigmentation patterns in different organs (Darlington & Janaki-Ammal 1945, Pinto Carrasco 2019), although such variability has been recorded in cultivation and not in the wild, as is the case of the cv. ‘Alba’ used in this study.

Nicandra yacheriana was the third species described for the genus, then considered to be allied to *N. physalodes*, according to morphological features (Leiva González 2010). In contrast, the current phylogenetic results based on DNA sequence data showed that *N. yacheriana* and *N. john-tyleriana* are more closely related, while *N. physalodes* forms the first splitting branch in the genus, sister group to the latter pair. Therefore, two main branches can be identified within *Nicandra*, one formed only by the widespread *N. physalodes*, and the other one by the sister species *N. yacheriana* and *N. john-tyleriana.* The origin of the three species was dated in the late Miocene (Neogene), separated by ca. 3 million years. Unlike this, the diversification within each species, i.e. their crown ages, were estimated in different periods/epochs, being in the early Pliocene in the Neogene for *N. physalodes*, and in the early-mid Pleistocene in the Quaternary for the younger pair *N. yacheriana* and *N. john-tyleriana*. Diverse evolutionary processes have contributed to increase the biodiversity in the Neotropics, as well as to maintain the biodiversity over long periods of time (Antonelli 2011). Based on the geological periods/epochs in which the diversification of the *Nicandra* species was hypothesized, along with the geographic areas currently occupied by each one, the most suitable diversification model for the genus would be the "cradle model" (Antonelli 2011), where high speciation rates could have been triggered by the formation of the eastern portion of the Andes during the Pliocene (Hughes & Eastwood 2006) or by climatic changes during the Pleistocene (Rull 2011).

