Research Article

Phylogeny and biogeography of the hollies (Ilex L., Aquifoliaceae)

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Abstract  The holly genus, Ilex L., in the monogeneric Aquifoliaceae, is the largest woody dioecious genus (>664 spp.), with a near-cosmopolitan distribution in mesic environments. We constructed a phylogeny based on two nuclear genes, representing 177 species spread across the geographical range, and dated using macrofossil records. The five main clades had a common ancestor in the early Eocene, much earlier than previously suggested. Ilex originated in subtropical Asia and extant clades colonized South America by 30 Ma, North America by 23 Ma, Australia by 8 Ma, Europe by 6 Ma, and Africa by 4 Ma. South and North America were colonized multiple times. Ilex also reached Hawaii (10 Ma) and other oceanic islands. Macrofossil and pollen records show the genus has tracked mesic climates through time and space, and had a wider distribution before late Miocene global cooling. Our phylogeny provides a framework for studies in comparative ecology and evolution.

Key words: Aquifoliaceae, biogeography, cosmopolitan distribution, dioecy, long distance dispersal.

1 Introduction

A key question in evolutionary biology is why some clades spread widely and generate many species while others do not. Many successful angiosperm clades have spread globally by giving rise to subclades that are able to colonize and speciate in novel environments (Hawkins et al., 2011). The genus Ilex L., however, has remained confined to the mesic growing conditions in which it presumably originated, and is absent from areas without year-round soil moisture or with prolonged winter cold. It has a very uniform reproductive biology and is instantly recognizable in flower or fruit, although leaf morphology is diverse and growth forms range from prostrate shrubs to tall trees. In favorable conditions the genus can form a major component of the plant community, with 10 or more species present in some subtropical forest plots. Regional diversity is very variable, however, with >200 species each in southwestern China and South America, and only single species in most of Europe, sub-Saharan Africa, and northern Australia. Ilex also reached Hawaii and other oceanic islands, reflecting efficient seed dispersal by birds attracted to large crops of small fruits, as well as unspecialized pollination (Tsang & Corlett, 2005). In several isolated areas with mesic climates that lack native Ilex species, including New Zealand, southeast Australia, and western North America, I. aquifolium L. is an invasive species.

Many Ilex species are utilized in various ways, some on a large scale. Diverse phytochemistry is reflected in the economically important use of different species for making beverages in South and North America, and East Asia, and many additional species have medicinal uses, including at least 27 in China (Yi et al., 2016). Tea from yerba mate (I. paraguariensis) is a billion US dollar industry in Argentina, Paraguay, Uruguay, and Brazil. Some larger species are locally used for timber, others are popular garden ornamentals, often with distinct cultivars, some are grown for honey production, the foliage and fruits of evergreen species are used as Christmas decorations, and deciduous species supply cut branches with bright red fruits in winter. A total of 150 species have been evaluated for the global IUCN Red List, of which two were considered extinct and 68 threatened; of 171 species evaluated in China, 26 were considered threatened. Some species that have not yet been formally evaluated are known from single populations or single collections.

A previous phylogeny, based on 108 species, found widespread discordance between plastid and nuclear trees, and suggested a mid-Miocene origin for the crown clade in East Asia or North America (Manen et al., 2010). This young date is hard to reconcile with the macrofossil record, with modern-looking leaf fossils since the Eocene, and Ilex-like
pollen widespread since the Cretaceous (see “Divergence times calibration with macrofossils” below). Extinction of sister lineages could account for this, but an earlier origin for the crown clade would be a simpler explanation. In order to improve our understanding of the evolution of this genus, we therefore sampled many additional species, particularly from the most likely area of origin, East Asia, and constructed a new phylogeny based on two nuclear genes and calibrated using three macrofossil records.

2 Material and Methods

2.1 Molecular sequencing and phylogenetic analyses

In total, 177 identified *ilex* species plus three *Helwingia* species (*H. chinensis* Batalin, *H. himalaica* Hook. f. & Thomson ex C. B. Clarke, and *H. japonica* (Thunb.) F. Dieter.) as outgroups were included in this study (Table S1). We included 127 species from Asia (defined here to include northern Australia, New Guinea, the Bonin Islands, and Micronesia), 23 species from North America (including Central America and the Caribbean), 19 species from South America, 3 from Europe (including the Mediterranean area), 2 from the Canaries-Azores, 2 from Oceania (including Micronesia, Hawaii, and New Caledonia), and 1 from Africa. Our coverage is good, except for South America, which is one of two centers of diversity of the genus, for which our sample included only approximately 10% of the known species.

