1916 - 2026

An Acad Bras Cienc (2026) 98(2): e20250861 DOI 10.1590/0001-3765202620250861
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

CELLULAR AND MOLECULAR BIOLOGY

Tracing the Maternal Lineages and Historical Biogeography of southern South American Hollies (*Ilex*, Aquifoliaceae)

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Abstract: This study aims to clarify the evolutionary history of maternal lineages of southern South American *Ilex* taxa and their divergence times and dispersal patterns. Through comprehensive phylogenetic analyses of complete plastomes, a core phylogenetic structure was established and subsequently employed 11 plastid markers as its proxy, allowing the inclusion of species lacking plastomic data. Robust clades, consistently supported by maximum likelihood and Bayesian analyses, were identified across datasets, suggesting that the markers retain phylogenetic signal. Four distinct maternal lineages were discerned within southern South America, alongside with five from North America and seven from Asia. Notably, *Ilex paraguariensis*-the yerba mate tree- demonstrates an ancestral relationship to northern American plastid lineages during the early Eocene to early Oligocene, indicating a separate dispersal event from other South American lineages, which originated in Asia in the early Eocene and likely diversified within South America. Furthermore, findings suggest that *Ilex* chloroplast genomes originated in the late Cretaceous, earlier than previously reported, supporting a scenario of extensive Neogene radiation. The utility of plastome data in resolving hybrid origins is also validated. This study underscores the necessity for further research on taxa from South, Central, and North America to comprehensively understand *Ilex* evolution.

Key words: Bayesian molecular dating, chloroplast genomes, intergenic spacers, maximum likelihood phylogenetics.

INTRODUCTION

The genus *Ilex* L. (Aquifoliaceae Bercht. & J. Presl) encompasses species with ornamental value, prized for their hardiness and showy fruits—especially for Christmas decorations, hence the common name 'hollies'—as well as species with medicinal or nutritional properties due to secondary metabolites accumulating in their leaves (Balkrishna et al. 2024, Berté et al. 2011, Choi et al. 2005, Hao et al. 2013, Heck & de Mejia 2007, Kong et al. 2021). They are distributed across temperate and tropical regions worldwide, with the highest diversity situated in East Asia and South America; few species are found in Europe, tropical Africa, and Australia (Loizeau et al. 2005, 2016, Manen et al. 2010, Su et al. 2020, Yao et al. 2016, 2021, 2022). South America holds approximately 250–357 species records, with Brazil and Venezuela concentrating most of *Ilex* biodiversity (Galle 1997, Giberti 1995, Rodríguez 2022), whereas Uruguay represents the southernmost distribution area with only two species recorded (Grela 2004). In southern South America, approximately 12 species have been recognized and characterized through morphological and anatomical traits (Giberti 1979, 1990, 1994a, b, 1998, 2001, 2006, 2008,

Giberti & Gurni 2008, Keller & Giberti 2011). Among these, the semidomesticated crop *Ilex paraguariensis* A. St. Hil., outstands being the source of the raw material for mate infusion, a culturally significant beverage consumed in Argentina, Brazil, Paraguay, and Uruguay (Giberti 2011a, b, Heck & de Mejia 2007).

Traditional taxonomic systems have been demonstrated artificial in *Ilex* (Cuénoud et al. 2000, Gottlieb et al. 2005, Manen et al. 2002, 2010, Xu et al. 2022, 2025, Yang et al. 2023) and most key characters were shown highly homoplastic (Cascales et al. 2017, Xu et al. 2025, Yang et al. 2023). In addition, interspecific hybridization and introgressions were vastly documented in *Ilex* (Andrews 1986, Cuénoud et al. 2000, Galle 1997, Manen 2004, Manen et al. 2002, 2010, Lee et al. 2006, Setoguchi & Watanabe 2000, Shi et al. 2016, Xu et al. 2025). These processes generate reticulate evolutionary relationships, which complicate molecular phylogenetic studies by producing extensive cytonuclear discordance and inconsistencies, even with morphology-based taxonomic classifications. Nevertheless, a general association of chloroplast markers and the geographic distribution of extant *Ilex* species has been documented (Cascales et al. 2017, Gottlieb et al. 2005, Manen et al. 2002, 2010, Xu et al. 2025, Yao et al. 2021). Previous phylogenetic studies employing a limited number of chloroplast markers often yielded insufficient resolution and ambiguous placements for southern South American *Ilex* species (Cuénoud et al. 2000, Manen et al. 2002, 2010, Selbach-Snadelbach et al. 2009). More recently, the use of multiple chloroplast regions—and even complete plastomes—combined with expanded taxon sampling, has improved our understanding of maternal lineage relationships within the genus (Cascales et al. 2017, Kong et al. 2021, Su et al. 2020, Xu et al. 2022, 2025, Yao et al. 2016). Like most free-living angiosperms (Borsch & Quandt 2009), *Ilex* plastomes exhibit a highly conserved quadripartite structure and synteny (Cascales et al. 2017, Kong et al. 2021, Su et al. 2020, Xu et al. 2025, Yao et al. 2016). The suitability of uniparentally inherited chloroplast markers, over biparentally transmitted nuclear information, for reconstructing maternal lineage dispersal and relationships has been widely discussed (Rendell & Ennos 2003).

With the recent availability of complete chloroplast genomes for multiple Asian and North American *Ilex* species, this study focuses on southern South American taxa to: (1) elucidate the origins of their maternal lineages, (2) estimate divergence times, and (3) reconstruct ancestral areas through comprehensive phylogenetic analyses. Regionally occurring species are analysed within a broader taxonomic framework than prior investigations. Complete plastomes were used to establish a robust core phylogenetic structure. From this foundation, 11 plastid markers were probed as a proxy for the full plastome, enabling the inclusion of southern South American species lacking chloroplast genome sequence data. This approach advances our understanding of the evolutionary history and dispersal processes that shaped *Ilex* diversity in the southernmost region of the Neotropics.

MATERIALS AND METHODS

Analyses of chloroplast genomes

Ninety-four complete plastomes from accepted *Ilex* species, varieties, and hybrids, along with one still undetermined taxon, were downloaded from GenBank (Supplementary material Table SI). In addition, two *Helwingia* Willd. (Helwingiaceae Decne., Aquifoliales) species´ plastomes were retrieved and used exclusively for rooting purposes, given that the monophyly of *Ilex* has been largely established (Cuénoud

et al. 2000, Gottlieb et al. 2005, Manen et al. 2002, 2010, Yang et al. 2023, Yao et al. 2021). Thus, a matrix with 94 *Ilex*´s plastomes plus two *Helwingia*´s plastomes (96-terminals matrix) and another of solely *Ilex* chloroplast genomes (94-terminals matrix) were generated as follows. The large and small single copy regions (LSC and SSC, respectively) plus a single inverted repeat region (i.e., IRa) were considered to avoid the redundancy from the other identical inverted repeat (i.e., IRb). Sequences were aligned using the slow iterative refinement method (FFT-NS-i algorithm) implemented in the online version of MAFFT ver. 7, with default settings (Katoh et al. 2019).

Maximum Likelihood (ML) was the optimality criterion used, as implemented in IQ-TREE ver. 2.1.3 (Nguyen et al. 2015). Both matrices were analysed under a single model of molecular evolution, as it was shown that plastome partitioning does not significantly improved results (Souza Casadinho 2023). The substitution model was selected with ModelFinder (Kalyaanamoorthy et al. 2017) in IQ-TREE. Group support values were estimated using the ultrafast Bootstrap algorithm (UFBoot; Hoang et al. 2018) with 10,000 pseudoreplicates. The 96-terminals matrix was analysed, to establish the rooting point, with the following commands: iqtree2 -s 96file.fas -bnni -bb 10000 -nt AUTO. For the 94-terminals matrix a more thorough search strategy was accomplished. Firstly, to enhance the likelihood of achieving the global optimum, ten independent runs (runX, where X = 0 to 9) were conducted, and secondly, the taxon input order was randomly changed. The command line used is: iqtree2 -s 94file.fas -pre runX -bnni -bb 10000 -nt AUTO. The selected most suitable model was TVM+F+R for both matrices, according to the Bayesian Information Criterion (BIC; Schwarz 1978). This substitution model represents the transversion model (where AG=CT), allowing the use of unequal and empirical base frequencies (F), and a rate site heterogeneity shaped by the FreeRate (R) model (Soubrier et al. 2012), with ten categories (R10) for the 96-terminals matrix and six categories (R6) for the 94-terminals. Resulting topologies were visualized with FigTree (Rambaut 2007). To select among the ten trees derived from the 94-terminals matrix, the Shimodaira - Hasegawa test (SH; Shimodaira & Hasegawa 1999) was performed with 10,000 replicates of resampling Log-likelihoods (Kishino et al. 1990). The command line used is: iqtree2 -s 94file.fas -z runX_10t.trees -m TVM+F+R6 -zb 10000 -pre topotest.test -nt AUTO. Additionally, Robinson-Foulds paired distances (RF; Foulds & Robinson 1981) were calculated with: iqtree2 -rf_all 10t_treefile.trees -pre file_RF. For this matrix, the nucleotide diversity was evaluated with DNAsp6 Ver. 6.12.03 (Rozas et al. 2017), employing the DNApolymorphism option, a window length of 100 and a step size of 25, considering haploid chloroplast DNA without counting IUPAC coded sites and gaps.

