Molecular Phylogeny and Biogeography of the Genus *Ilex* L. (Aquifoliaceae)

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The chloroplast *atpB-rbcL* spacer was sequenced for 115 species of *Ilex* and for *Nemopanthus mucronatus* (Aquifoliaceae). In addition, *rbcL* sequences were obtained for 47 selected species. Taken together with fossil records in the literature, the data indicate that: (1) the lineage *Ilex* was already cosmopolitan long before the end of the Cretaceous. A relative test of the rate of nucleotide substitution indicates that extant species do not represent the entire lineage because of the extinction of its basal branches. The common ancestor of the extant species probably appeared at the upper Tertiary. (2) Several Asian/North American disjunctions are observed, as well as North American/South American relationships. The directions of these relationships are yet to be determined. The African species *Ilex mitis* is closely related to Asian species. *Ilex anomala* (Hawaii and Tahiti) is related to American species. *Ilex perado* and *Ilex canariensis* (both in Macaronesia) have totally different relationships, the former being related to Eurasian species, while relationships of the latter remain obscure. (3) Gene trees substantially contradict the systematics of Loesener. *Nemopanthus*, closely related to *Ilex amelanchier*, is nested in the genus *Ilex*. The family Aquifoliaceae is organized in four groups, each of them having a geographic or ecological peculiarity.

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INTRODUCTION

The genus *Ilex* L. comprises more than 500 species of dioecious trees and shrubs distributed throughout temperate and tropical regions of the world (Galle, 1997). Together with the monospecific genus *Nemopanthus* Raf. of eastern North America, it forms the family Aquifoliaceae Bartl. (holly family). The classification of the Angiosperm Phylogeny Group (1998) places Aquifoliaceae together with Helwingiaceae Decne. (East Asia) and Phyllonomaceae Small (Central America) in the order Aquifoliales, which is sister to the asterids. The genera *Phelline* Labill. and *Sphenostemon* Baillon, often associated with Aquifoliaceae (Cronquist, 1988), are definitively more distantly related. Based on *rbcL* and *atpB* data, some members of the polyphyletic Icacinaceae (Benth.) Miers, such as *Gonocaryum* Miq. (East Asia), seem to be related to the Aquifoliales (V. Savolainen pers. comm.)

The pollen of *Ilex* is very characteristic (Muller, 1981) and its fossil remains can be traced as far back as the Turonian in Australia [90 million years ago, (Mya)]. The genus was apparently already cosmopolitan by the early Tertiary (Martin, 1977, but see Fig. 1). The main areas of extant diversification are East Asia and South America. The genus is also well represented in South-east Asia and North America. Only one species is found in tropical Africa,

another is found in northern tropical Australia and four related species are found in Europe, although the fossil record indicates a much higher diversity in these three regions during the Tertiary. A few species occur in Hawaii, the Caribbean, the Canary Islands, the Azores, Madeira, New Caledonia and Fiji (see Fig. 1 for an overview of the present geographical distribution of the genus and its fossil record). In fact, the lineage was already cosmopolitan much before the end of the Cretaceous.

The holly family represents a challenge for molecular systematics. Although this family has a good fossil record, the interpretation of its evolutionary and biogeographical history is still a mystery. Moreover, several recent biosystematic studies show that the classification of the Aquifoliaceae requires re-examination. The last comprehensive treatment of the genus, with the definition of five sub-genera further divided into sections, is that of Loesener (1901, 1908, 1942). Hu (1949, 1950), in her revision of the Chinese species, adapted Loesener's system, including substantial modifications. Further large-scale studies, mainly anatomical, have been undertaken (Baas, 1973, 1975; Lobreau-Callen, 1975; Loizeau and Spichiger, 1992), and most of these did not fully agree with Loesener's system. Thus, a molecular study should provide further understanding of *Ilex* evolution, which is one of the aims of this study.

In this paper, the results obtained from chloroplast sequence data and a broad sampling of Aquifoliaceae are

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presented. The trees inferred are not congruent with the systematics of Loesener. These data open new areas for further taxonomic and biogeographic investigations in the genus *Ilex*.

MATERIALS AND METHODS

Specimens and DNA sequences

Nemopanthus mucronatus (L.) Trel. and 115 different species of *Ilex* were screened by DNA sequencing (63 species from Asia, 23 from North and Central America and the Caribbean, 22 from South America, three from Europe, two from Macaronesia, one from Africa, one from Hawaii). Some related genera (*Helwingia* Willd., *Phyllonoma* Willd. and *Gonocaryum*) were included in the analysis to help with the placing of the root within the Aquifoliaceae. For many species, we produced sequences from several accessions to scan for intraspecific variability. The sequence data set represents 142 specimens. Vouchers for most of these are deposited at Geneva (G). Table 1 shows the specimens studied together with voucher information, geographic distribution and habitat (temperate, subtropical, tropical or montane tropical).

Plastid *atpB-rbcL* spacer sequences, as well as the first 124 nucleotides of *rbcL* were determined for all 142 specimens. As the amount of variability was rather weak, 49 specimens (representing 47 species) were selected to determine the sequence of the entire *rbcL* gene. There are 886 nucleotide sites for 142 specimens representing 116 species and three hybrids (83 variables sites and 30 potentially informative sites in the ingroup). In addition, there are 2070 nucleotide sites for 49 specimens representing 47 species (246 variables sites and 93 potentially informative sites in the ingroup). For outgroups, the *rbcL* sequence is available for *Helwingia*, *Phyllonoma* and *Gonocaryum*, while the *atpB-rbcL* spacer sequence is available only for *Helwingia* and *Phyllonoma*. Both data sets are available as aligned DNA sequences (http://www.cjb.unige.ch).

DNA analysis

DNA was extracted from silica gel-dried leaves or, in a few cases, from herbarium specimens (sample numbers beginning with H). The *atpB-rbcL* spacer and *rbcL* were amplified and sequenced according to Manen and Natali (1995). Some sequencing was performed on a 310 ABI PRISM or a 373 ABI sequencer. Alignments were done manually. A few gaps were introduced in the matrix of the *atpB-rbcL* spacer and were treated as fifth character states.

All analyses were performed using the heuristic search option of PAUP 3·1 (Swofford, 1991). Trees were produced with tree bisection reconnection (TBR) swapping, CLOSEST addition and MULPARS options.

The DNA matrices produced a quasi-infinite number of most parsimonious trees. To avoid the production of only one island of solutions, 20 replications of RANDOM addition of taxa were carried out, saving no more than 100 trees equal to the number of steps observed in preliminary longer analyses without random addition of sequences. No shorter trees were found.

Because of the low variation observed in Aquifoliaceae and confronted with low phylogenetic signals, all trees shown (Figs 2 and 3) are strict consensus trees obtained after successive weighting according to the maximum RC values of characters. The branches observed in the initially equally-weighted trees are indicated by a thick bar. The support of clades was estimated by 100 bootstrap replications with TBR branch swapping, ten trees being kept at each replicate.

Biogeographic analysis

Firstly, the phylogenies obtained were analysed with the software TASS (Nelson and Ladiges, 1995, 1996) in order to investigate relationships between areas. TASS allows the isolation of informative parts of a phylogeny by ignoring paralogous (overlapping) nodes in a first step, and coding the information in a matrix in a second step. The algorithm used proceeds from the tips of the branches and moves towards the root of the tree. If a node relates taxa whose distributions do not overlap, it is conserved. If a node relates taxa that overlap geographically, it is considered paralogous and thus ignored. The subtrees thus obtained summarize the information contained in the phylogeny. One advantage of this method is that it allows discussion of the biogeographic information prior to the computation of an area cladogram. Morever, since this method considers that paralogous information accumulates as one moves from the tips of the tree to its root, it is relatively robust to inaccuracies in the innermost nodes of the tree.