Two nuclear markers, internal transcribed spacer (ITS) and chloroplast glutamine synthetase gene (*nepGS*), were chosen because of the known problems with cytonuclear discordance in *ilex* (Manen et al., 2010). All available data for these two markers was downloaded from GenBank and included in our analyses, and 81 additional species, mostly from China, were sequenced as part of this study, plus two species were half sequenced. Voucher information and GenBank accession numbers are in Table S1.

Total DNA was extracted from silica gel-dried leaves according to the modified CTAB method (Doyle & Doyle, 1987). The primers for ITS amplification were ITS5 (GTAGTGAACCTGCGG AAG) and ITS4 (TCCTCCGGATATTTGATAGC) (Gu, 2011), and for *nepGS* amplification were GScp687f (CGATGCTACCTACAAGCTT) and GScp994r (AATTGCTCTTTGTGGCGAAG) (Emshwiller & Doyle, 1999). Each amplification was carried out in 50 µL of a reaction mixture containing 30 µL 2× PCR Master Mixture (containing 2x Taq DNA polymerase, 2x polymerase chain reaction buffer, 2x dNTP, and MgCl2), 14 µL ddH2O, 1 µL of each primer, and 4 µL DNA template. The amplification was undertaken at 95°C for 4 min, 35 cycles of 95°C for 45 s, 58°C for 1 min, and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. The amplified products were sequenced in an ABI 3730 sequencer (Thermo Fisher Scientific, Waltham, MA, USA) in the Germplasm Bank of Wild Species, Kunming, China. The raw sequences were initially edited with Geneious version 9.1.7 (Kease et al., 2012) and then aligned in MAFFT (Katoh & Standley, 2013), followed by manual adjustment where necessary. All sequences produced were uploaded in GenBank (Table S1).

The Bayesian inference method was used for phylogenetic analyses, as “rogue taxa” were identified in these two nuclear markers in Manen et al. (2010). The nucleotide substitution model of the alignment was determined by jModelTest2 (Darriba et al., 2012). *BEAST* version 2.4.3 was used for phylogenetic analysis (Bouckaert et al., 2014), with the strict clock model and Birth Death model in the speciation process selected. The analysis ran for 200,000,000 generations and sampled one tree every 1,000 generations. The effective sample size values were checked in Tracer version 1.6, in which all effective sample size values were >220 and the burn-in value was 30% (http://tree.bio.ed.ac.uk/software/tracer/). LogCombinor version 2.4.3 was used for discarding 30% of trees as burn-in and resampling trees to less than 10,000. Finally, a maximum clade credibility tree was generated in TreeAnnotator version 2.4.3 using the rest of the resampled trees.

2.2 Divergence times calibration with macrofossils

Divergence times were estimated based on three macrofossil records. First, we chose the oldest fossil record for the Corneas, the most basal order of the asterids, as the crown age of Aquifoliales. This fossil, *Hironoa fusiformis* M. Takahashi, P. R. Crane & S. R. Manchester, was described from coalified fruits of Coniacian age (86.3–89.8 Ma) (Atkinson et al., 2016). The base of the Coniacian, 89.8 Ma, was used as the root age and set as the maximum bound of uniform prior distribution in this constraint. The minimum bound of this constraint was based on the oldest macrofossil (a seed) of *ilex* from Central Europe, dated at 69 Ma (Knobloch & Mai, 1986). Second, leaf macrofossils of *i. protocornuta* Xiang-Chuan Li & Bai-Nian Sun from the late Miocene (5.3–11.63 Ma) were used as the minimum bound of the stem node of the least clade containing *i. cornuta* Lindl. & Paxton, because *i. protocornuta* is close to living *i. cornuta* (Li et al., 2010). This least clade containing *i. cornuta* in our phylogeny included three species, *i. cornuta, i. dimorphophylla* Koidz., and *i. zhejiangensis* C. J. Tseng. The ages of 5.3 and 69 Ma were used as the minimum and maximum bound of the uniform prior distribution in this constraint. Finally, a leaf macrofossil record of *i. castellii* Kvaček & Walther from the Oligocene of Europe was used as the stem age of subsection Oxyodonta in section Aquifolioideae in Loesener’s taxonomic system (Loesener, 1942; Kvaček et al., 2009). Ages of 23.03 and 69 Ma were used as the minimum and maximum bound of the stem node of uniform prior distribution, in which this stem node included all sampled species belonging to subsection Oxyodonta in the Loesener system.