Phylogenetic concordance between plastomic and intergenic spacers datasets_

To assess the phylogenetic concordance between results derived from the LSC+IR+SSC data (i.e., the 94-terminals matrix) and from a panel of intergenic spacer regions (IGS) with suitable variation (Cascales et al. 2017), an analysis was conducted as indicated above but in a single run and involving the set of 94 *Ilex* terminals and 11 IGS. For this, each IGS (*trnH-psbA, trnK-rps16, trnS-trnG, atpH-atpI, trnT-psbD, trnL-trnF, ndhC-trnV, atpB-rbcL, psbE-petL, ndhF-rpl32*, and *rpl32-trnL)* was extracted as an independent matrix from aligned plastomes using Geneious 7.1.7 (Biomatters, http://www.geneious.com). Then, IGS matrices were analysed simultaneously in IQ-TREE, as follows: iqtree2

-p C:\directory_route\matrices_folder -pre 94_11IGS -bb 10000 -nt AUTO. The phylogenetic concordance was assessed by comparing both topologies by inspecting them visually in FigTree and by RF distances calculation, as follows: iqtree2 -rf_all compar_treefile.trees -pre compar_RF. These analyses subsequently enabled the use of the IGS panel as a proxy for chloroplast genomes, facilitating the inclusion of species for which plastomes are currently unavailable.

Taxonomically expanded analyses with the IGS panel

One hundred nucleotide sequences of the 11 IGS panel, corresponding to 11 South and North American species and one Asian species, were retrieved from the Genbank (Table SII). Additionally, 21 sequences were newly generated from genomic DNA of three South American species obtained previously by our research group (Cascales et al. 2017) or from newly extracted DNA from herbarium specimens of three South and North American species. The latter species were *I. affinis* Gardner (voucher specimen BAF nº 15177, Museo de Farmacobotánica JA Domínguez, FB-UBA, Buenos Aires, Argentina), *I. glabra* (L.) Gray (voucher specimen BAF nº 13929) and *I. opaca* Aiton (voucher specimen SI 206906, Instituto Darwinion, San Isidro, Buenos Aires, Argentina) (Table SII). DNA extraction was done as in Cascales et al. (2017) and amplification conditions remained consistent with that study, and following the recommendations of Savolainen et al. (1995) for herbarium specimens. The sequencing was performed by Macrogen Inc. (Korea); chromatograms were inspected and edited with Bioedit (Hall 1999). Further attempts were made to amplify IGSs from the following herbarium specimens, albeit with negative results: *I. amygdalina* Reissek ex Loes. (voucher specimen BAF nº 13857), *I. chamaedryfolia* Reissek (voucher specimens BAF nº 13850 and SI 125959), and *I. coriacea* (Pursh.) Champ. (voucher specimen BAF nº 13906).

The IGS matrices were re-aligned to accommodate the additional species, using Muscle (Edgar 2004) with default settings as implemented in MEGA-X ver. 10.2.0 (Tamura et al. 2021). Then, ten independent runs were carried-out in IQ-TREE, each consisting in the simultaneous analysis of the 11 IGS matrices and 103-106 taxa (Table SI and SII), as follows: iqtree2 -p C:\directory_route\matrices_folder -pre IGS_runX -bb 10000 -nt AUTO. Resulting topologies were compared as described above, by using the SH test [iqtree2 -p C:\directory_route\matrices_folder -z IGS_10trees.treels -n 0 -zb 10000 -pre topoSH -nt AUTO] and the RF distances [iqtree2 -rf_all IGS_10trees.treels -pre RF_IGS]. Based on this information, the best run was selected, visualised in FigTree and rooted as previously determined. As well, the gene concordance factor (gCF) was estimated in IQ-TREE. This was done by using the topology of the best run as the reference tree, and by generating a topology for each IGS [iqtree2 -S C:\directory_route\matrices_folder -pre loci -nt AUTO]. All these were used in the calculation of gCFs, as follows: iqtree2 -t IGS_run_Best.treefile --gcf loci.treefile -p C:\directory_route\matrices_folder -pre GCF. Additionally, the file with topologies for each IGS (loci.treefile) was imported in SplitsTree 6.4.13 (Huson & Bryant, 2024) to graphically represent topological conflicts through a super-network (Huson et al. 2004). In the same program, a test of recombination (Phi test; Bruen et al. 2006) was carried out on the IGS dataset.

Molecular Dating

For this analysis, artificial hybrids and the unidentified specimen were excluded. The adjustment of the data to a strict or a relaxed molecular clock was accomplished through

the likelihood ratio test, as implemented in MEGA11. For this, topologies were obtained for 103 taxa based on 11 IGS sequences under the same model [GTR+G, alpha= 0.15], with and without constraints of the molecular clock. The null hypothesis of equal evolutionary rates throughout the tree (i.e., strict clock) was rejected ($P= 1.67 \times 10^{-28}$) for this data set.

Divergence times among extant chloroplast lineages were estimated in BEAST v.2.7 (Bouckaert et al. 2014, 2019) using the Birth Death process as the tree prior (which accommodates extinctions and is suitable for incomplete taxon sampling), linked sites, clock and tree models, and an Optimized Relaxed Clock model (ORC v.1.2.0; Douglas et al. 2021). The clock model assumed a lognormal distribution with default settings (mean, M= 1). The nucleotide substitution model was selected using bModeltest v.1.3.3 (Bouckaert & Drummond 2017). Four calibration points were employed. As none of the fossils of *Ilex* have ever been included in phylogenetic analyses, the alliances of *Ilex* fossils with extant species relies on paleontological studies (Walther & Kvaček 2008, Kvaček et al. 2009, Doweld 2019, 2022). Following suggestions of Parham et al. (2012) and Wikström et al. (2015), the upper bound age of the sediment in which the fossils were found (actualized with the International Chronoestratigraphic Chart v2023) was used as the minimum age constraint. For the whole group, the minimum age was set with the macrofossil endocarp of *Ilex antiquorum* Doweld (Doweld 2022) from the late Cretaceous, European Maastrichian (Germany), comprising the range 66–72.1 million years (Myr). The maximum bound was set in 86.8 Myr, based on the maximum age of the Ericales (Diapensiaceae) macrofossil (flower) *Actinocalyx bohrii* Friis (Friis, 1985) [Asen, Scania, Sweden] (Martínez-Millan 2010), dated in late Santonian-early Campanian (86.3 ± 0.5 - 83.6 ± 0.2 Myr). Thus, a uniform distribution constraint to 66-86.8 Myr was used for the whole group. For the following calibrations points, the maximum bound was set to 83.6 Myr. The leaf macrofossil of *I. opacina* Doweld from the European lower Oligocene (Rupelian; 27.82–33.9 Myr), allied to extant *I. opaca* by Doweld (2019), was used for dating the clade formed by *I. opaca, I. cassine, I. x attenuata* and *I. rubra,* applying a uniform distribution with a minimum bound of 27.82 Myr. The species *I. opaca, I. cassine* and *I. rubra* appeared closely related in nuclear phylogenies (Yang et al. 2023, Yao et al. 2021). The leaf macrofossil of *I. subrotunda* Doweld (Doweld 2019) from the Asian Middle Miocene (Utto formation, Japan; 15.98–20.44 Myr) was used for constraining the age of a strongly supported clade involving *I. rotunda,* to which is putatively related (Doweld 2019). Yet, the extant sister taxon of *I. rotunda* is not well established (Xu et al. 2025, Yang et al. 2023, Yao et al. 2021). A uniform distribution with a minimum bound of 15.98 Myr was applied. The late Miocene macrofossil of *I. protocornuta* X-C Li & B-N Sun (Li et al. 2010), dated to 5.3–11.63 Myr, was used to calibrate a clade that includes *I. cornuta,* as was done in Yao et al. (2021) but applying a uniform distribution with a minimum bound of 5.3 Myr. The analyses were performed twice for 50 million generations, sampling every 5000 generations. Convergence was assessed using Tracer v.1.7.2 (Rambaut et al. 2018), verifying that effective sample sizes exceeded 200 for all statistics. Results were combined using LogCombiner (Drummond & Rambaut 2007); a burn-in of 10% was applied to each run. Post burn-in trees and their parameters were summarized to generate a maximum clade credibility (mcc) chronogram using TreeAnnotator v.1.8.0 (Drummond et al. 2012), when an additional 20% burn-in was applied. Thus, 5600 topologies were discarded in total. Divergence times estimates with 95% highest posterior density (HPD) intervals, and

posterior probabilities (PP) for each node were visualized using FigTree.

Ancestral Area Reconstructions

To generate reconstructions of ancestral areas, the Statistical Dispersal-Extinction Cladogenesis analysis (S-DEC; Beaulieu et al. 2013, Ree & Smith 2008, Yu et al. 2015) and the Statistical Dispersal-Vicariance Analysis (S-DIVA, Yu et al. 2010) were used, as implemented in RASP4 (Yu et al. 2020). Four geographic areas were defined (A = Asia, B = South America, C = North America, D = Europe). Each species was assigned to a single region based on current distributions as cited in published data (Tables SI and SII), maximum ancestral areas were established as two and the probability of dispersal between areas was set to one. Analyses were conducted using 100 trees randomly taken from 1000 post burn-in trees derived from the Bayesian dating. The mcc chronogram was used to plot reconstructed areas.

RESULTS

The alignment of complete plastomes (LSC+IRa+SSC) for the 96-terminals matrix reached a length of 136182 bp. Of these, 125974 sites (92.5%) are conserved. The two *Helwingia* plastomes show differences solely in 143 sites but did not form a clade (Figure S1). Subsequent analyses were rooted according to this placement of the root.