Secondly, the phylogenies obtained were analysed with Bremer's (1992) method to investigate the area of origin of the group. In the ancestral areas analysis of Bremer (1992), each area is treated as a single character, which may be optimized onto the cladogram using either Camin-Sokal or Dollo parsimony. By comparing the numbers of necessary gains and losses under the two optimizations, respectively, it is possible to estimate which areas were most probably part of the ancestral area.

These analyses were performed on both phylogenies depicted in Figs 2 and 3, including all nodes in a first analysis, and only those supported by bootstrap percentages higher than 50 % in the second case.

RESULTS

Interspecific relationships in Aquifoliaceae based on gene trees

The first part of the strategy was to obtain an overview of the available Aquifoliaceae species. For this purpose, 142 specimens representing 116 species with a wide geographic distribution were examined using the data of the plastid *atpB-rbcL* spacer. As the level of divergence was found to be relatively low, 49 representative specimens were selected from the trees produced (several in unresolved parts of the trees) for which additional sequence data from *rbcL* were obtained. The results obtained from these 49 specimens using combined sequence data of the *atpB-rbcL* spacer and *rbcL* will be discussed first (Fig. 2). The data from the 142

A

B
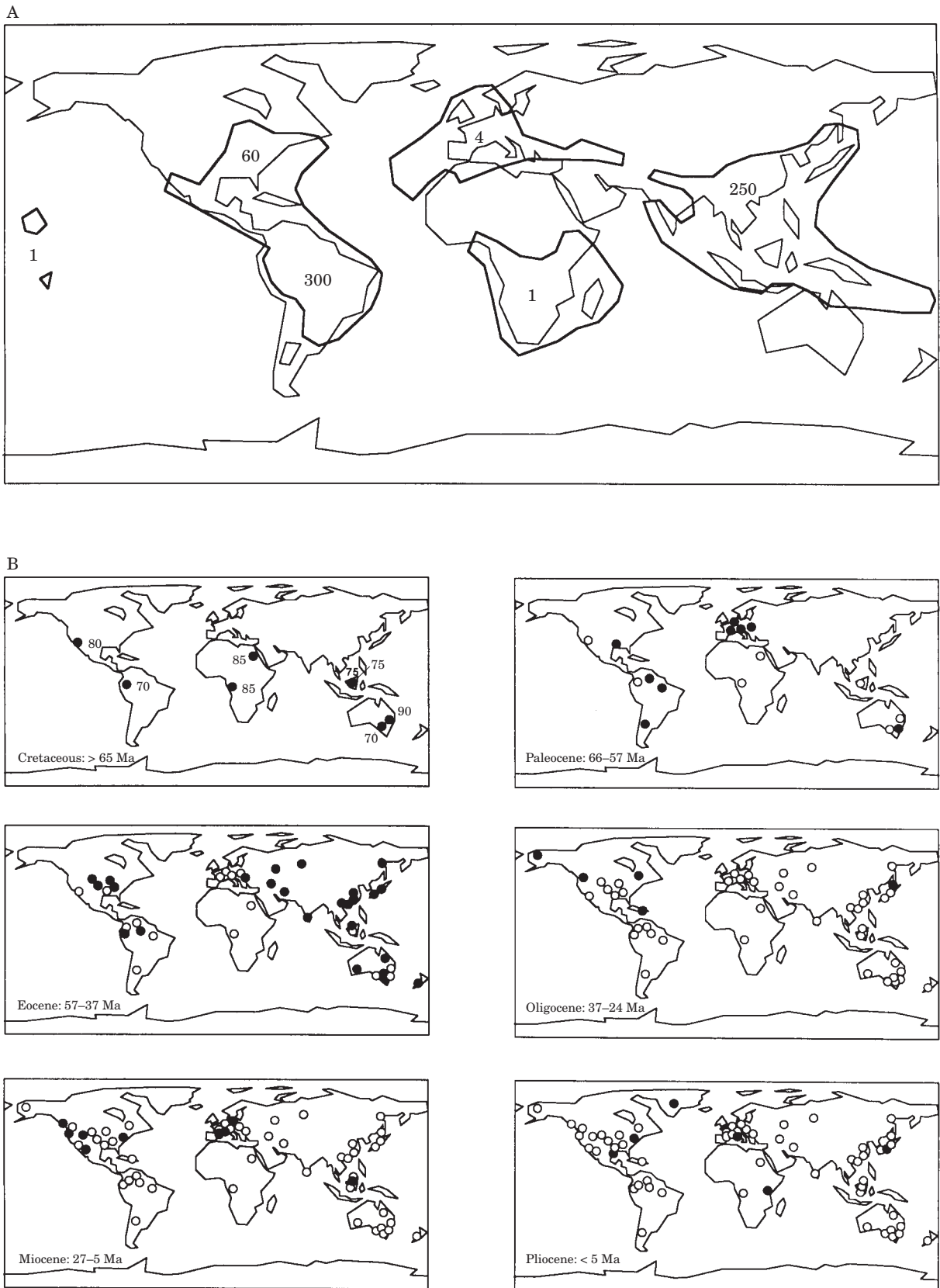
FIG. 1. A, Geographical distribution of the genus *Ilex*, according to Hu (1967) and Lobreau-Callen (1975). The approximate number of species in the different areas is indicated according to the authors. B, Geographical distribution of *Ilex* fossil records at different geological periods. These records have been verified or cited by Lobreau-Callen (1975), Martin (1877) and Muller (1981). (●) New records for the indicated periods; (○) older records.

TABLE 1. *List of* Ilex *specimens and species from which sequence data are available, with voucher information, geographical distribution and habitat*