2.3 Ancestral area reconstructions

Data on distributions of sampled species were obtained from multiple online open sources, including the *Flora of China* (Chen et al., 2008), Monographia Aquifolioideae (Loizeau & Barriera, 2007), Global Biodiversity Information Facility (GBIF, 2018), and Plants of the World Online (POWO, 2019). To reconstruct the ancestral area of *ilex*, seven distribution regions were defined: (i) Asia (including north Australia, New Guinea, Bonin Islands, and Micronesia); (ii) Oceania (including New Caledonia, Vanuatu, Hawaiian Islands, and French Polynesia); (iii) Africa (including Madagascar); (iv) Europe (including the Mediterranean area); (v) Canaries-Azores (including Azores, Madeira, and the Canaries); (vi) North America (including Central America and the Caribbean); and (vii) South America (see Fig. 2B). The ancestral area reconstruction of each node in the phylogeny was inferred.
in package “BioGeoBEARS” based on the best-fit model calculated (Matzke, 2012). All analyses were based on the seven distribution regions and the maximum clade credibility tree with fossil calibration produced in BEAST analyses. This analysis was undertaken with the maximum number of region constraints (<2) in each node.

2.4 Ancestral state reconstruction of ecological traits

Data on leaf margin, fruit color, fruit diameter, and maximum tree height of each species in the phylogeny were obtained from online sources, including their type specimen, the Flora of China (Chen et al., 2008), the Monographia Aquifoliacearum website (Loizeau & Barriera, 2007), the eFlora of India (eFlora of India, 2019), and others. The ancestral state reconstruction for fruit color and leaf margin were carried out using single-rate models in package “phytools” (Revell, 2012) in the software R project (R Core Team, 2019). The ancestral state reconstruction for fruit diameter and maximum tree height were carried out using the maximum likelihood estimation method in function “fastAnc()” in “phytools”. Reconstruction results were also visualized in a heatmap by using the package “phytools”. Phylogenetic signals for leaf margin and fruit color were tested by using the EM-Mantel test in R, which is an evolutionary model-based test that has appropriate type I error and strong power to detect phylogenetic signals for both continuous and categorical traits (Debastiani & da Silva, 2017). Phylogenetic signals of fruit diameter and maximum tree height were tested by using “phylosignal()” in package “picante” (Kembel et al., 2010) in R.

2.5 Evolutionary test of climatic tolerances

We obtained georeferenced specimen records from the Global Biodiversity Information Facility (GBIF, 2018), the Chinese Virtual Herbarium (CVH, 2015), and our own fieldwork. In total, 35 004 distribution records for 163 out of the 177 identified species were collected and checked manually against natural distribution ranges in Plants of the World Online (POWO, 2019) and GBIF (GBIF, 2018). Geocoding of locality data from CVH was undertaken using Amap API (2019) in Python 3.6. These data were further cleaned by comparing with the natural global distribution of Ilex in Python 3.6, leaving 21 657 unique records after removal of duplicates. Nineteen bioclimatic variables in CHLSA (Karger et al., 2017) were extracted for each record in R package “raster” (Hijmans, 2019). Phylogenetic signals of the selected bioclimatic variables were tested using the “phylosignal()” function in package “picante” (Kembel et al., 2010).

3 Results

Ilex consists of five clades with 22 major subclades (Fig. 1). Posterior probabilities for most nodes were higher than 0.8, except near the tips of the most diverse clades (Figs. 1, S1). The species in East Asia, Europe, North America, and South America do not form monophyletic groups, but the three most basal clades (A–C) are entirely Eurasian and the three species sampled in clades A and B all grow at 1000–3000 m in Southwest China. Clade D includes Asian and American species, whereas clade E includes species from across the modern global range of the genus. The topology is basically similar to that of the nuclear tree of Manen et al. (2010), but the addition of 61 more species gives higher resolution, particularly in the basal, Asian clades.