Alignment of the 94-terminals matrix (solely *Ilex* terminals and LSC+IRa+SSC data) reached a length of 133218 bp, of which 128096 sites (96.16%) are conserved, 5122 are variable, 2483 are parsimony-informative, and 2639 singletons. The G+C content is 37%. The average nucleotide diversity is Pi= 0.0031; the LSC region (up to position 88590) has Pi =0.0036, the SSC (between 115350-133218) has Pi=0.0046, and the intervening inverted repeat region show Pi=0.0006 (Figure S2). The ten independent ML runs yielded phylograms with no visual topological differences. Both the SH test (Table SIII) and the zero RF distances obtained (not shown) confirm convergence. Therefore, the topology from Run 2 was selected based on its lowest BIC value (Table SIII). In the most likely phylogram four majors strongly supported clades are considered (bootstrap support value, BSV ≥95%; Figure 1a, Figure S1). Only ten nodes show BSV <80%. Clade I encompasses most maternal lineages of American species plus Asian *I. nitidissima* (100% BSV). The clade II consists of chloroplastidial genomes of 34 Asian species plus Eurasian *I. aquifolium;* this clade is distinguished by very short branches. Clade III, comprising plastomes of 21 Asian taxa, exhibits comparatively longer branches and near-complete resolution. Clade IV (95% BSV) is composed of lineages of 28 Asian and two American taxa (*I. decidua* and *I. verticillata*). The plastome of *I. decidua* is highly similar with that of Japanese *I. serrata* (p-distance= 0.00007). Indeed, the representatives of *I. chapaensis* and *I. asprella* var. *tapuensis* possess the most similar plastomes (p-distance= 0.00004), whereas those of *I. dumosa* and *I. purpurea* exhibit the greatest differences (p-distance= 0.00589).

The alignment of the panel of 11 IGS for the set of 94 *Ilex* taxa reached a total of 9742 bp, of which 91.68% are constant sites, and shows no missing data (Table SIV). In the resultant phylogram, the four same clades are clearly recognized (Figure S3). The RF distance between the multilocus IGS phylogram and that derived from the complete plastome (Figure 1b) is 180, being the main differences based on shorter branches and lesser resolution in clades with already short branches, that now appear collapsed (e.g., clade II), as clade composition

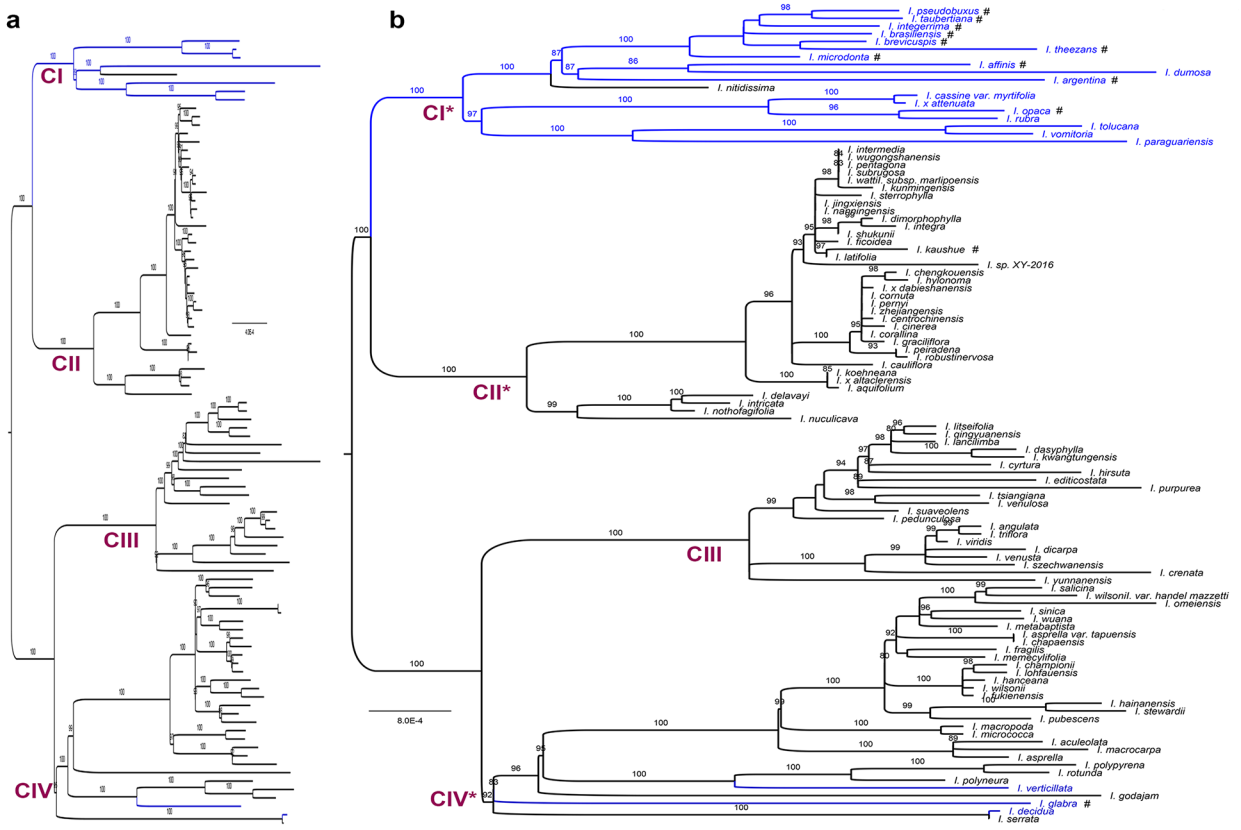


Figure 1. Maximum likelihood phylograms of *Ilex* species´ maternal lineages. a. Rooted phylogram for the chloroplastidial genome dataset (LSC+IRa+SSC) of 94 extant taxa (LogL = -240644.9188). b. Rooted phylogram derived from the panel of 11 IGS regions and 106 taxa (LogL = -22752.1143). Both phylograms were rooted in accordance with the outgroup-rooted phylogram in Figure S1. American taxa are shown in blue, Asian and Eurasian taxa, in black. Solely support values ≥80% are displayed. Clades (denoted as CI, CII, CIII and CIV) are described in the text; the asterisk denotes the inclusion of taxa in comparison with 1a, these taxa are denoted with a #. The scale bar represents substitutions per site.

remains the same. As well, 27 nodes show BSV <80%.

The inclusion of IGS data from nine southern South American, two North American and an Asian species resulted in matrices with 103–106 terminals (Tables SII and SV); globally, the missing data reach 1.22% and 90.4% of constant sites. The SH test found no significant differences among the topologies from the ten independent runs thus, run 3 is selected based on its lower BIC value (Table SVI). The RF distances globally ranged between 18–38, and the topology of run 3 shows RF distances between 22–34 against the others. Nevertheless, visual inspection shows no considerable topological differences. The alternative relationships displayed by ML topologies from each IGS, are graphically condensed by a super-network (Figure S4). The Phi test failed to find recombination signals (P= 0.3894). Selected phylogram (run 3) shows 28 nodes with BSV <80%, and that all maternal lineages from southern South American taxa plus *I. opaca* (from North America) relate to those of clade I, here referred to as clade I* (Figure 1b). This clade shows complete support (100% BSV) but a low gCF (36.4%), this means that four IGS support that branch while somehow six contradicts it (Table SV). The maternal lineages

of *I. pseudobuxus, I. taubertiana, I. integerrima, I. brasiliensis, I. brevicuspis, I. theezans,* and *I. microdonta* form a fully supported clade, but only three IGS favouring it (27.3% gCF); while some internal relationships are less supported (50–77% BSV; 0-36.4% gCF), that of *I. pseudobuxus* and *I. taubertiana* lineages received high support (98% BSV), but low gCF (45.5%). The lineage of *I. affinis* appears as sister to that of *I. dumosa* (86% BSV; 50% gCF), and these appear as sister to the lineage of *I. argentina* (87% BSV; 9% gCF). The latter lineage showed an unclear phylogenetic position (Table SV). All these taxa have complete IGS occupancy, except *I. affinis* and *I. theezans* that have 91% occupancy (Table SII). The sister plastid lineage to the afore mentioned southern South America taxa, is *I. nitidissima* with 100% BSV and 45.6% gCF. *Ilex paraguariensis* appears sharing a common maternal ancestor with North and Central American taxa (*I. vomitoria* and *I. tolucana)* with 100% BSV and 54.6% gCF; whereas *I. opaca* (with 55% of IGS occupancy) and *I. rubra* form a highly supported clade (96% BSV; 66.7% gCF). The maternal lineages of *I. x attenuata* and *I. cassine* (91% gCF) appear as sister clade of the latter (63.6% gCF). Plastid data of *I. kaushue* locate this terminal as sister to *I. latifolia* (97% BSV) in clade II* (100% BSV, 63.6% gCF). Clade III (100% BSV, 72.7% gCF) shows identical lineage composition as in Figure 1a. Instead, clade IV* (92% BSV, 18.2% gCF) incorporates the maternal lineage of North American *I. glabra* (with 64% of IGS occupancy), with 83% BSV and 28.6% gCF (Figure 1b). This clade also includes the lineages of *I. decidua* and *I. verticillata* (North America), each sharing a most recent common ancestral lineage with Asian representatives than to each other, as in Figure 1a. All members of clade IV* share a 116 bp synapomorphic deletion within IGS *trnK-rps16* (not shown).