Species	Sample No.	Collector	Voucher	Distribution	Habitat
aculeolata Nakai	140	Zhong Shu Hua	(−)	East Asia	t, sT
affinis Gardn.	H300	P.E. de Silva *et al.*	R6996	South America	T
amara (Vell.) Loes.	H301	R. Harley	26229	South America	T
amelanchier Curtis	100	S. Andrews	SA1475	USA	t
amelanchier Curtis	299	S. Andrews	SA1657	USA	t
anomala Hook. & Arn.	101	C. Imada *et al.*	(−)	Hawaii	T
anomala Hook. & Arn.	127	S. Andrews	SA1547	Hawaii	T
anomala Hook. & Arn. **(1)**	155	S. Andrews	SA1525	Tahiti	T
aquifolium L.	136	S. Andrews	SA1487	Europe, N.Africa	t
argentina Lillo	139	G. Giberti	507	South America	sT, Tm
asperula Reiss.	H303	J.N. Nakajima	1350	South America	T
asprella (Hook. & Arn.) Champ.	171	S. Andrews	SA1532	Esat Asia	t, T
x *attenuata* Ashe	74	S. Andrews	SA 1515	USA	t, T
bioritsensis Hayata	142	S. Andrews	SA1480	East Asia	t
brasiliensis (Spreng.) Loes.	102	G. Giberti	BACP105	South America	T, Tm
brevicuspis Reiss.	105	G. Giberti	BACP99	South America	T
buergerii Miq.	11	H. Nagamasu	5369	East Asia	t, sT
buxifolia Gardn.	H304	Glaziou	16747	South America	T
canariensis Poir.	90	S. Andrews	SA1488	Canary Islands	sT
canariensis Poir.	H305	S. Andrews *et al.*	459	Canary Islands	sT
cassine L.	145	S. Andrews	SA1476	USA, Mexico, Bahamas, Cuba	sT
cassine L.	162	S. Andrews	SA1545	USA, Mexico, Bahamas, Cuba	sT
cassine L.	6	P.-A. Loizeau	no voucher	USA, Mexico, Bahamas, Cuba	sT
chamaedryfolia Reiss.	117	G. Hatschbach	61·222	South America	Tm
ciliospinosa Loes.	55	Bot. Gard. Munich	(−)	East Asia	t
ciliospinosa Loes.	168	S. Andrews	SA1484	East Asia	t
cissoidea Loes.	163	S. Andrews	SA860	South East Asia	T
colchica Pojark.	67	S. Andrews	SA1458	Europe, Asia	t
collina Alexander	81	S. Andrews	SA1479	USA	t
conocarpa Reiss.	H306	J.A. Ratter *et al.*	R5222	South America	T
corallina Franch.	88	S. Andrews	SA1517	East Asia	t
coriacea (Pursh) Chapm.	147	S. Andrews	SA1536	USA, Mexico	t, sT
cornuta Lindl. & Paxt.	57	P. Ferard	(−)	East Asia	t
cornuta Lindl. & Paxt.	61	S. Andrews	SA1452	East Asia	t
crenata Thunb. ex Murray **(2)**	14	H. Nagamasu	(−)	East Asia	t
cumulicola Small	160	S. Andrews	SA1537	USA	sT
curtisii (Fern.) Small	122	S. Andrews	SA1543	USA	t
cymosa Blume	172	S. Andrews	SA1570	South East Asia	T
cf. *cymosa* Blume	164	S. Andrews	SA1575	South East Asia	T
cyrtura Merr.	84	S. Andrews	SA1492	East Asia	t, sT, T
cf. *cyrtura* Merr.	60	S. Andrews	SA1455	East Asia	t, sT, T
decidua Walt.	73	S. Andrews	SA1474	USA	t
dimorphophylla Koidz.	8	H. Nagamasu	5370	East Asia	t, sT
dipyrena Wall.	96	S. Andrews	SA1478	East Asia	t
discolor Hemsl.	108	A.M. Olivo	(−)	Mexico	sT, Tm
dugesii Fernald	98	S. Andrews	SA1497	Mexico	sT, Tm
dumosa Reiss.	103	G. Giberti	no voucher	South America	Tm
fargesii Franch. **(3)**	68	S. Andrews	SA1453	East Asia	t, sT
fargesii Franch. **(4)**	124	S. Andrews	SA1495	East Asia	t, sT
?*ficoidea* Hemsl.	85	S. Andrews	SA1505	East Asia	sT
fragilis Hook. f.	152	C.J. Wingfield	(−)	East Asia	t
geniculata Maxim.	125	S. Andrews	SA1527	East Asia	t
georgei Comber	65	S. Andrews	SA1454	East Asia	t
glabra (L.) A. Gray	143	S. Andrews	SA1468	Canada, USA	t
goshiensis Hayata	10	H. Nagamasu	5035	East Asia	sT
guianensis (Aubl.) Kuntze	H7	D'Arcy et al.	15500	South America-Mexico	sT, T
guianensis (Aubl.) Kuntze	H307	A. Sugden	1207	South America-Mexico	sT, T
havilandii Loes.	131	S. Andrews	SA1586	Borneo	Tm
hippocrateoides Kunth	H8	Nuñez	9904	South America	Tm
hookeri King	72	S. Andrews	SA1464	East Asia	t
hylonoma Hu & Tang	138	Zhong Shu Hua	(−)	East Asia	t, sT
integerrima (Vell.) Reiss.	106	G. Giberti	BACP114	South America	T
integra Thunb. ex Murray	9	H. Nagamasu	5073	East Asia	t, sT
integra Thunb. ex Murray	5	P.-A. Loizeau	no voucher	East Asia	t, sT
intricata Hook. f.	150	S. Andrews	SA1568	East Asia	t
kinabaluensis S. Andrews	166	S. Andrews	SA1580	Borneo	Tm
kingiana Cockerell	144	S. Andrews	SA1494	East Asia	t
x *kiusiana* Hatusima	170	S. Andrews	SA1552	East Asia	t
kusanoi Hayata	158	S. Andrews	SA1512	East Asia	sT
laevigata (Pursh) A. Gray	114	S. Andrews	SA1507	USA	t
latifolia Thunb. ex Murray	62	S. Andrews	SA1462	East Asia	t
laurina Kunth	H1	Wallnöfer	15-5788	South America	Tm
leucoclada (Maxim.) Makino	159	S. Andrews	SA1542	East Asia	t, sT
liebmannii Stanley	71	S. Andrews	SA1463	Central America, Mexico	Tm
?*liukiuensis* Loes.	17	H. Nagamasu	5034	East Asia	sT
longipes Chapman ex Trel.	77	S. Andrews	SA1472	USA	t

TABLE 1. (*cont.*)