Among the island endemics, the Hawaiian I. anomala Hook. & Arn. is in American subclade E5, the New Caledonian I. sebertii Pancher is in Asian subclade E4, and the Canary Islands I. canariensis Poir. is basal in South American subclade E1 (Fig. 1). In contrast, the non-endemic Canary Islands species, I. perado Soland. ex Ait., is in Eurasian subclade C5. The four endemics in the Bonin Islands, I. matanoana Makino, I. mertensii Maxim., I. percoriacea Tuyama, and I. beecheyi (Loes.) Makino, form a monophyletic subclade in clade C6 (Fig. 1), whereas the four endemics in the Ryukyu Islands had three different origins, with I. maximowiciziana Loes. in subclade C6, I. dimorphophylla Koidz. in subclade C4, and I. warburgii Loes. and I. liukiensis Loes. as sister species in subclade C6 (Fig. 1). The four non-endemics in the Ryukyu Islands, I. rotunda Thunb., I. integra Thunb., I. goshiensis Hayata, and I. macrocarpa Oliv., all belong to different subclades (E7, C3, E3, and E6, respectively).

Our dating suggests a Late Cretaceous stem age for Ilex of 84.7 Ma (76.63–89.80 Ma) in 95% highest posterior density, whereas the crown clade originated in the Eocene at 50.8 Ma (43.53–57.63 Ma) (Figs. 1, S2; Table 1). The phylogeny (Fig. 1) and ancestral area reconstruction analysis (Fig. 2) show that crown-clade Ilex originated in Asia. The oldest clade in the Americas (D) diverged from Eurasian ancestors in the Oligocene at 30.4 Ma (26.21–34.3 Ma) and its earliest branches are in South America, whereas the oldest clade (D2/D3) in North America diverged in the late Oligocene/Early Miocene at 23.1 Ma (16.66–31.61 Ma). The Canary Islands endemic (I. canariensis) diverged at 27.7 Ma (23.59–31.74 Ma), the New Caledonian endemic (I. sebertii) at 18.7 Ma (14.39–22.67 Ma), and the Hawaii endemic (I. anomala) at 10.2 Ma (7.81–12.86 Ma). The only Australian species (I. arnhemensis Loes.) diverged at 8.3 Ma (6.41–10.22 Ma), the four European species at 5.9 Ma (4.35–7.61 Ma), and the only African species (I. mitis (L.) Radlk.) at 4.2 Ma (2.47–5.98 Ma). The Bonin Island subclade diverged at 1.1 Ma (0.15–4.23 Ma).

The deciduous species in East Asia (I. aculeolata Nakai, I. asprella (Hook. & Arn.) Champ. ex Benth., I. chapaensis Merr., I. fragilis Hook., I. kusanoi Hayata, I. macrocarpa Oliv., I. macropoda Miq., I. micrococca Maxim., I. polyneura (Hand.-Mazz.) S. Y. Hu, I. serrata Thunb., and I. tsoll Merr. & Chun) form a monophyletic group (E6), but the eight North American species do not, with I. montana Torr. & A. Gray, I. longipes Chapm. ex Trel., I. decidua Walter, and I. collina Alexander in D2, I. opaca Alton in D3, and I. verticillata (L.) A. Gray, I. laevigata (Pursh) A. Gray, and I. geniculata Maxim. in E8. Extant species of Ilex usually have red fruits (110/177 in our sample), followed by black, with a few recorded as other colors (Table S3). The EM-Mantel test showed a phylogenetic signal for fruit color (P = 0.05) and the most recent common ancestor was reconstructed as having red fruits with highest likelihood value (0.624) (Fig. 3A). Most extant species of Ilex have leaves that are serrate or entire (Table S4), and fewer than 30 species globally have spiny leaves. The EM-Mantel test showed a phylogenetic signal for leaf margin type (P < 0.05) and the most recent common ancestor (MRCA) was reconstructed as having serrate leaves with highest likelihood value (0.535). Serrate leaves are found throughout...
Fig. 1. Chronogram of 183 Ilex species with three macrofossil constraints and three Helwingiaceae species as outgroups estimated in BEAST2. Positions of the fossil constraints are indicated with red arrows and letters (a, b, and c). Median ages are presented near nodes as million years (Ma). Nodes with posterior probability lower than 0.80 are labeled by empty blue circle.
Table 1: Ages of main lineages of the Aquifoliaceae based on BEAST analysis