The Bayesian mcc chronogram (Figure 2) is topologically congruent with that of the ML phylogram (Figure 1b). Molecular dating estimates divergence of the ancestral plastome of clade I* (node 171) at around 60.8 million years ago (Mya) during the Paleocene (Table I). Biogeographic reconstructions (S-DEC and S-DIVA) indicate that the ancestral plastid lineage originated in either Asia + South America or Asia + North America. The chloroplast genome of the common ancestor of *I. nitidissima* and southern South American species (excluding *I. paraguariensis*) diverged at approximately 46.9 Mya (95% HPD: 35.78–58.42; PP=1) in the early Eocene, with Asia + South America as the ancestral area. Some divergences within southern South American subclades are not reliable (PP=0.49); however, the ancestral plastid lineage of *I. affinis* and *I. dumosa* split in the region around 31.78 Mya (95% HPD: 20.21–42.54; early Oligocene; PP=1) and that of *I. pseudobuxus, I. taubertiana, I. integerrima, I. brasiliensis, I. brevicuspis, I. theezans,* and *I. microdonta,* ca. 25 Mya (95% HPD: 16.68–33.68; late Oligocene; PP=1). After the divergence of the lineage *I. microdonta*, the ancestral plastid lineage of the remaining species radiated around 20 Mya (95% HPD: 13.18–27.11; early Miocene; PP=0.95), while that of *I. brevicuspis* and *I. theezans* occurred at 12.5 Mya (95% HPD: 7.03-18.8; middle Miocene; PP=1), and *I. pseudobuxus* and *I. taubertiana* diverged more recently at 9.63 Mya (95% HPD: 4.61-15.15; late Miocene; PP=1). The ancestral plastome of North American taxa plus *I. paraguariensis* (node 170; PP=0.84) dates in the late Paleocene (ca. 57.3 Mya), with its ancestral area inferred as North America + South America (S-DEC), or solely North America (S-DIVA) (Table I). Particularly, the ancestral plastome of *I. paraguariensis, I. tolucana* and *I. vomitoria* diverged around 42.39 Mya (95% HPD: 30.26–54.58; PP=1) during early Eocene to early Oligocene. The other northern American lineage conformed by *I. cassine, I x attenuata, I. rubra* and *I. opaca,* diverged later in

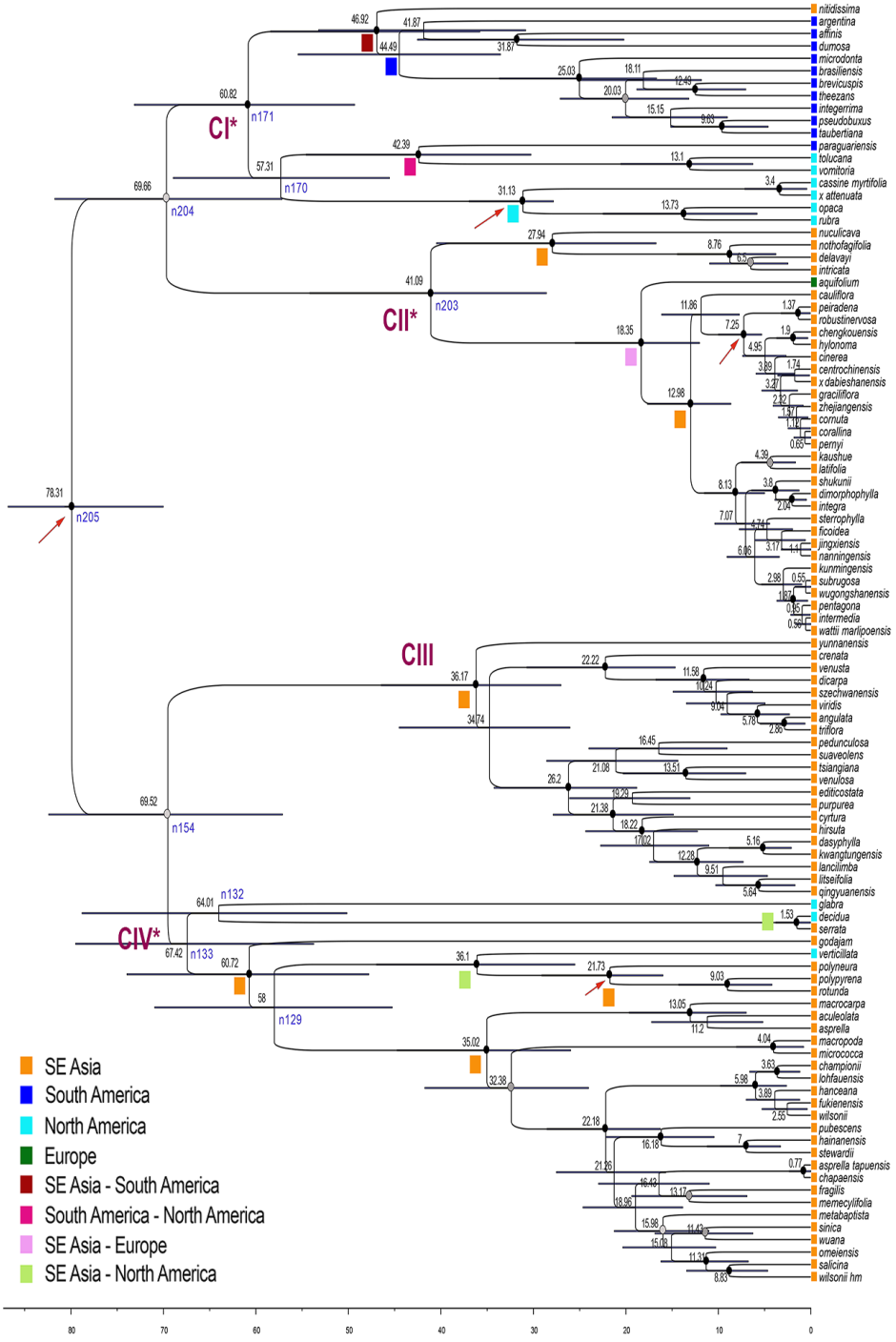


Figure 2. Time calibrated tree for maternal lineages of 103 extant *Ilex* taxa. Numbers to the left of each node are the estimated ages in million years (Myr). Node bars give the 95% highest posterior density for the node age. Black dots indicate posterior probabilities (PP) between 0.95-1; grey dots, PP= 0.90-0.94; light grey dots, PP= 0.85-0.89. Posterior probabilities are average values estimated from post burn-in trees. Nodes with ambiguously reconstructed ancestral areas are indicated in blue font and detailed in Table II; otherwise, reconstructed ancestral areas are depicted with coloured squares. The scale at the bottom represents millions of years before present; the arrows mark calibrated nodes (see materials and methods). Clades (denoted as CI, CII, CIII and CIV) are described in the text; the asterisk denotes the inclusion of taxa in comparison with Figure 1a.

late Eocene-Oligocene (ca. 31.13 Mya; 95% HPD: 27.82–36.96; PP=1). Clade II* (node 203; PP=1) is dated to about 41 Mya (late Eocene), with Southeast Asia as the most likely ancestral area. The split of clades III–IV* (node 154; PP=0.87) occurred ca. 69.5 Mya, with Asia as the ancestral area. Clade III dates to around 36.2 Mya (95% HPD: 25.97–44.75, upper Eocene; PP=1), in Southeast Asia. Clade IV* (node 133) radiated in the late Cretaceous (ca. 67.42 Mya), however its credibility is low (PP=0.62). Likewise, node 132 (PP=0.32) is estimated to split in the Paleocene (64 Mya), with S-DEC reconstructing the ancestral area as Asia + North America, while S-DIVA suggests either southeast Asia + North America or solely North America with equal probability. This node encompasses the two distinct American plastid lineages of *I. glabra* and *I. decidua*, plus Asian *I. decidua*. The divergence of chloropastidial lineage of American *I. verticillata* and Asian *I. polypyrena, I. polyneura* and *I. rotunda* (PP=1) was estimated in the late Eocene (ca. 36 Mya; 95% HPD: 25.51–46.97) with southeast Asia + North America as the most likely ancestral area. The chronogram reveals diversification peaks in the Neogene (with 47 events in the Miocene and 14 in the Pliocene), followed by the Paleogene (with 20 events: six during Oligocene, nine in the Eocene and five in the Paleocene), the Quaternary (with 16 events) and three splits in the late Cretaceous (Figure 2).

DISCUSSION

The present study identifies that at least seven main maternal lineages are discernible in Asia, five in North America, four in southern South America, and a single in Eurasia among specimens considered. Regarding southern South American taxa, Manen et al. (2002) proposed two plastid lineages based on three markers, whereas Manen et al. (2010), using the same markers but broader taxon sampling, identified three cytoplasmic lineages in the region. Under a more restricted taxonomical scheme but using multilocus markers, Cascales et al. (2017) also proposed the occurrence of three maternal lineages in the region. Grounded on multilocus plastid markers and expanded taxonomic sampling, present findings indicate that *I. brasiliensis, I. brevicuspis, I. integerrima, I. microdonta, I. pseudobuxus, I. taubertiana* and *I.*

Table I. Results of the ancestral area reconstruction algorithms (S-DEC and S-DIVA) based on plastid data, for the nodes numbered in Figure 2. The most likely ancestral area is depicted in boldface. A= Southeast Asia, B= South America, C= North America, and D= Europe.