Species	Sample No.	Collector	Voucher	Distribution	Habitat
lundii Warm.	H308	H.S. Irwin *et al.*	5836	South America	T
macrocarpa Oliver	58	P. Ferard	(−)	East Asia	t, sT
macrocarpa Oliver	76	S. Andrews	SA1479	East Asia	t, sT
macrophylla Wall.	154	S. Andrews	SA1572	Borneo	T
macropoda Miq.	91	S. Andrews	SA1514	East Asia	t
maingayi Hook. f.	148	S. Andrews	SA1571	South East Asia	T
x makinoi Hara	169	S. Andrews	SA1520	East Asia	t
matanoana Makino	12	A. Soejima	930210	East Asia	sT
maximowicziana Loes.	15	H. Nagamasu	5204	East Asia	T
maximowicziana Loes. (**5**)	2	H. Nagamasu	5036	East Asia	T
mertensii Maxim.	3	A. Soejima	930208	East Asia	sT, T
micrococca Maxim.	79	S. Andrews	SA1498	East Asia	sT, T
microdonta Reiss.	118	G. Hatschbach	61·224	South America	T, Tm
mitis (L.) Radlk.	63	S. Andrews	SA1456	Africa	Tm
montana Torrey & A. Gray	121	S. Andrews	SA1546	USA	t
mutchagara Makino	95	S. Andrews	SA1506	East Asia	T
myrtifolia Walt.	94	S. Andrews	SA1504	USA	t, sT
nervulosa (Loes.) S. Andrews	165	S. Andrews	SA1573	South East Asia	T
nipponica Makino	161	H. Nagamasu	5534	East Asia	t
nitida (Vahl) Maxim.	80	G.J. Breckon	(−)	Jamaica, Puerto Rico	T
nothofagifolia Kingdon-Ward	82	S. Andrews	SA1499	East Asia	t
opaca Ait.	92	S. Andrews	SA1483	USA	t
oppositifolia Merr.	134	S. Andrews	SA1594	Borneo	Tm
paraguariensis A. St. Hil.	146	S. Andrews	SA1490	South America	T
pedunculosa Miq.	75	S. Andrews	SA1503	East Asia	t
perado Ait. ssp. *perado*	64	S. Andrews	SA1451	Canaries	t, sT
perado Ait.(6)	56	P. Ferard	(−)	Canaries	t, sT
perado Ait.(6)	120	S. Andrews	SA1489	Canaries	t, sT
pernyi Franch.	53	Youg-Ming Yuan	(−)	East Asia	T
pseudobuxus Reiss.	104	G. Giberti	BACP113	South America	T, Tm
pubescens Hook. & Arn.	69	S. Andrews	SA1459	East Asia	T
purpurea Hassk.	123	S. Andrews	SA1529	East Asia	sT, T
purpurea Hassk.	97	S. Andrews	SA1470	East Asia	sT, T
?quercetorum I.M. Johnston	126	S. Andrews	SA1531	America	?
repanda Griseb.	119	S. Andrews	SA1521	Bahamas, Cuba	T
retusa Klotzsch	H309	A.S. Tavares *et al.*	101	South America	T
revoluta Stapf	132	S. Andrews	SA1584	Borneo	Tm
rotunda Thunb. ex Murray	4	H. Nagamasu	5221	East Asia	sT, T
rotunda Thunb. ex Murray	167	S. Andrews	SA1569	East Asia	sT, T
rubra S. Watson	83	S. Andrews	SA1496	Mexico	t, Tm
rugosa F. Schmidt	16	H. Nagamasu	(−)	East Asia	t
rupicola Kunth	H310	M. Giler *et al.*	E-395 8A	South America	Tm
serrata Thunb. ex Murray	78	S. Andrews	SA1477	East Asia	t
shennongjiaensis Dudley & Sun	156	S. Andrews	SA1530	East Asia	t
spicata Blume	133	S. Andrews	SA1592	South East Asia	T, Tm
spinigera (Loes.) Loes.	93	S. Andrews	SA1502	Caucasus	t
sugerokii Maxim.	149	S. Andrews	SA1523	East Asia	t
teratopis (Loes.) Loes.	H10	Smith *et al.*	13150	South America	Tm
theezans Mart. ex Reiss.	107	G. Giberti	BACP116	South America	T, Tm
theezans Mart. ex Reiss.	115	G. Hatschbach	61·225	South America	T, Tm
theezans Mart. ex Reiss.	116	G. Hatschbach	61·223	South America	T, Tm
tolucana Hemsl.	89	S. Andrews	SA1491	Mexico, Honduras, Salvador	t, sT
triflora Blume	129	S. Andrews	SA1578	South East Asia	T, Tm
tsoii Merr. & Chun	151	S. Andrews	SA1528	East Asia	sT, T
verticillata (L.) A. Gray	59	P. Ferard	(−)	Canada, USA	t
viridis Champ.	113	Carl Tao Chen	no voucher	East Asia	sT, T
vomitoria Ait.	66	S. Andrews	SA1461	USA, Mexico	sT
wallichii Hook. f.	173	S. Andrews	SA1576	South East Asia	T
warburgii Loes.	1	H. Nagamasu	5033	East Asia	sT
wilsonii Loes.	86	S. Andrews	SA1500	East Asia	t, sT
yunnanensis Franch.	54	Bot. Gard. Munich	(−)	East Asia	t
yunnanensis Franch. (**7**)	157	S. Andrews	SA1508	East Asia	t
zhejiangensis C.J. Tseng	99	S. Andrews	SA1493	East Asia	sT
zygophylla Merr.	130	S. Andrews	SA1581	Borneo	Tm

Other specimens: *Nemopanthus mucronatus* (L.) Trel. (Savolainen nmuc G); *N. mucronatus* 153 (SA 1466); *Helwingia japonica* Dietr. (Kew 1953–24705); *Phyllonoma ruscifolia* Willd. (Asplund 10669).

cf, *confer*; x, hybrid; (−), voucher without number; T, tropical; t, temperate; sT, subtropical; Tm, tropical montane.

(1): f. *taitensis* (A. Gray) Nadeaud
(2): var. *paludosa* (Nakai) Hara
(3): ssp. *fargesii* var. *brevifolia* S. Andrews
(4): ssp. *melanotricha* (Merr.) S. Andrews
(5): var. *kanehirae* (Yamamoto) Yamazaki
(6): ssp. *platyphylla* (Webb. & Berth.) Tutin var. *platyphylla*
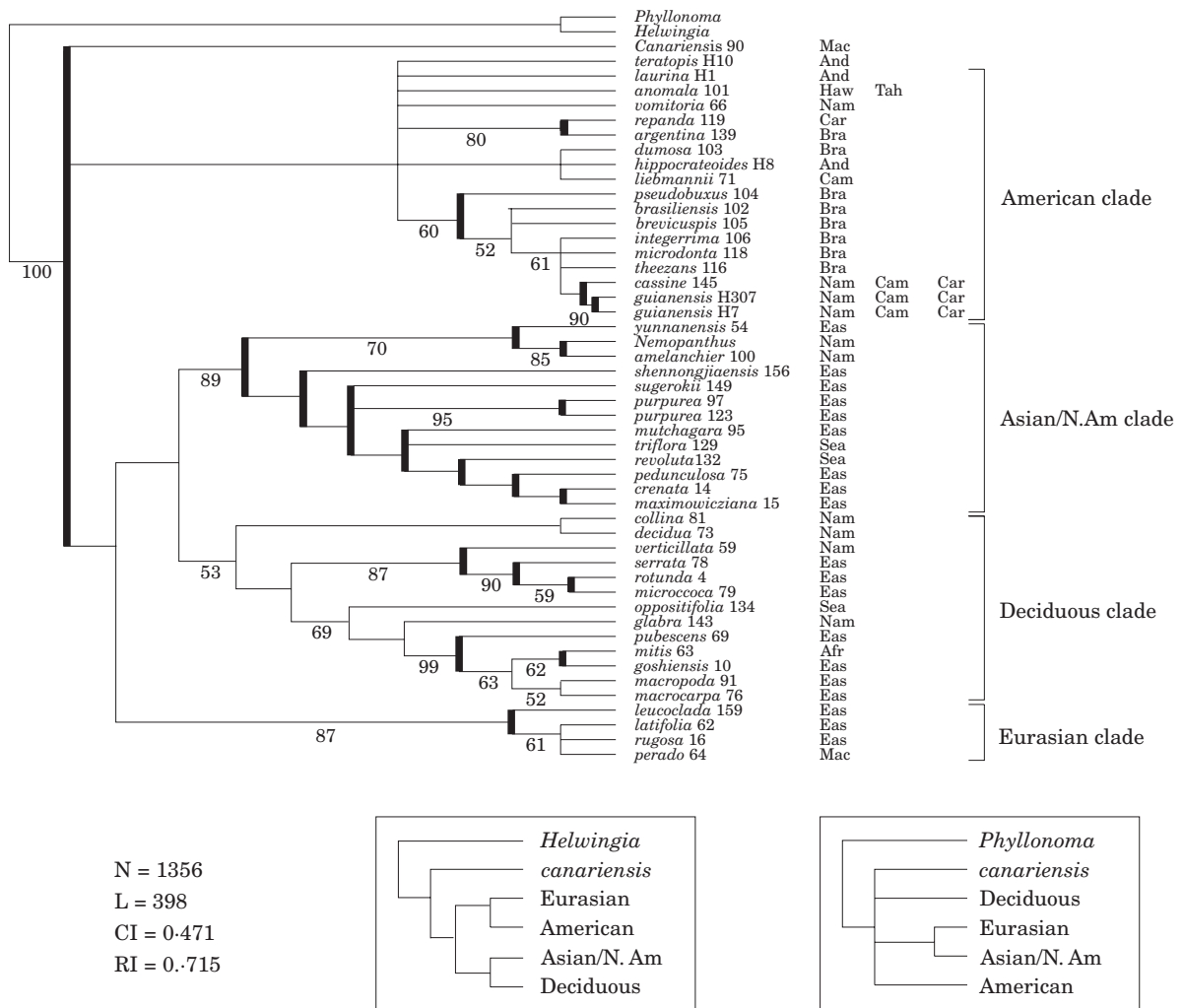(7): var. *gentilis* (Franch.) Loes.