| Lineages | Stem age (Ma) | 95% HPD of stem age (Ma) | Crown age (Ma) | 95% HPD of crown age (Ma) |
|----------|--------------|--------------------------|---------------|--------------------------|
| Outgroup | 84.66        | 76.63–89.80              | 4.77          | 2.43–7.35                |
| A        | 50.75        | 43.53–57.63              | 38.41         | 30.30–46.02              |
| B        | 50.75        | 43.53–57.63              | 41.80         | 34.15–50.93              |
| C        | 32.69        | 28.13–37.19              | 25.18         | 23.03–28.43              |
| C1       | 32.69        | 28.13–37.19              | 25.18         | 23.03–28.43              |
| C2       | 24.03        | 21.91–28.10              | 23.54         | 20.26–26.99              |
| C3       | 18.51        | 14.82–22.65              | 17.68         | 13.60–22.21              |
| C4       | 8.46         | 6.33–10.87               | 5.85          | 5.30–6.80                |
| C5       | 8.46         | 6.33–10.87               | 5.87          | 4.35–7.61                |
| C6       | 12.55        | 9.05–18.09               | 12.43         | 8.77–18.18               |
| D        | 30.39        | 26.21–34.73              | 28.12         | 23.42–32.87              |
| D1       | 30.39        | 26.21–34.73              | 28.12         | 23.42–32.87              |
| D2       | 12.81        | 9.90–15.75               | 6.86          | 4.53–9.48                |
| D3       | 12.81        | 9.90–15.75               | 11.74         | 8.95–14.79               |
| D4       | 19.33        | 15.95–22.65              | 11.50         | 9.13–14.11               |
| D5       | 19.33        | 15.95–22.65              | 12.37         | 9.95–14.86               |
| E        | 30.39        | 26.21–34.73              | 27.93         | 23.88–31.75              |
| E1       | 27.93        | 23.88–31.75              | 27.74         | 23.59–31.74              |
| E2       | 26.43        | 22.61–30.20              | 21.20         | 17.74–24.76              |
| E3       | 23.18        | 19.83–26.63              | 21.92         | 18.58–25.52              |
| E4       | 23.18        | 19.83–26.63              | 21.44         | 18.09–24.81              |
| E5       | 17.07        | 13.68–20.65              | 12.22         | 9.81–14.79               |
| E6       | 16.86        | 13.34–20.64              | 12.36         | 9.86–15.15               |
| E7       | 15.13        | 12.11–18.08              | 12.40         | 9.92–14.96               |
| E8       | 14.62        | 11.71–17.76              | 6.16          | 4.19–8.20                |
| E9       | 14.62        | 11.71–17.76              | 13.86         | 11.18–17.45              |

HPD, highest posterior density; Ma, million years ago.

the phylogeny, but spiny leaves in our sample are found mostly in Eurasian subclade C4/C5/C6, where they do not form a monophyletic group, and American subclade D3; and entire-leaved species are mostly tropical and subtropical lowland species in clade E (Fig. 3B). There were no significant phylogenetic signals for fruit size and plant maximum height, suggesting both of these are evolutionarily labile, but 89% of Ilex fruits are < 10 mm diameter.

Phylogenetic signals (P < 0.05) were also detected in the bioclimatic preferences of Ilex species, including the maximum temperature of the warmest month (MTWMM), minimum temperature of coldest month (MTCM), temperature annual range (TAR), and the precipitation of the driest quarter (PDQ). Ancestral state reconstruction of climatic preferences suggests that the MRCA occupied a subtropical climate with MTWMM of 31.6°C, MTCM of 4.1°C, TAR of 27.1°C, and PDQ of 184.5 mm/month, which is similar to the median for extant species (31.7°C, 1.7°C, 29.9°C, and 124.1 mm, respectively), although slightly less seasonal (Fig. 53).