Node	PP [a]	Age range (95% HPD) (Myr)	S-DEC Ancestral areas [b] (%)	S-DEC Marginal probability	S-DIVA Ancestral areas [b] (%)	S-DIVA Marginal probability
129	0.67	45.26—70.96	**A (81.01)** AC (18.45)	0.79	**A (100)**	1
132	0.32	50.15—78.8	**AC (81.57)** C (11.61)	0.82	**AC (50.00) C (50.00)**	0.5
133	0.62	53.73—79.5	**A (57.79)** AC (41,34)	0.38	**A (64.08)** AC (35.92)	0.32
154	0.87	57.11—82.41	**A (68.24)** AC (31.02)	0.39	**A (72.31)** AC (27.69)	0.46
170	0.84	45.55—68.94	**BC (89.88)**	0.67	**C (69.14)** BC (30.86)	0.69
171	1	49.32—73.15	**AB (83.93)** AC (14.64)	0.75	**AC (66.49)** BC (31.94)	0.46
203	1	28.58—54.18	**A (80.38)** AD (18.74)	0.8	**A (100)**	1
204	0.89	57.15—81.75	**AB (85.30)**	0.58	**A (46.10)** AC (27.14) AB (26.77)	0.31
205	1	70.01—86.8	**AB (83.56)** AC (14.06)	0.49	**A (45.10)** AC (30.41) AB (21.79)	0.15

[a]posterior probabilities; [b]solely proportions >10% are presented.

theezans, conformed one such maternal lineage (L1). The second southern South American plastid lineage (L2) is represented by *I. argentina,* while the third lineage (L3) involves *I. dumosa* and *I. affinis*. The fourth maternal lineage (L4) involves *I. paraguariensis*. Species of L1 are predominantly from the Brazilian Paranaense biogeographic province, except for *I. brevicuspis* and *I. theezans* which are also found in the Paranaense province that reaches the lowlands of northeastern Argentina (Giberti 1994a, b, 1995). *Ilex argentina* thrives in the sub-Andean rainforests of the Yungas phytogeographic province in northwestern Argentina and in Bolivia, between 900-2300 m.a.s.l. (Giberti 1994a, 2006), and it is the only verified polyploid species (2n= 4*x* = 80) in southern South America (Garberoglio et al. 2023, Gottlieb et al. 2005, Greizerstein et al. 2004). These features set it apart, and potentially reproductively as well. Furthermore, present outcomes showing its alternative phylogenetic relationships with other southern South American or Asian taxa in the IGS panel analysis, support *I. argentina* as a distinct maternal lineage (L2). A clearer phylogenetic picture should emerge when multilocus or complete chloroplast genome data from other Andean species become available. Nuclear information displays *I. argentina* as sister taxa of the seven species from L1 (Manen et al. 2010, Yang et al. 2023), supporting its distinction. The inclusion of multilocus plastid data gathered here from an herbarium specimen of *I. affinis* helped clarifying not only the position of its own cytoplasmic lineage, but also that of *I. dumosa*. Differing from prior results (Cascales et al. 2017), this study suggests that *I. dumosa* and *I. affinis* may represent another maternal lineage (L3) distinct from *I. argentina*. Both species exhibit overlapping distributions in northeastern Argentina, Brazil, and Paraguay and resemble morphologically (De Egea Elsam et al. 2018, Keller & Giberti 2011). Although only half of the IGSs support this relationship, it is further corroborated by Yang et al. (2023) ´s taxon-rich nuclear marker analysis, which groups them with other South American species forming a sister clade to Asian taxa with strong support. Noteworthy, the southern South American cytoplasmic lineages (L1-L3) share a most recent common maternal ancestor with that of Asian *I. nitidissima*. The nuclear-based clade XIV in Yang et al. (2023) depicts *I. brasiliensis, I. brevicuspis, I. integerrima, I. microdonta, I. pseudobuxus, I. theezans,* and *I. argentina* related with *I. nitidissima,* in partial agreement with current results. Although Xu et al. (2025) did not include South American representatives, their chloroplast-based phylogenetic tree places *I. nitidissima* in a sister clade to North American species, a relationship not recovered in their SNP-based analysis. Whether this represent another example of cyto-nuclear discordance caused by recent hybridization/introgression events and/or incomplete lineage sorting in the genus (Manen et al. 2010, Xu et al. 2025), remains to be elucidated. For *I. paraguariensis,* present analyses reveal that its distinct cytoplasmic lineage (L4) shows clear phylogenetic connections to North American lineages. This agrees with results from nuclear data (Manen et al. 2020, Yang et al. 2023, Yao et al. 2021), which reinforce current proposal. Chloroplastidic data, either partial or complete, also suggested such relationship (Manen et al. 2020, Xu et al. 2022). Particularly, *I. paraguariensis* and northern American *I. vomitoria* formed a robust clade when their plastomes where jointly analysed (Xu et al. 2022). *Ilex vomitoria* involves a species complex with intermediate forms (Clark 2023) and is distinguished by its use as a high-caffeine beverage (Wainwright & Putz 2014). This feature is shared with *I. paraguariensis* and *I. guayusa* Loes., an interesting Amazon Andean species

(Barriera 2023) whose phylogenetic allies are utterly unknown. Even though herbarium specimen processing yielded multilocus plastid data for only two North American taxa, one (*I. glabra*) was associated with Asian maternal lineages and the other (*I. opaca*) with American maternal lineages. Among the eight North American *Ilex* that could be examined, the five plastid lineages identified suggest recurrent dispersals between Asia and North America, consistent with the patterns inferred by Yang et al. (2023) from nuclear markers.

Traditional taxonomic studies of southern South American species have been conducted over the years (Giberti 1979, 1990, 1994a, b, 1998, 2001, 2006, 2008, Giberti & Gurni 2008). Subsequent research on phytochemical compounds (Filip et al. 2001), metabolomics (Choi et al. 2005, Kim et al. 2010), AFLP fingerprinting and ITS phylogenetics (Gottlieb et al. 2005), cytogenetics (Greizerstein et al. 2004), proteinaceous secretions (González & Tarrago 2009), seed dormancy (Galíndez et al. 2018), and genome sizes (Garberoglio et al. 2023) revealed groupings that partially overlap with one another and with the plastid lineages postulated in this study. These inconsistencies emphasise (a) the need for assessing intraspecific variation, and (b) the necessity of reliable diagnostic characters for delimiting monophyletic taxa to characterize the continent´s remarkable *Ilex* diversity. Character mapping analyses underscored extensive homoplasy in total-evidence approaches or across chloroplast, nuclear data or SNP-based phylogenetic reconstructions (Cascales et al. 2017, Xu et al. 2025, Yang et al. 2023).

In addition to the Asian origin for southern South American lineages L1-L3, the present study suggests that they likely diversified within the subcontinent between the early Oligocene and late Miocene. Remarkably, the most relevant *Ilex* species in the region, the yerba mate tree -*I. paraguariensis*-, reveals a distinct ancestral link to North American maternal lineages during the early Eocene to early Oligocene which ultimately trace back to Asia (in the Paleocene). Ancestral area reconstructions were inconclusive for that subclade (node 170; Figure 2), but because from the early Jurassic until the emergence of the Isthmus of Panama in the late Pliocene (around 3.5 Mya; Burnham & Graham 1999) North America was isolated from South America (Ezcurra & Agnolín 2012), the most plausible ancestral area would be North America. Overall, these results suggest that at least two separate dispersal events have shaped current *Ilex* diversity in the Southern Cone of South America. One event originating in Southeast Asia, gave rise to the common ancestor of L1-L3 lineages. The other, from North America to South America, involving the ancestor of L4 lineage. Further investigations with broader taxon representation are anticipated to validate these hypotheses.

The genus *Ilex* is considered globally distributed based on the pollen records from the late Cretaceous Australian deposits (Barreda et al. 2019, Fernández et al. 2021, Loizeau et al. 2005, 2016, Manen et al. 2002) and further corroborated by macrofossils (Doweld 2022). Molecular dating by Manen et al. (2010) suggested a Miocene-Eocene origin for the crown clade of *Ilex* in Eastern Asia or North America. The authors attributed the discrepancy with macrofossils and pollen records to the extinction of various sister lineages. Yao et al. (2021) proposed the origin of *Ilex* in the early Eocene in subtropical Asia and multiple bidirectional dispersals to South America, North America, Southwest China (since the Paleogene), among other regions, without ruling out the possibility of extinctions. Even though Manen et al. (2010) and Yao et al. (2021) used identical nuclear markers, their methodological choices (e.g., molecular clocks, tree priors, calibration points, and distribution

functions) differ significantly influencing their results. Present findings are based on a set of explicit choices that yielded agreement with fossil records, proposing that maternal lineages of extant *Ilex* species diverged during the late Cretaceous, and that they underwent extensive radiation during the Neogene (particularly, the Miocene). Biogeographical analyses performed here revealed some inconsistencies with the predicted continental drift patterns (Scotese 2004). For instance, the dates estimated for the splitting of the whole group as well as for clades I* and II*, are only compatible with Southeast Asia being the most feasible ancestral area for the most recent common ancestral plastid lineage. During the estimated time span (49–82 Mya), Asia and North America were connected through Laurasia or Asiamerica, from which South America remained isolated (Ezcurra & Agnolín 2012). For the same reasons, the most likely ancestral area for the clade I* would be Asia and/or North America, excluding South America as a possibility. Fossil remains showing fruit consumption by birds from the early Cretaceous suggest their key role as early seed dispersers (Hu et al. 2022). The morphophysiological dormancy of *Ilex* pyrenes (Galíndez et al. 2018, Tsang & Corlett 2005) may represent an adaptation for long-distance endozoochorous dispersal by birds (Giberti 2001, Loizeau et al. 2005). Being dioecious plants, successful dispersal requires the establishment of both sexes. However, facultative apomixis has been suggested in *I. aquifolium* (Obeso 1996) and *I. paraguariensis* (Klaus et al. 2024). This raises the likelihood for single-plant colonization (Clark, 2023), enhancing long-distance dispersal possibilities, as those proposed in Yao et al. (2021) and herein.

Until 2018, there were solely nine chloroplast genomes published from Chinese and southern South American species of *Ilex* (Cascales et al. 2017, Yao et al. 2016). Afterwards, plastomes for Asian species were released at an incremental pace (Chen et al. 2019, Kong et al. 2021, Park et al. 2019a, b, Shan et al. 2020, Su et al. 2020, Xu et al. 2022, 2025, among others). These studies encountered synteny and low diversity even among disjunct taxa and showed partial discordance with nuclear-based phylogenies and, as well, with traditional classification systems (Xu et al. 2025, for a review). The present study includes 84 representatives from the ten clades obtained by Xu et al. (2025) for Asian *Ilex*, and highlights the notable underrepresentation of American species, considering that South America is a major centre of biodiversity (Barriera 2023, Rodríguez 2022). Despite this disparity, the four principal clades identified here are recovered regardless of the dataset (i.e., full plastome or the IGS set) and the analytical method employed (i.e., ML or Bayesian inference). The datasets produced consistent topologies, suggesting that the IGS panel retained phylogenetic signal, despite constituting only 7.3% of the plastome length (excluding the IRb). The implementation of super-network graphics, which show both topological conflicts and consistencies, combined with gene concordance factor (gCF) estimation, enabled a detailed dataset analysis and revealed insights beyond simple bifurcating topologies. Even though low gCF values were observed for certain phylogenetic relationships using the IGS markers, several of those relationships received high support from the complete plastome dataset. This suggests that some reasonable information loss occurs within the IGS panel. Therefore, the employment of multiple IGS regions as a pragmatic proxy for the complete plastome, represents a balance between reducing information content and the economically challenging NGS sequencing.