FIG. 2. Strict consensus of successively weighted most parsimonious trees of 46 *Ilex* species and *Nemopanthus* produced by the combined data of the *atpB-rbcL* spacer and *rbcL*. Clades present in the equally weighted strict consensus tree are indicated by a thick bar. Bootstrap values are indicated below the branches. Clades described in the text are bracketed. N, Number of most parsimonious trees; L, length of the trees; CI, consistency index excluding uninformative sites; RI, retention index. Abbreviations of the geographic distribution are: Afr, Africa; And, Andes; Bra, Brazil; Cam, Central America; Car, Caribbean Islands; Eas, East Asia; Eur, Europe; Gui, Guiana; Haw, Hawaii/Tahiti; Mac, Macaronesia; Nam, North America; Sam, South America; Sea, South-east Asia.

specimens using only the *atpB-rbcL* spacer will be presented subsequently (Fig. 3).

Combined spacer and rbcL *trees for* 49 *representative specimens.* Figure 2 shows the strict consensus trees obtained with the combined *atpB-rbcL* spacer and *rbcL* data, with *Helwingia* and *Phyllonoma* as outgroups. The position of *Ilex canariensis* Poir. is unresolved within Aquifoliaceae. For the sake of convenience, the four major clades observed in the strict consensus are named according to geographical or ecological peculiarities of the component taxa: American clade, Eurasian clade, Asian/North American clade and Deciduous clade (this nomenclature is validated in the subsequent analysis of Fig. 3). The American clade, statistically poorly sustained, comprises all the South American species selected, a Mexican species, a North American species and the Hawaiian species. The Eurasian clade (*I. perado* Ait., *I. leucoclada* (Maxim.) Makino, *I.*

latifolia Thumb. ex Murray and *I. rugosa* F. Schmidt) is supported by a bootstrap value of 90 %. The monospecific genus *Nemopanthus*, closely related to *I. amelanchier* Curtis, is nested in the Asian/North American clade (bootstrap 79 %). Finally, the Deciduous clade (bootstrap 53 %) comprises all the Asian and North American deciduous species (excluding the tandem *Nemopanthus*/*I. amelanchier*) together with some evergreen species.

The core branches connecting the above-mentioned clades have no statistical support. Moreover, the use of either *Helwingia* or *Phyllonoma* as a unique outgroup affect the internal topology of the tree without changing the composition of the previously defined four clades (inset in Fig. 2). Nevertheless, except for the unresolved position of *I. canariensis*, this analysis suggests the division of Aquifoliaceae into four main groups.

Spacer trees for 142 specimens. Are these four main

groups conserved in the analysis using 142 specimens and the *atpB-rbcL* spacer? Figure 3 shows the consensus tree obtained with 142 specimens (116 species) using the *atpB-rbcL* spacer with *Helwingia* and *Phyllonoma* as outgroups. Specimens used in Fig. 2 (combined analysis) are indicated by filled circles to aid the comparison between Figs 2 and 3.

Contrary to expectations, when 142 specimens (instead of 49) are studied, with less DNA data, the large one-gene consensus tree obtained (Fig. 3) is relatively congruent with the small two-gene consensus tree of Fig. 2. This reinforces the soundness of the previously described clades.

The strict consensus tree shows that the three clades having a bootstrap value above 50 % in Fig. 2 (Eurasian clade, Asian/North American clade and Deciduous clade) are conserved in the large spacer tree. The American clade (poorly sustained in Fig. 2) is not resolved in Fig. 3. The only major incongruence between both trees is that *I. guianensis* (Aubl.) Kuntze (two specimens sequenced), previously nested in the American clade, is now a sister species of the Eurasian clade.

The Asian/North American clade comprises *Nemopanthus* closely related to *I. amelanchier* (two specimens sequenced) and to the Chinese *I. yunnanensis* Franch. This clade also contains 14 other Asian species, including *I. yunnanensis* var. *gentilis* (Franch.) Loes.

The Deciduous clade comprises North American and Asian species plus the African species *I. mitis* (L.) Radlk. This clade was already observed in the two-gene tree of Fig. 2. A feature of this clade is that it comprises all the deciduous species included in the analysis (22 deciduous species), except *I. amelanchier*, which is allied to *Nemopanthus* (also deciduous). It also contains 19 evergreen species together with the deciduous species.

The Eurasian clade is also observed in Fig. 2. It comprises exclusively Eurasian species represented by the clade *I. perado*, *I. leucoclada*, *I. latifolia* and *I. rugosa* observed above.

Unresolved in any particular clade, the last group comprises all the South American species (except *I. guianensis*, sister of the Eurasian clade), all Mexican species, all the evergreen species of North America (except the closely related species *I. glabra* (L.) A. Gray and *I. coriacea* (Pursh) Chapm.), all the Caribbean species, *I. anomala* Hook. & Arn. from the Pacific Islands and *I. canariensis* (two specimens sequenced), whose position was unresolved in Fig. 2. This group of species, whose position is unresolved, correspond to the American clade defined in Fig. 2 (in Fig. 3 it is named American group instead of American clade). It does not contain any Asian species.

Measure of the rate of nucleotide substitution in Aquifoliaceae

The molecular data could be used to answer an important question: are the extant *Ilex* species studied representative of the entire *Ilex* lineage indicated by the fossil record, or for only a part of it, because of past extinctions? In other words, is the common ancestor of the extant species at least 90 My old as indicated by the fossil record or is it more recent?

The mean rate of nucleotide substitution for *rbcL* in the 19 pairs of woody lineages studied by Albert *et al.* (1994) is $2 \cdot 05 \times 10^{-10}$ substitutions per site per year (range = $3 \cdot 01 \times 10^{-10}$, s.d. = $0 \cdot 75 \times 10^{-10}$). Taking the minimum value of 90 Mya for the ancestry of the Aquifoliaceae, the interspecific rate of nucleotide substitution for *rbcL*, calculated from Table 2, is rather low. For each of the pairs among *Nemopanthus*, *Ilex crenata* Thumb., *I. vomitoria* Ait., *I. perado* and *I. rotunda* Thumb. (a representative phylogenetic range of Aquifoliaceae, see above), the interspecific mean rate of nucleotide substitution is $1 \cdot 31 \times 10^{-10}$ substitutions per site per year (range $1 \cdot 16 \times 10^{-10}$, s.d. $0 \cdot 38 \times 10^{-10}$). This apparently rather slow rate of nucleotide substitution in *Ilex* was observed previously in a comparison with interspecific variation within the genus *Nothofagus* Blume, a woody lineage of approximatively the same age (Manen, Cuénoud and Martinez, 1998). If the value of the rate of nucleotide substitution in *Ilex*, calculated above, is used to calculate the time of divergence of the *Helwingia/Phyllonoma* pair, a value of at least 198 Mya is obtained, which is highly unlikely. Two possibilities could explain this low rate: either the rate of substitution of *Ilex* is actually relatively low compared with other lineages, or the extant (and/or sampled) species do not represent the entire 90 My old lineage but a more recent part of it, indicating past extinctions.