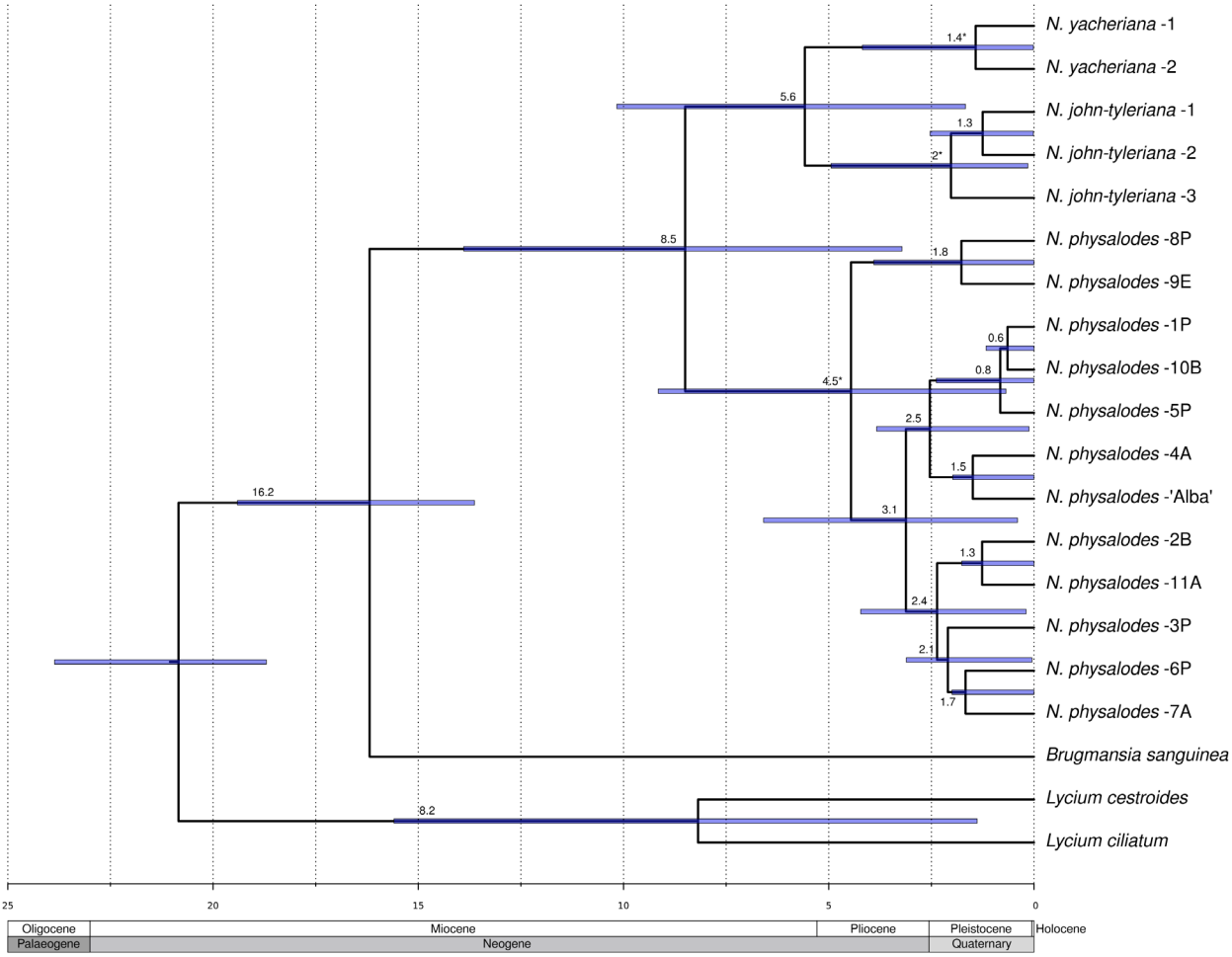

FIGURE 2. Divergence times estimated within *Nicandra* (main splits specified). Maximum clade credibility tree using a sequence dataset of four DNA markers: *trn*L-*trn*F, *ndh*F-*rpl*32, ITS and *waxy*. Species crown ages indicated by asterisks. Blue bars at nodes indicate 95% HPD. The outgroup was excluded. Samples identified as in the Suppl. Table 1.

Nicandra physalodes inhabits in a broad range of environments, such as dry (usually disturbed) soils up to 3000 masl, mountain rainforests such as the Argentinean Yungas, or the lowland *lomas* (MacBride 1962, Brako & Zarucchi 1993, Dillon 2005, Carrizo García 2013, Lleellish *et al.* 2015). *Nicandra physalodes* is one of the Solanaceae species reported in the peculiar vegetation formations called *lomas*, found in foggy valleys across the Peruvian and Atacama (Chile) deserts, which are characterized by a specialized floristic community (Dillon 2005). Given that *N. physalodes*

is hypothesized to have originated in Peru, it has been defined as a possible autodisjunt species from the Andes (Dillon 2005). This species was reported for a few northern coastal *lomas* in Peru (Dillon 2005) and later also for central Peruvian sites (Lleellish *et al.* 2015). Our own collections include a site found in more southern *lomas*, in Arequipa (sample 8P), where it was also previously reported by Leiva González (2010), although no particular attention was given to this relevant fact. Therefore, *N. physalodes* is present all along the coastal line of Peru. The native habitats of the sister species *N. john-tyleriana* and *N. yacheriana* present some differences, with *N. john-tyleriana* growing in xerophytic environments at elevations from 600 to 2450 masl, which also include *lomas* formations (Leiva González & Pereyra Villanueva 2007; voucher Barboza GE 4813), and *N. yacheriana* found only in *lomas*, around 550–600 masl (Leiva González 2010). Both sister species are, separately, sympatric with *N. physalodes*. A number of autodisjunt Solanaceae species representative of different subfamilies, such us *Browallia americana* Linnaeus (1753: 631), *Nicotiana glauca* Graham (1828: pl. 2837) and *Acnistus arborescens* (Linnaeus 1756: 10) Schlechtendal (1832: 67), have been reported in the *lomas* formations from Peru and Chile, although it remains uncertain whether they have originated from extant populations found in the adjacent Andes or they have dispersed upland from the *lomas* (Dillon 2005). Considering the available specimens collected to date together with the pattern of phylogenetic diversification recovered within *Nicandra*, a similar question can be raised for the genus as a whole. However, it is possible that gaps exist across field collections, particularly for *N. yacheriana* and *N. john-tyleriana*, as well as misidentifications of *N. physalodes* herbarium specimens, and thus this topic would need more comprehensive studies. The phylogenetic reconstructions recovered very short branches within *N. physalodes*, with no observable eco/biogeographic component in the branching topology (Figures 1 and 2). For instance, the two samples collected in the Peruvian *lomas* (8P and 3P) were not resolved as sister, while the cv. 'Alba' was resolved nested with other samples collected in the wild (Figures 1 and 2). Even if the sampling may be considered limited, the entire known native range of the three species was covered. Nevertheless, more exhaustive field collections for all three species, as well as a larger source of DNA data and deeper phylogeographic analyses may provide more information to better understand the biogeographic diversification of *Nicandra*.

Conclusion

Analyses designed to unravel the taxonomic, phylogenetic and functional components of biodiversity are fundamental frameworks for other studies, whether basic or applied. In this context, the study carried out has contributed to the knowledge on the diversity and evolution of the genus *Nicandra*, a phylogenetically isolated member of the large Solanaceae family. In addition to the morphological diagnosis, a new source of evidence was provided to achieve a more integrative approach to delimit the three *Nicandra* species. The data and results reported set a reliable phylogenetic framework (along with a first estimate of divergence ages) for other studies, such as those phytochemicals, considering that *Nicandra* species produce valuable and exclusive withanolides, or biogeographic, given the distribution pattern of all three *Nicandra* species.

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