Weak reproductive barriers in *Ilex* have led to hybrid species and horticultural varieties (Galle

1997, Lee et al. 2006, Manen et al. 2002, 2010, Setoguchi & Watanabe 2000, Shi et al. 2016, Yao et al. 2022). Herein, four hybrids were included to attempt recognizing the female parental through the chloroplastidial data. Thus, the natural hybrid *I. x attenuata* 'Sunny Foster' shows its chloroplast genome allied to that of *I. cassine*, as in Manen et al. (2010). For *I.* x *dabieshanensis*, *I. cornuta* appears as the maternal donor, as was depicted in Xu et al. (2022) and Zhou et al. (2022). Nonetheless, asymmetric bidirectional hybridizations between the parental species *I. cornuta* and *I. latifolia* have been proposed (Shi et al. 2016). Artificial hybrid *I. x koehneana* '*Win. L. Winn*', was previously linked to *I. latifolia* based on plastome data (Chong et al. 2021). With both parental lineages included herein, *I. aquifolium* is established as the maternal donor of this cultivar. Andrews (1986) suggested that *I. aquifolium* contributed the pollen in the first cross and subsequent backcrosses with *I. perado*; these crosses gave rise to *I. x altaclerensis* 'Belgica Aurea' and other cultivars (Yao et al. 2022). Present findings indicate that this hybrid´s plastome is closely related to that of *I. aquifolium*, contradicting Andrews´ (1986) proposal. These results further highlight the practical utility of plastomic data analyses.

This study focused on elucidating phylogenetic relationships among maternal lineages of southern South American *Ilex* through an expanded taxonomic context, aiming to clarify their biogeographic origins, divergence timing, and ancestral distributions. While efforts to enhance species representation using herbarium materials proved only partially successful, our comprehensive analytical framework has successfully addressed key evolutionary questions.

Acknowledgments

I am a member of the Researcher Career of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. I would like to express my gratitude to all the teaching and non-teaching staff who support the daily activities of the FCEN-UBA, even during these challenging times for scientific endeavours. I extend my thanks to the dedicated personnel of the BAF Herbarium, housed at the Museo de Farmacobotánica JA Dominguez (FB-UBA, Buenos Aires, Argentina) and of the SI Herbarium, housed at the Instituto Darwinion (San Isidro, Buenos Aires, Argentina), and to Dra. MS Rodriguero and anonymous reviewers who help improving this manuscript. I gratefully acknowledge past grants from the Universidad de Buenos Aires (UBACYT 20020170100614BA) and the Agencia Nacional de Promoción Científica y Técnica (BID PICT 2020-serie A- 1978).

REFERENCES

ANDREWS S. 1986. Clones of *Ilex* × *altaclerensis*. I International Symposium on Taxonomy of Cultivated Plants 182: 377-380.

BALKRISHNA A, RAI P, VERMA R, ROHELA A, KUMAR A, ARYA V, KHAN N, AMAROWICZ R, KUMAR D & VERMA R. 2024. Mechanistic insight into anti-inflammatory potential, phytochemistry and ethnomedicinal status of *Ilex* species: A review. Phytochem Rev: https://doi.org/10.1007/s11101-024-09968-2.

BARREDA VD, PALAZZESI L & OLIVERO EB. 2019. When flowering plants ruled Antarctica: Evidence from Cretaceous pollen grains. New Phytol 223: 1023-1030.

BARRIERA G. 2023. Taxonomy and nomenclature of South American Aquifoliaceae I: *Ilex* from Ecuador with the description of two new taxa. Candollea 78: 53-78. http://dx.doi.org/10.15553/c2023v781a6.

BEAULIEU JM, TANK DC & DONOGHUE MJ. 2013. A Southern Hemisphere origin for campanulid angiosperms, with traces of the break-up of Gondwana. BMC Evol Biol 13: 80-97.

BERTÉ KAS, BEUX MR & SPADA PKWDS. 2011. Chemical composition and antioxidant activity of yerba-mate (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) extract as obtained by spray drying. J Agric Food Chem 59: 5523-5527.

BORSCH T & QUANDT D. 2009. Mutational dynamics and phylogenetic utility of noncoding chloroplast DNA. Pl Syst Evol 282: 169-199.

BOUCKAERT R ET AL. 2019. BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. PLoS Comp Biol 15: e1006650. https://doi.org/10.1371/journal.pcbi.1006650.

BOUCKAERT R, HELED J, KÜHNERT D, VAUGHAN T, WU CH, XIE D, SUCHARD MA, RAMBAUT A & DRUMMOND AJ. 2014. BEAST 2: A software platform for Bayesian evolutionary analysis. PLoS Comp Biol 10: e1003537. https://doi.org/10.1371/journal.pcbi.1003537.

BOUCKAERT RR & DRUMMOND AJ. 2017. bModelTest: Bayesian phylogenetic site model averaging and model comparison. BMC Evol Biol 17: 1-11.

BRUEN TC, PHILLIPE H & BRYANT D. 2006. A simple and robust statistical test for detecting the presence of recombination. Genetics 172: 2665-2681. https://doi.org/10.1534/genetics.105.048975.

BURNHAM RJ & GRAHAM A. 1999. The history of neotropical vegetation: new developments and status. Ann Miss Bot Gar 86: 546-589.

CASCALES J, BRACCO M, GARBEROGLIO MJ, POGGIO L & GOTTLIEB AM. 2017. Integral phylogenomic approach over *Ilex* L. species from southern South America. Life 7: 47. https://doi.org/10.3390/life7040047.

CHEN Y, CHEN HX, LI HL, CHENG XL & WANG LY. 2019. Complete plastome sequence of *Ilex asprella* (Hooker and Arnott) Champion ex Bentham (Aquifoliaceae), a Chinese folk herbal medicine. Mit DNA Part B 4: 2341-2342.

CHOI YH, SERTIC S, KIM HK, WILSON EG, MICHOPOULOSA F, LEFEBER AWM, ERKELENS C, KRICUN SDP & VERPOORTE R. 2005. Classification of *Ilex* species based on metabolomic fingerprinting using nuclear magnetic resonance and multivariate data analysis. J Agric Food Chem 53: 1237-1245.

CHONG X, LI Y, ZHOU Y, ZHOU Y, CHEN H, LI X, LI N, LU X & ZHANG F. 2021. The complete chloroplast genome sequence and phylogenetic analysis of *Ilex* × *koehneana* 'Wirt L. Winn' (Aquifoliaceae). Mit DNA Part B 6: 796-797. https://doi.org/10.1080/23802359.2021.1882904.

CLARK RC. 2023. Hollies (*Aquifoliaceae*) of North America north of Mexico. *Phytoneuron* 13: 1-19.

CUÉNOUD P, DEL PERO MARTÍNEZ MA, LOIZEAU PA, SPICHIGER R, ANDREWS S & MANEN JF. 2000. Molecular phylogeny and biogeography of the genus *Ilex* L. (Aquifoliaceae). Ann Bot 85: 111-122.

DE EGEA ELSAM J, CÉSPEDES G, PEÑA-CHOCARRO M, MERELES F & ROLÓN C. 2018. Recursos Fitogenéticos del Paraguay: Sinopsis, Atlas y Estado de Conservación de los Parientes Silvestres de Especies de Importancia para la Alimentación y la Agricultura (Parte I). Rojasiana 4: 1-228.

DOUGLAS J, ZHANG R & BOUCKAERT R. 2021. Adaptive dating and fast proposals: Revisiting the phylogenetic relaxed clock model. PLoS Comp Biol 17: e1008322. https://doi.org/10.1371/journal.pcbi.1008322.

DOWELD AB. 2019. New names for *Ilex* and *Ilexpollenites* (Aquifoliaceae), extant and fossil. Notulae Systematicae ad Palaeofloram Europaeam spectantes II. Phytotaxa 388: 179. https://doi.org/10.11646/phytotaxa.388.2.5.

DOWELD AB. 2022. New names of *Ilex* and *Ilexpollenites* (Aquifoliaceae), extant and fossil: Addendum Notulae Systematicae ad Palaeofloram Europaeam spectantes II. Phytotaxa 531: 143-146. https://doi.org/10.11646/phytotaxa.531.2.7.

DRUMMOND AJ & RAMBAUT A. 2007. BEAST: Bayesian Evolutionary Analysis by Sampling Trees. BMC Evol Biol 7: 214. https://doi.org/10.1186/1471-2148-7-214.

EDGAR RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nuc Acids Res 32: 1792-1797. https://doi.org/10.1093/nar/gkh340.

EZCURRA MD & AGNOLÍN FL. 2012. A new global palaeobiogeographical model for the Late Mesozoic and Early Tertiary. Syst Biol 61: 553-566.

FILIP R, LÓPEZ P, GIBERTI GC, COUSSIO J & FERRARO G. 2001. Phenolic compounds in seven South American *Ilex* species. Fitoterapia 72: 774-778.

FOULDS LR & ROBINSON DF. 1981. Comparison of phylogenetic trees. Math Biosc 53: 131-147. https://doi.org/10.1016/00255564(81)900432.