To answer this question, the relative test of the rate of nucleotide substitution (Wu and Li, 1985) was used. Using *Gonocaryum* as the reference taxon, Fig. 4 shows that the Aquifoliaceae do not seem to evolve more slowly than their close relatives *Helwingia* and *Phyllonoma*. Indeed, a divergence of 40 and 42 is observed in the comparison of *Gonocaryum/Phyllonoma* and *Gonocaryum/Helwingia*, respectively. On the other hand, a divergence of 45·6 is observed in the *Gonocaryum*/Aquifoliaceae comparison. Other reference taxa in the Asteridae give the same results, and this test calculated according to a more statistically reliable method (Li and Bousquet, 1992; Robinson *et al.*, 1998), gives the same conclusion (M. Robinson, pers. comm.). Thus, it is demonstrated that the 90 My old *Ilex* lineage is not fully represented by the extant species studied and that basal branches of the lineage are extinct. An examination of the fossil record of *Ilex* (Fig. 1) shows that the Eocene was an important time for the diversification of *Ilex*. This date might, in fact, be the time of ancestry for the extant *Ilex*. A close study of the many fossil records of *Ilex*-like pollen may show the nature of the extinct lineages. As expected, a study of the complete history of *Ilex* needs to include not only a phylogenetic study of the extant species, but also a precise study of the fossil record.

Biogeographic investigations

All geographic localities are included in this analysis except: North Australia (*I. arnhemensis* (F. Muell.) Loes.— closely related to extant South-east Asian species and probably not related to the ancient fossils found in South
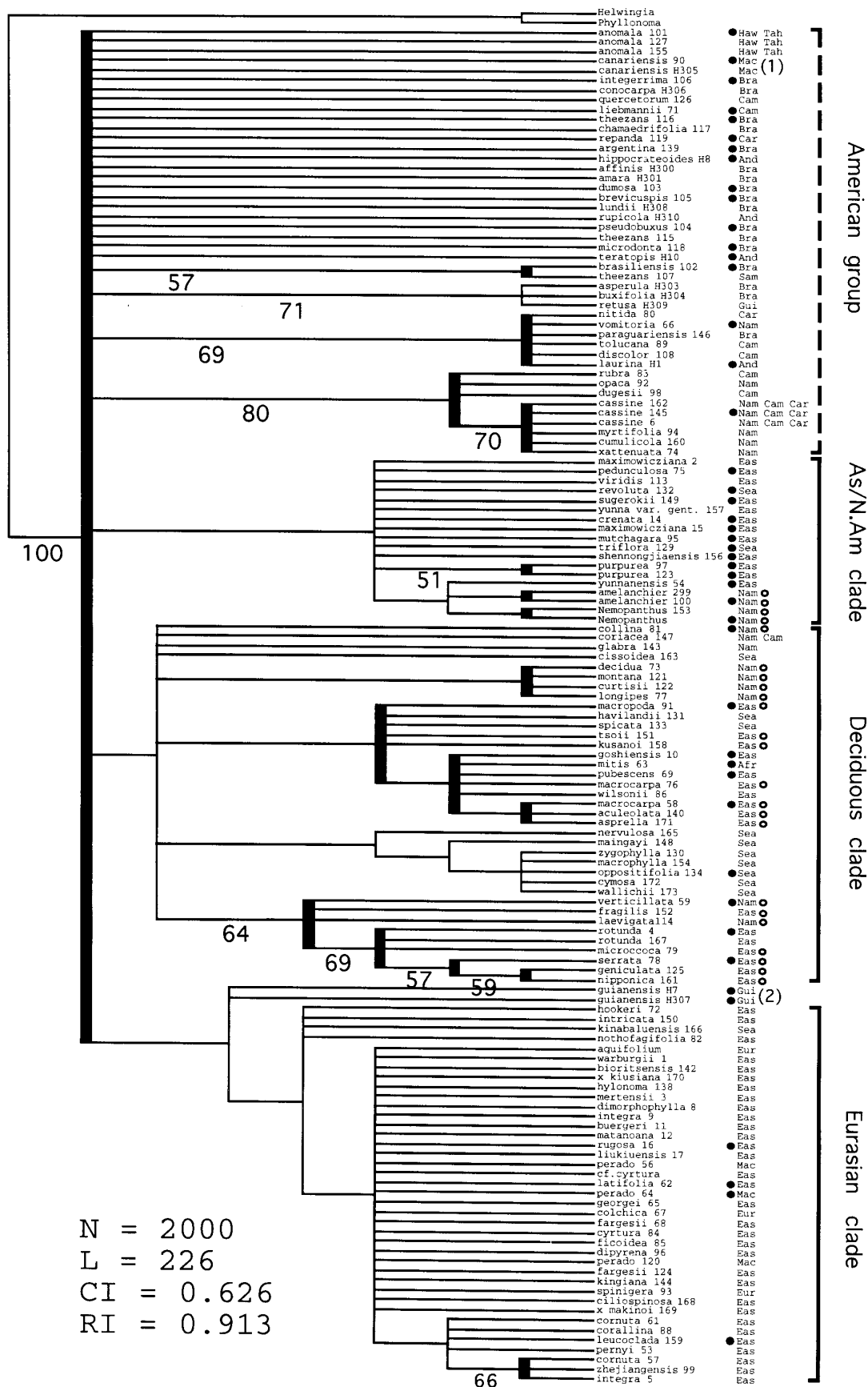
Helwingia
Phyllonoma
anomala 101 ● Haw Tah
anomala 127   Haw Tah
anomala 155   Haw Tah
canariensis 90 ● Mac
canariensis H305   Mac
integerrima 106 ● Bra
conocarpa H306   Bra
quercetorum 126   Cam
liebmannii 71 ● Cam
theezans 116 ● Bra
chamaedrifolia 117   Bra
repanda 119 ● Car
argentina 139 ● Bra
hippocrateoides H8 ● And
affinis H300   Bra
amara H301   Bra
dumosa 103 ● Bra
brevicuspis 105 ● Bra
lundii H308   Bra
rupicola H310   And
pseudobuxus 104 ● Bra
theezans 115   Bra
microdonta 118 ● Bra
teratopis H10 ● And
brasiliensis 102 ● Bra
theezans 107   Sam
asperula H303   Bra
buxifolia H304   Bra
retusa H309   Gui
nitida 80   Car
vomitoria 66 ● Nam
paraguariensis 146   Bra
tolucana 89   Cam
discolor 108   Cam
laurina H1 ● And
rubra 83   Cam
opaca 92   Nam
dugesii 98   Cam
cassine 162   Nam Cam Car
cassine 145 ● Nam Cam Car
cassine 6   Nam Cam Car
myrtifolia 94   Nam
cumulicola 160   Nam
xattenuata 74   Nam

(1)

American group

57
71
69
80
70

maximowicziana 2   Eas
pedunculosa 75 ● Eas
viridis 113   Eas
revoluta 132 ● Sea
sugerokii 149 ● Eas
yunna var. gent. 157 ● Eas
crenata 14 ● Eas
maximowicziana 15 ● Eas
mutchagara 95 ● Eas
triflora 129 ● Sea
shennongjiaensis 156 ● Eas
purpurea 97 ● Eas
purpurea 123 ● Eas
yunnanensis 54 ● Eas
amelanchier 299   Nam ○
amelanchier 100   Nam ○
Nemopanthus 153   Nam ○
Nemopanthus ● Nam ○