4 Discussion

Although our 50.8 Ma date for the origin of the crown clade is much earlier than the ca. 15 Ma date proposed by Manen et al. (2010), it still leaves a ca. 34-Ma gap after the late Cretaceous origin of the genus and family based on fossil data (Fig. 1). Other recent, well-calibrated, dates for the origin of the Aquifoliaceae are in the range 68–95.5 Ma (Beaulieu et al., 2013; Magallón et al., 2015; Wikström et al., 2015; Zeng et al., 2017), so our 84.7 Ma date is reasonable. Extensive lineage extinction between the Cretaceous and Eocene could account for this gap, and this is supported by the continuous presence of Ilex-like fossils during this period.

The fossil record of Ilex has been reviewed multiple times (Martin, 1977; Manen et al., 2002; Loizeau et al., 2005) and new records continue to be added (Kvaček et al., 2009; Carpenter et al., 2015; Barreda et al., 2019). Ilex-like pollen, in the fossil genus Ilexpollenites, has been quite widely recorded in the late Cretaceous, when it was present in Antarctica (Barreda et al., 2019) and Australia (Martin, 1977; Carpenter et al., 2015), and at scattered localities across the Northern Hemisphere (Loizeau et al., 2005). This pollen is very distinctive, even in comparison with the other two families in the Aquifoliales, Helwingiaceae and Phyllonomaceae (Schori & Furness, 2014). The early presence of pollen in Australia and Antarctica is intriguing, as it has been suggested that the Aquifoliaceae and other campanulids originated in the Southern Hemisphere (Beaulieu et al., 2013), and the long stem length for Ilex means that this is not impossible. However, the much greater separation between East Asia and Australia in the late Cretaceous suggests that a Gondwanan origin followed by greater separation between East Asia and Australia in the late Cretaceous is unlikely.

Macrofossils (pyrenes) are first recorded in the upper Maastrichtian or early Paleocene of Europe (Knobloch & Mai, 1986; Knobloch et al., 1993). Leaf macrofossils have also been identified as Ilex, but the widely accepted records are
Fig. 2. Ancestral area reconstruction result for 177 identified Ilex species based on the best fit model (BAYAREALIKE+J) in package “BioGeoBEARS.” A, Capital letters right of the phylogeny show phylogenetic clade names. Lowercase letters in the phylogenetic nodes and next to phylogenetic tips show reconstructed ancestral area with the maximum likelihood: a (dark blue), Asia, including north Australia, New Guinea, Bonin Islands, and Micronesia; b (blue green), Oceania, including New Caledonia, Vanuatu, Hawaii Islands, and French Polynesia; c (green), Africa, including Madagascar; d (yellow), Europe and the Mediterranean area; e (orange), Canaries and Azores; f (red), North America, including central America and the Caribbean; g (pink), South America. B, Map showing the geographic sets used for ancestral area reconstruction in subfigure (A).
all for spiny species, which make up less than 5% of the extant species in the genus. Non-spiny Ilex leaf fossils would almost certainly be overlooked. Reliable leaf fossils are mostly post-Eocene and thus consistent with the extant crown clade, but the pyrenes suggest that Ilex itself has been around since at least the Paleocene, and the pollen suggests an even earlier spread of additional clades – in Ilex or other, now extinct, genera – in the Aquifoliaceae.

After the origin of the crown clade in the Eocene, Ilex-like pollen continues to be found in areas where the genus does not occur today, such as late Oligocene/early Miocene New Zealand (Lee et al., 2012; Mildenhall et al., 2014) and southern Australia (Korasidis et al., 2017). There is also evidence for at least one extinction event in Europe, where Ilex leaf macrofossils (Kvaček et al., 2009) date back to the Oligocene, but the extant species belonging to a clade which diverged in the late Miocene (Fig. 1). Extinctions might also help explain the anomalous occurrence of a single member of an otherwise South American subclade, I. canariensis, in the Canary Islands, as the forests in which it occurs today are depleted remnants of the extensive mesic subtropical forests of Miocene North Africa (Corlett & Hughes, 2015), which could well have been rich in Ilex species.