GALÍNDEZ G, CECCATO D, BUBILO R, LINDOW-LÓPEZ L, MALAGRINA G, ORTEGA-BAES P & BASKIN CC. 2018. Three levels of simple morphophysiological dormancy in seeds of *Ilex* (Aquifoliaceae) species from Argentina. Seed Sc Res 28: 131-139.

GALLE FC. 1997. *Hollies: The Genus Ilex*. Holly Society of America. Timber Press, Portland, Oregon, 536 p.

GARBEROGLIO MJ, GONZÁLEZ GE, KRYVENKI MA & GOTTLIEB AM. 2023. Genome size variation of southern South American species of *Ilex* (Aquifoliaceae). Darwiniana 11: 167-179.

GIBERTI GC. 1979. Las especies argentinas del género *Ilex* L. (Aquifoliaceae). Darwiniana 22: 217-240.

GIBERTI GC. 1990. Clave de las especies argentinas del género *Ilex* (Aquifoliaceae). Bol Soc Argent Bot 26: 159-162.

GIBERTI GC. 1994a. Aquifoliaceae. In: Flora Fanerogámica de Argentina, Fascículo 1 (157). Programa PROFLORA (CONICET), Museo Botánico, IMBIV, Córdoba, Argentina, 8 p.

GIBERTI GC. 1994b. Aquifoliaceae. In: Flora del Paraguay, vol. 24. Spichiger R & Ramella L (Dirs.). Ed Conser Jard Bot Ville de Geneva - Miss Bot Gar, Switzerland, 34 p.

GIBERTI GC. 1995. *Ilex* en Sudamérica: florística, sistemática y potencialidades con relación a un banco de germoplasma para la yerba mate. In: Erva-mate: biologia e cultura no Cone Sul. Editora da Universidade /UFRGS, 303-312 p.

GIBERTI GC. 1998. Hallazgo de *Ilex brasiliensis* (Aquifoliaceae) en la Argentina. Bol Soc Argent Bot 33: 137-140.

GIBERTI GC. 2001. Diferentes aspectos del género *Ilex* (Aquifoliaceae). Corología, arquitectura floral, posición sistemática. Tesis. Doctorado en Farmacobotánica y Farmacognesia. FFyB - UBA.

GIBERTI GC. 2006. Notas en Aquifoliaceae Sudamericanas I: *Ilex herzogii* sinónimo de *Ilex argentina*. Kurtziana 32: 53-54.

GIBERTI GC. 2008. Aquifoliaceae. in Catálogo de las Plantas vasculares del Cono Sur (Argentina, Sur de Brasil, Chile, Paraguay y Uruguay), Vol. 2. Dicotyledoneae: Acanthaceae-Fabaceae (Abarema - Schizolobium). In: Zuloaga FO, Morrone O & Belgrano MJ (Eds), Monographs in Systematic Botany, Missouri Botanical Garden, 1143-1146 p.

GIBERTI GC. 2011a. La "yerba mate" (*Ilex paraguariensis*, Aquifoliaceae) en tempranos escritos rioplatenses de Bonpland y su real distribución geográfica en Sudamérica austral. Bonplandia 20: 203-212.

GIBERTI GC. 2011b. South American *Ilex* raw materials for regional infusions: Advanced arboreal crop systems versus wild forest extractive production. In: Filip R (Ed), Multidisciplinary Approaches on Food Science and Nutrition for the XXI Century, Kerala, India, 17-27 p.

GIBERTI GC & GURNI AA. 2008. Anatomía floral comparada de once especies sudamericanas de *Ilex* L. (Aquifoliaceae) relacionadas con la yerba mate. Dominguezia 24: 77-94.

GONZÁLEZ AM & TARRAGÓ J. 2009. Anatomical structure and secretion compounds of colleters in nine *Ilex* species (Aquifoliaceae) from southern South America. Bot J Linn Soc 160: 197-210.

GOTTLIEB AM, GIBERTI GC & POGGIO L. 2005. Molecular analyses of the genus *Ilex* (Aquifoliaceae) in southern South America, evidence from AFLP and ITS sequence data. Amer J Bot 92: 352-369. https://doi.org/10.3732/ajb.92.2.352.

GREIZERSTEIN EJ, GIBERTI GC & POGGIO L. 2004. Cytogenetic studies of southern South American *Ilex* species. Caryologia 57: 19-23. https://doi.org/10.1080/00087114.2004.10589367.

GRELA IA. 2004. Geografía florística de las especies arbóreas de Uruguay: propuesta para la delimitación de dendrofloras. MSc Thesis, Programa de Desarrollo de las Ciencias Básicas, Ministerio de Educación y Cultura, Universidad de la República, Montevideo, Uruguay. (Unpublished).

HALL TA. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nuc Acids Symp Series 41: 95-98.

HAO D, GU X, XIAO PG, LIANG Z, XU L & PENG Y. 2013. Research progress in the phytochemistry and biology of *Ilex* pharmaceutical resources. Acta Pharm Sinica B 3: 8-19. https://doi.org/10.1016/j.apsb.2012.12.008.

HECK CI & DE MEJIA EG. 2007. Yerba Mate Tea (*Ilex paraguariensis*): A comprehensive review on chemistry, health implications, and technological considerations. J Food Sci 72: R13851. https://doi.org/10.1111/j.1750-3841.2007.00535.x.

HOANG DT, CHERNOMOR O, VON HAESELER A, MINH BQ & VINH LS. 2018. UFBoot2: Improving the ultrafast bootstrap approximation. Mol Biol Evol 35: 518-522. https://doi.org/10.1093/molbev/msx281.

HU H, WANG Y, MCDONALD PG & WROE SW. 2022. Earliest evidence for fruit consumption and potential seed dispersal by birds. eLife 11: e74751. https://doi.org/10.7554/eLife.74751.

HUSON DH & BRYANT D. 2024. The SplitsTree App: Interactive analysis and visualization using phylogenetic trees and networks. Nature Meth 21: 1773-1774. doi: 10.1038/s41592-024-02406-3.

HUSON DH, DEZULIAN T, KLOEPPER T & STEEL MA. 2004. Phylogenetic super-networks from partial trees. In: Jonassen I & Kim J (Eds), Algorithms in Bioinformatics. WABI 2004. Lecture Notes in Computer Science, vol 3240. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-30219-3_33.

KALYAANAMOORTHY S, MINH BQ, WONG TKF, VON HAESELER A & JERMIIN LS. 2017. ModelFinder: Fast model selection for accurate phylogenetic estimates. Nature Meth 14: 587-589. https://doi.org/10.1038/nmeth.4285.

KELLER HA & GIBERTI GC. 2011. Primer registro para la flora Argentina de *Ilex affinis* (Aquifoliaceae), sustituto de la "yerba mate". Bol Soc Arg Bot 46: 187-194.

KIM HK, KHAN S, WILSON EG, KRICUN SDP, MEISSNER A, GORALER S & VERPOORTE R. 2010. Metabolic classification of South American *Ilex* species by NMR-based metabolomics. Phytochemistry 71: 773-784.

KISHINO H, MIYATA T & HASEGAWA M. 1990. Maximum likelihood inference of protein phylogeny and the origin of chloroplasts. J Mol Evol 31: 151-160. https://doi.org/10.1007/BF02109483.

KLAUS MVV ET AL. 2024. Apomictic seed formation and viability in *Ilex paraguariensis* A. St.-Hil. (Aquifoliaceae). Cien Natura Santa Maria 46: e84934.

KONG B, PARK H, LAU T, LIN Z, YANG T & SHAW P. 2021. Comparative analysis and phylogenetic investigation of Hong Kong *Ilex* chloroplast genomes. Nature 11: 5153. https://doi.org/10.1038/s41598-021-84705-9.

KVAČEK Z, TEODORIDIS V & WANG Q. 2009. *Ilex geissertii* sp. n. (Aquifoliaceae), a fossil ancestor of *Ilex* sect. *Ilex* in the upper Miocene and Pliocene of Europe. Rev Palaeobot Palynol 157: 192-210.

LEE NS, YEAU SH & PARK JO. 2006. Molecular evidence for hybridization of *Ilex* × *wandoensis* (Aquifoliaceae) by RAPD analysis. J Pl Biol 49: 491-497. https://doi.org/10.1007/BF03031131.

LI X, SUN B, XIAO L, WU J & LIN Z. 2010. Leaf macrofossils of *Ilex protocornuta* sp. nov. (Aquifoliaceae) from the Late Miocene of East China: Implications for palaeoecology. Rev Palaeobot Palynol 161: 87-103.

LOIZEAU P, BARRIERA G, MANEN JF & BROENNIMANN O. 2005. Towards an understanding of the distribution of *Ilex* L. (Aquifoliaceae) on a worldwide scale. Biol Skrifter 55.

LOIZEAU PA, SAVOLAINEN V, ANDREWS S & SPICHIGER R. 2016. Aquifoliaceae. In: Kadereit JW & Bittrich V (Eds), Flowering plants. Eudicots, the families and genera of vascular plants 14. Springer International Publishing, Basel, Switzerland, p. 31-36.

MANEN JF. 2004. Are both sympatric species *Ilex perado* and *Ilex canariensis* secretly hybridizing? Indication from nuclear markers collected in Tenerife. BMC Evol Biol 4: 46. https://doi.org/10.1186/1471-2148-4-46.

MANEN JF, BARRIERA G, LOIZEAU PA & NACIRI-GRAVEN Y. 2010. The history of extant *Ilex* species (Aquifoliaceae): Evidence of hybridization within a Miocene radiation. Mol Phyl Evol 57: 96-177. https://doi.org/10.1016/j.ympev.2010.09.006.

MANEN JF, BOULTER MC & NACIRI-GRAVEN Y. 2002. The complex history of the genus *Ilex* L. (Aquifoliaceae): Evidence from the comparison of plastid and nuclear DNA sequences and from fossil data. Pl Syst Evol 235: 79-98.