51

As/N.Am clade

collina 81   Nam Cam
coriacea 147   Nam
glabra 143   Sea
cissoidea 163   Nam ○
decidua 73   Nam ○
montana 121   Nam ○
curtisii 122   Nam ○
longipes 77 ● Eas ○
macropoda 91   Sea
havilandii 131   Sea
spicata 133   Eas ○
tsoii 151   Eas ○
kusanoi 158 ● Eas
goshiensis 10 ● Afr
mitis 63 ● Eas
pubescens 69   Eas ○
macrocarpa 76   Eas
wilsonii 86 ● Eas ○
macrocarpa 58   Eas ○
aculeolata 140   Eas ○
asprella 171   Sea
nervulosa 165   Sea
maingayi 148   Sea
zygophylla 130   Sea
macrophylla 154 ● Sea
oppositifolia 134   Sea
cymosa 172   Sea
wallichii 173 ● Nam ○
verticillata 59   Eas ○
fragilis 152   Nam ○
laevigata114 ● Eas
rotunda 4   Eas
rotunda 167   Eas ○
microccoca 79 ● Eas ○
serrata 78   Eas ○
geniculata 125   Eas ○
nipponica 161 ● Gui

Deciduous clade

64
69
57
59

guianensis H7 ● Gui
guianensis H307 ● Gui
hookeri 72   Eas
intricata 150   Eas
kinabaluensis 166   Sea
nothofagifolia 82   Eas
aquifolium   Eur
warburgii 1   Eas
bioritsensis 142   Eas
x kiusiana 170   Eas
hylonoma 138   Eas
mertensii 3   Eas
dimorphophylla 8   Eas
integra 9   Eas
buergeri 11   Eas
matanoana 12   Eas
rugosa 16 ● Eas
liukiuensis 17   Eas
perado 56   Mac
cf.cyrtura   Eas
latifolia 62   Eas
perado 64 ● Mac
georgei 65   Eas
colchica 67   Eur
fargesii 68   Eas
cyrtura 84   Eas
ficoidea 85   Eas
dipyrena 96   Mac
perado 120   Mac
fargesii 124   Eas
kingiana 144   Eas
spinigera 93   Eur
ciliospinosa 168   Eas
x makinoi 169   Eas
cornuta 61   Eas
corallina 88   Eas
leucoclada 159 ● Eas
pernyi 53   Eas
cornuta 57   Eas
zhejiangensis 99   Eas
integra 5   Eas

(2)

Eurasian clade

100

N = 2000
L = 226
CI = 0.626
RI = 0.913

66

FIG. 3. For legend see facing page.

TABLE 2. *Subtrees isolated by TASS from the trees of Figs 2 and 3*

	All branches	Supported branches only
Subtrees isolated from the combined analysis of 49 specimens corresponding to Fig. 2.		
American clade	(Car, Nam/Cam) Haw	idem
	(Bra, And) Haw	idem
	[(Cam/Car/Gui, Nam) Bra] Haw	idem
Eurasian clade	none	(Eas, Mac) Haw
A./N.Am. clade	none	[(Nam, Eas) Sea] Haw
Deciduous clade	[(Eas, Afr) Nam] Sea	[(Eas, Afr) Nam, Sea] Haw
		(Nam, Eas) Haw
Subtrees isolated from the spacer analysis of 142 specimens corresponding to Fig. 3.		
American clade	(Nam, Cam, Bra, And, Car) Haw	(Nam, Cam, Bra, And, Car) Haw, Sea, Eur, Mac
	(Cam/Car, Nam) Haw	(Cam/Car, Nam) Haw, Sea, Eur, Mac
	(Bra, Gui) Haw	(Bra, Gui) HT, Sea, Eur, Mac
Eurasian clade	{[(Eas, Eur, Mac) Sea] Cam/Gui/Car} Haw	none
A./N.Am. clade	[(Nam, Eas) Sea] Haw	none
Deciduous clade	[(Nam, Eas) Cam] Haw	[(Nam, Eas) Cam] Haw, Sea, Eur, Mac
	{[(Eas, Afr) Sea] Cam} Haw	(Eas, Afr) Haw, Sea, Eur, Mac

Areas separated by a slash (e.g. Cam/Car/Gui) mean that the information is given by a species for which the distribution overlaps the three areas. Abbreviations: as in Fig. 2.

FIG. 4. Pairwise patristic distance of *rbcL* (1428 nucleotides) between *Gonocaryum*, *Phyllonoma*, *Helwingia*, *Nemopanthus* and four representative species of *Ilex*. The mean distance of *Gonocaryum*/Aquifoliaceae is indicated, as well as the mean interspecific distance in the Aquifoliaceae. Following the calculation of Albert *et al.* (1994) and 90 Mya for the ancestry of the Aquifoliaceae, the interspecific mean rate of nucleotide substitution is: $16\cdot8/1428/90 \times 10^6 = 1\cdot31 \times 10^{-10}$ substitutions per site per year.

Australia; Martin, 1977); New Caledonia (*I. sebertii* Pancher); and Fiji (*I. vitiensis* A. Gray). We were unable to obtain fresh material or to extract DNA from herbarium specimens of these three species.

The phylogenies obtained were analysed with the software TASS (Nelson and Ladiges, 1996) and with the method of Bremer (1992). The principles of these analyses are explained in the Materials and Methods. The analyses were performed on both phylogenies depicted in Figs 2 and 3.

Subtree analysis using TASS. Results are shown in Table 2. Most of the subtrees have *I. anomala* at their base since it is the only species in its area, and because of its unresolved position. Consequently, the basal position of Hawaii has no significance as the ancestral area of origin of Aquifoliaceae (see the ancestral areas analysis below). On the other hand, the relationships observed between the brackets are informative.

A common East Asian/North American relationship

FIG. 3. Strict consensus of successively weighted most parsimonious trees of 140 *Ilex* specimens (116 species) and two specimens of *Nemopanthus* produced by the data of the *atpB-rbcL* spacer. Clades present in the equally weighted strict consensus tree are indicated by a thick bar. Bootstrap values are indicated below the branches. The 49 specimens studied in Fig. 2 are indicated by ●. Deciduous taxa are indicated by ○. Clades or groups described in the text are bracketed. (1) The position of *I. canariensis* is unresolved in Fig. 2. (2) *I. guianensis* belongs to the American clade in Fig. 2. Abbreviation as in Fig. 2.

TABLE 3. *Bremer coefficients for the different areas deduced from both analyses, using all branches (A) or supported branches only (B). The highest coefficient is given a value of 1.*

	49-specimen tree		142-specimen tree	
	A	B	A	B
Asia	0·83	1·00	0·79	1·00
East Asia	0·78	0·69	0·57	0·71
South-East Asia	0·11	0·11	0·09	0·07
N. + C. America	0·24	0·25	0·12	0·14
North America	0·20	0·20	0·14	0·12
Central America	0·14	0·15	0·14	0·06
South America	1·00	0·94	1·00	0·02
Guiana	0·04	0·05	0·04	0·01
Brazil	0·59	0·60	0·56	0·15
Andes	0·28	0·25	0·09	0·04
Hawaii/Tahiti	0·09	0·08	0·02	0·01
Macaronesia	0·26	0·14	0·02	0·01
Caribbean Islands	0·08	0·10	0·05	0·02
Europe	0·04	0·05	0·04	0·03
Africa	0·06	0·05	0·01	0·01

(Boufford and Spongberg, 1983) is found in both analyses and is observed in two different nodes defining the Asian/North American clade and the Deciduous clade. The 142-specimen tree gives further information by relating these two areas more closely to each other than to Central America, whereas the 49-specimen tree relates them more closely to each other than to South-east Asia.

Several complex relationships within the Americas and the Caribbean Islands are shown in the American clade in both analyses: North and Central America/South America, North and South America/Caribbean Islands, Brazil/ Guiana, Brazil/Andes.

Africa is more closely linked to East Asia than to South-east Asia as shown in the Deciduous clade (the position of North and Central America in these subtrees is congruent with the East Asian/North American relations discussed above).