More generally, the combination of exceptional powers of long-distance dispersal (e.g., in the colonization of Hawaii), with high extinction following late Miocene cooling and...
Novel defensive chemistry would be particularly backed up by detailed studies of the chemical components by the many species used for teas and medicines, and this is phytochemistry is diverse and distinctive in to explain the exceptional diversi

much more variable, but not exceptionally so, and seem unlikely contributed little to diversi

reproductive biology reached an early adaptive plateau and pollination and seed dispersal systems, suggest that the uniform flower and fruit morphology, associated with generalist pollination and seed dispersal systems, suggest that the reproductive biology reached an early adaptive plateau and contributed little to diversification. Vegetative structures are much more variable, but not exceptionally so, and seem unlikely to explain the exceptional diversification of the genus. However, phytochemistry is diverse and distinctive in *Ilex* species, as shown by the many species used for teas and medicines, and this is backed up by detailed studies of the chemical components (Yi et al., 2016). Novel defensive chemistry would be particularly beneficial in the colonization of new areas, and subsequent diversification of this chemistry might allow multiple species to coexist. This hypothesis could potentially be tested by looking for associations between specific chemical traits and clade diversification, and for both phylogenetic and chemical trait overdispersion in coexisting species (Weber & Agrawal, 2012).

Informative comparisons with *Ilex* are provided by four other large, widespread genera of woody plants with good phylogenies. *Diospyros* L. (Ebenaceae) is another well-dispersed, dioecious genus (Duangjai et al., 2009; Samuel et al., 2019), but its modern distribution differs from that of *Ilex* because it has also diversified in dry forests, with resulting high diversities in Africa, India, and New Caledonia, where the few species of *Ilex* are confined to more mesic sites (Paun et al., 2016). *Diospyros* is also more diverse than *Ilex* in tall, dense, tropical rainforests and less diverse in frost-prone areas. In *Rhododendron* L., the tiny seeds are dispersed by wind and pollination is relatively unspecialized, but the genus does not seem capable of crossing large marine gaps and different clades colonized each region (Shrestha et al., 2018). *Ficus* L. (Moraceae) has excellent seed dispersal but an obligate pollinator mutualism, which reduces the chance of establishment after long-distance seed dispersal, leading to its absence from oceanic islands and a tighter relationship between clades and areas than seen in *Ilex* (Cruaud et al., 2012; Zhang et al., 2018). In *Quercus* L. (Fagaceae), pollination is by wind, but seed dispersal is by scatter-hoarding and was probably always across land connections, leading again to a tight match between clades and areas (Xu et al., 2019).

In conclusion, this study has pushed back the origin of the existing *Ilex* crown clade into the early Eocene, with multiple lines of evidence suggesting that the MRCA grew in moist tropical East Asia. The genus has subsequently used efficient seed dispersal to track this mesic climate type through space and time, colonizing suitable areas rapidly, but disappearing when the climate changed. Over 50 million years, *Ilex* appears to have expanded its range of tolerance to temperature, but not to moisture. The current near-cosmopolitan distribution,
with extreme variation in regional diversity, thus reflects a balance between dispersal, diversification, and extinction.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12567/suppinfo:

Fig. S1. Phylogeny of 183 Ilex species estimated in BEAST2. Numbers near phylogenetic nodes are posterior probabilities.

Fig. S2. Chronogram tree of 183 Ilex species estimated in BEAST2 with three macrofossil constraints. The numbers in square brackets next to each node are the lower and upper bounds of the 95% highest posterior density intervals for the age.

Fig. S3. Evolutionary reconstruction of Ilex tolerance of the maximum temperature of the warmest month based on package “phytools.”

Fig. S4. Evolutionary reconstruction of Ilex tolerance of the minimum temperature of the coldest month based on package “phytools.”

Fig. S5. Evolutionary reconstruction of Ilex tolerance of the temperature annual range based on package “phytools.” Fig. S6. Evolutionary reconstruction of Ilex climatic tolerance of the precipitation of the driest quarter based on package “phytools.”

Table S1. Information on Ilex species included in this study.

Table S2. Comparison of the best-fit biogeographical models for Ilex based on log likelihood value (lnLik) and AIC value in package “BioGeoBEARS” of R project.

Table S3. Fruit color, fruit diameter, leaf margin, and maximum tree height of the 177 identified Ilex species. In the color column, na means data not available.

Data S1. The chronogram based on the two nuclear sequences (ITS and ncpGS) and three fossil constraints, available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.kop2ngf4x