MARTÍNEZ-MILLÁN M. 2010. Fossil record and age of the Asteridae. Bot Rev 76: 83-135. https://doi.org/10.1007/s12229-010-9040-1.

NGUYEN LT, SCHMIDT HA, VON HAESELER A & MINH BQ. 2015. IQTREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Mol Biol Evol 32: 268-274. https://doi.org/10.1093/molbev/msu300.

OBESO JR. 1996. Producción de frutos y semillas en *Ilex aquifolium* L. (Aquifoliaceae). An J Bot Madrid 54: 533-539.

PARHAM JF ET AL. 2012. Best practices for justifying fossil calibrations. Syst Biol 61: 346-359. https://doi.org/10.1093/sysbio/syr107.

PARK J, KIM Y, KWON W, NAM S & XI H. 2019a. The complete chloroplast genome of Nepal holly, *Ilex integra* Thunb. (Aquifoliaceae). Mit DNA Part B 4: 1257-1258.

PARK J, KIM Y, NAM S, KWON W & XI H. 2019b. The complete chloroplast genome of horned holly, *Ilex cornuta* Lindl. and Paxton (Aquifoliaceae). Mit DNA Part B 4: 1275-1276.

RAMBAUT A. 2007. FigTree. Institute of Evolutionary Biology, University of Edinburgh. Available at: http://tree.bio.ed.ac.uk/.

RAMBAUT A, DRUMMOND AJ, XIE D, BAELE G & SUCHARD MA. 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Syst Biol 67: 901-904. https://doi.org/10.1093/sysbio/syy032.

REE RH & SMITH SA. 2008. Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. Syst Biol 57: 4-14.

RENDELL S & ENNOS RA. 2003. Chloroplast DNA diversity of the dioecious European tree *Ilex aquifolium* L. (English holly). Mol Ecol 12: 2681-2688. https://doi.org/10.1046/j.1365-294X.2003.01934.x.

RODRÍGUEZ A. 2022. Una nueva especie de *Ilex* L. (Aquifoliaceae) de Costa Rica. Phytoneuron 63: 1-9.

ROZAS J, FERRER-MATA A, SÁNCHEZ-DELBARRIO JC, GUIRAO-RICO S, LIBRADO P, RAMOS-ONSINS SE & SÁNCHEZ-GRACIA A. 2017. DnaSP v6: DNA sequence polymorphism analysis of large datasets. Mol Biol Evol 34: 3299-3302.

SAVOLAINEN V, CUÉNOUD P, SPICHIGER R, MARTÍNEZ MDP, CREVECOEUR M & MANEN JF. 1995. The use of herbarium specimens in DNA phylogenetics: Evaluation and improvement. Pl Syst Evol 197: 87-98.

SCHWARZ GE. 1978. Estimating the dimension of a model. Ann Stat 6: 461-464. https://doi.org/10.1214/aos/1176344136.

SCOTESE CR. 2004. A continental drift flipbook. J Geol 112: 729-741.

SELBACH-SCHNADELBACH A, SMITH CAVALLI S, MANEN JF, COELHO GC & DE SOUZA-CHIES TT. 2009. New information for *Ilex* phylogenetics based on the plastid *psbA-trnH* intergenic spacer (Aquifoliaceae). Bot J Linn Soc 159: 182-193.

SETOGUCHI H & WATANABE H. 2000. Intersectional gene flow between insular endemics of *Ilex* (Aquifoliaceae) on the Bonin Islands and the Ryukyu Islands. Am J Bot 87: 793-810.

SHAN Z, ZHOU B, LI Y, LIU D, LI W, MA J & SU T. 2020. Complete chloroplast genome sequence and phylogenetic analysis of *Ilex viridis* Champ. ex Benth. Mit DNA B Resour 5: 914-915. https://doi.org/10.1080/23802359.2020.17199139.

SHI L, LI N, WANG S, ZHOU Y, HUANG W, YANG Y, MA Y & ZHOU R. 2016. Molecular Evidence for the Hybrid Origin of *Ilex dabieshanensis* (Aquifoliaceae). PLoS One 25: e0147825. https://doi.org/10.1371/journal.pone.0147825.

SHIMODAIRA H & HASEGAWA M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol Biol Evol 16: 11-14. https://doi.org/10.1093/oxfordjournals.molbev.a026201.

SOUBRIER J, STEEL M, LEE MSY, SARKISSIAN CD, GUINDON S, HO SYW & COOPER A. 2012. The influence of rate heterogeneity among sites on the time dependence of molecular rates. Mol Biol Evol 9: 345-3358. https://doi.org/10.1093/molbev/mss140.

SOUZA CASADINHO A. 2023. Análisis filogenómico de linajes maternos de *Ilex paraguariensis* y otras especies co-genéricas. Tesis de grado. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. (Unpublished).

SU T, ZHANG M, SHAN Z, LI X, ZHOU B, WU H & HAN M. 2020. Comparative survey of morphological variations and plastid genome sequencing reveals phylogenetic divergence between four endemic *Ilex* species. Forests 11: 964. https://doi.org/10.3390/f11090964.

TAMURA K, STECHER G & KUMAR S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis version 11. Mol Biol Evol 38: 3022-3027. https://doi.org/10.1093/molbev/msab120.

TSANG AC & CORLETT RT. 2005. Reproductive biology of the *Ilex* species (Aquifoliaceae) in Hong Kong, China. Botany 83: 1645-1654.

WALTHER H & KVAČEK Z. 2008. Die Gattung *Ilex* L. (Aquifoliaceae) im Paläogen von Mitteleuropa. Feddes Repert 119(3-4): 172-190. https://doi.org/10.1002/fedr.200811160.

WAINWRIGHT AE & PUTZ FE. 2014. A misleading name reduces marketability of a healthful and stimulating natural product: A comparative taste test of infusions of a native Florida holly (*Ilex vomitoria*) and yerba mate (*I. paraguariensis*). Econ Bot 68: 350-354.

WIKSTRÖM N, KAINULAINEN K, RAZAFIMANDIMBISON SG, SMEDMARK JEE & BREMER B. 2015. A revised time tree of the Asterids: Establishing a temporal framework for evolutionary studies of the coffee family (Rubiaceae). PLoS One 10: e0126690. https://doi.org/10.1371/journal.pone.0126690.

XU K, LIN C, LEE S, MAO L & MENG K. 2022. Comparative analysis of complete *Ilex* (Aquifoliaceae) chloroplast genomes: Insights into evolutionary dynamics and phylogenetic relationships. BMC Genomics 23: 203. https://doi.org/10.1186/s12864022083979.

XU K, YANG Y, CHEN H, LIN CX, JIANG L, GUO ZL, LI M, HAO MZ & MENG K. 2025. Extensive cytonuclear discordance revealed by phylogenomic analyses suggests complex evolutionary history in the holly genus *Ilex* (Aquifoliaceae). Mol Phyl Evol 204: 108255. https://doi.org/10.1016/j.ympev.2024.108255.

YANG Y, JIANG L, LIU ED, LIU WL, CHEN L, KOU YX, FAN DM, CHENG SM, ZHANG ZY & PENG H. 2023. Time to update the sectional classification of *Ilex* (Aquifoliaceae): New insights from *Ilex* phylogeny, morphology, and distribution. J Syst Evol 61: 1036-1046.

YAO X, SONG Y, YANG JB, TAN YH & CORLETT RT. 2021. Phylogeny and biogeography of the hollies (*Ilex* L., Aquifoliaceae). J Syst Evol 59: 73-82 https://doi.org/10.1111/jse.12567.

YAO X, TAN YH, LIU YY, SONG Y, YANG JB & CORLETT RT. 2016. Chloroplast genome structure in *Ilex* (Aquifoliaceae). Sci Rep 6: 28559. https://doi.org/10.1038/srep28559.

YAO X, ZHANG F & CORLETT RT. 2022. Utilization of the hollies (*Ilex* L. spp.): A review. Forests 13: 94 https://doi.org/10.3390/f13010094.

YU Y, HARRIS AJ, BLAIR C & HE XJ. 2015. RASP (Reconstruct Ancestral State in Phylogenies): A tool for historical biogeography. Mol Phyl Evol 87: 46-49.

YU Y, BLAIR C & HE XJ. 2020. RASP 4: Ancestral state reconstruction tool for multiple genes and characters. Mol Biol Evol 37: 604-606.

YU Y, HARRIS AJ & HE XJ. 2010. S-DIVA (statistical dispersal-vicariance analysis): A tool for inferring biogeographic histories. Mol Phyl Evol 56: 848-850.

ZHOU T, NING K, MO Z, ZHANG F, ZHOU Y, CHONG X, ZHANG D, EL-KASSABY Y, BIAN J & CHEN H. 2022. Complete chloroplast genome of *Ilex dabieshanensis*: Genome structure, comparative analyses with three traditional *Ilex* tea species, and its phylogenetic relationships within the family Aquifoliaceae. PLoS One 17: e0268679. https://doi.org/10.1371/journal.pone.0268679.

SUPPLEMENTARY MATERIAL

Tables SI-SVI.
Figures S1-S4.

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How to cite

GOTTLIEB AM. 2026. Tracing the Maternal Lineages and Historical Biogeography of southern South American Hollies (*Ilex*, Aquifoliaceae). An Acad Bras Cienc 98: e20250861. DOI 10.1590/0001-3765202620250861.

Manuscript received on August 8, 2025;
accepted for publication on February 2, 2026

Handling editor

María del Carmen Coscarón

Data Availability

The data underlying this article are available in the GenBank Nucleotide Database at [https://www.ncbi.nlm.nih.gov/genbank/] and can be accessed with the accession numbers indicated in Supplementary materials Table SI and SII.

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