Macaronesia is represented here by *I. canariensis* and *I. perado*. As the former species appears in an isolated unresolved phylogenetic position, it is considered paralogous information. The algorithm only keeps the information from *I. perado* that has a defined position. As such, the Eurasian clade indicates that Macaronesia is linked with Eurasia. In the 142-specimen tree, a relationship between Eurasia and the Americas is due to the placement of *I. guianensis* at the base of a large Eurasian clade. This information can be compared to an East Asian/North American relationship provided that only Central America is retained as relevant in the range of *I. guianensis*.

To summarize, regarding the main areas, several links exist between East Asia and North America on one hand, and between North and South America on the other, but no information is given about the relative position of these three areas to each other. Secondary areas are always closely related to one main area, except Hawaii, which is only distantly linked to the Americas.

Ancestral areas analysis. The results of ancestral areas analysis using the method of Bremer (1992) are shown in Table 3. The area with the highest coefficient is either Asia or South America depending whether calculations are performed with supported branches only or all branches, respectively. North America always has lower coefficients, and the remaining areas lie far behind, with the exception of Macaronesia in the analysis using the 49-specimen tree. As in the TASS analysis, the data cannot discriminate between Asia or South America as the area of origin.

DISCUSSION

Based on morphological and anatomical data, the study of general evolutionary trends in the Aquifoliaceae is rather difficult. Since the last monograph on the genus *Ilex* (Loesener, 1901, 1908, 1942), several botanists have tried to elucidate these trends from: (1) morphological and bio-geographical considerations (Hu, 1967; Martin, 1977); (2) leaf anatomy (Baas, 1975); (3) wood anatomy (Baas, 1973); (4) pollen morphology (Lobreau-Callen, 1975, 1977); and (5) a study of inflorescence structure (Loizeau and Spichiger, 1992; Loizeau, 1994; Coelho and Mariath, 1996). These authors all found disagreements when their data sets were compared against Loesener's work. The results presented here are also in disagreement with Loesener. The results presented here are based on a large DNA data set, yielding new independent evidence.

Taxonomic significance

The species analysed are representative of all five subgenera, all the series and most of the sections described by Loesener (1942). Sections which are missing only include *Myrsinoides* from East Asia and *Ledifoliae* from Brazil (both from the series *Aquifolium*). This work indicates that the general lines of the Loesener's system are inconsistent with the sequence data and opens new lines of investigation.

Both analyses show the nesting of *Nemopanthus* among the *Ilex* species, in association with *I. amelanchier*. The inclusion of *Nemopanthus* in the genus *Ilex* was suggested by Clark (1974). However he placed it close to *I. collina* Alexander rather than to *I. amelanchier*. Few characters (reduced and caducous calyx, free petals) differentiate this taxon, and one (free petals) is found in some *Ilex* species e.g. *I. collina*. Baas (1973, 1975), in his studies of wood and vegetative anatomy of the Aquifoliaceae, found almost no character unique to *Nemopanthus*. The nesting of this genus within *Ilex* is therefore not surprising.

The monospecific subgenus *Rybonia* Loes., consisting of *I. oppositifolia* Merr. is not isolated but nested in the Deciduous clade. It seems, therefore, that the opposite leaves characterizing this species are not an indication of a major separate lineage.

The species *I. teratopis* (Loes.) Loes., representing the monospecific subgenus *Yrbonia* Loes., characterized by the presence of two ovules per locules (instead of one), occurs in a position (the American clade) that gives no particular information on the validity of this subgenus.

Finally, the infrageneric classifications of Loesener and

Hu show only a few examples of agreement with the molecular phylogeny.

Geographical significance

North America, having links with both East Asia and South America, and a lower Bremer's coefficient than these two areas, has obviously been colonized from one or both of these areas. However, the presence of apparently ancestral species in both Asia and South America is not in agreement with the estimation that the common ancestor of the extant species studied dates from the Tertiary, since the two regions have been separated from each other since the Cretaceous.

If phylogeny is responsible for this discrepancy, it could be due to either inaccuracy of the phylogeny, or inadequate taxonomic sampling. It could also be due to a displacement of tropical North American species to South America during the late Tertiary/Quaternary, so that their assignment to South America is misleading in the identification of their origin. The latter hypothesis is congruent with the low abundance of fossils in South America throughout the Tertiary, relative to Asia (as seen in the Aquifoliaceae pollen fossil record database; Boulter and Manen, unpubl. res.), which contrasts with the very high diversity seen today.

The comparison of the results of both analyses (TASS and the ancestral areas analyses) shows that Africa and Europe have obviously been recently colonized from East Asia. Macaronesia shows a less clear pattern since its association with Europe and East Asia (due to *I. perado*) contrasts with its fairly high Bremer's coefficient (due to *I. canariensis*). The origin of *I. canariensis*, because of its phylogenetic isolation, is hard to determine. Takhtajan (1967) thought that this species, because of alleged relationships with some South American species, was indicative of trans-atlantic relationships dating from before the opening of this oceanic basin. However, this is not compatible with the estimation of the age of the species sampled for molecular analysis. In the absence of more information, *I. canariensis* can be thought of as associated with the mediterranean arcto-tertiary flora, which seems true of most of the flora of the region (Sundig, 1979). Some specimens from Tertiary deposits in the Rhône valley have indeed been assigned to this species (Bramwell, 1976), but the specific identification of fossil *Ilex* seems highly questionable.

CONCLUSIONS AND PERSPECTIVES

Excluding *I. canariensis* whose position is unresolved, the cladogram of Fig. 2 shows that the Aquifoliaceae is organized into four distinct groups, the hierarchy of which is still a matter of discussion. The four distinct groups have geographic and ecological peculiarities: one group is exclusively American, another is exclusively Eurasiatic, the last two are in accordance with the well-documented Asian/North American disjunction, one of them comprising all the deciduous species analysed (except the tandem *Nemopanthus*/*I. amelanchier*). On the other hand, geographic correlations are not apparent in the classification of Loesener, whereas the deciduous habit diagnoses its subgenus *Prinus*. However, in this study, the deciduous species are grouped in one clade, but together with other evergreen species. This would indicate that the deciduous habit represents an ancestral state that is lost in other taxa, or a derived state appearing frequently in the Deciduous clade.

The exact position of the root of Aquifoliaceae is still to be refined. An interesting discrepancy exists concerning the position of *I. guianensis*, which is nested in the American clade in Fig. 2, but at the base of the Eurasian clade in Fig. 3. Moreover, the use of *Helwingia* or *Phyllonoma* as an outgroup gives exactly the same subdivisions of Aquifoliaceae, but with a different hierarchy. In the case of *Helwingia* alone, a relationship between the Eurasian clade and the American clade is observed (inset in Fig. 2). This is to be correlated with the position of *I. guianensis* at the base of the Eurasian clade in Fig. 3. More data are necessary to clarify the hidden phylogeny of Aquifoliaceae.

Croizat (1952), Hu (1967), Martin (1977) and Aubréville (1976) have discussed the phytogeographic hypotheses concerning the diversification of the genus *Ilex*. But their hypotheses are based mainly on the classification system of Loesener, which appears incorrect. Thus, these hypotheses must be re-examined in the light of data presented here and further data accumulating.

No information can be drawn from the phylogeny for the moment regarding early events. Similar analyses performed on the Asterales by Bremer and Gustafsson (1997) indicate an origin of the group (which is sister to the Aquifoliales) in east Gondwana. The same could be true for *Ilex*. But since we have demonstrated that the ancestry of the sampled extant species dates from the Tertiary only, traces for a Gondwanian origin of the Aquifoliaceae are difficult to investigate directly using molecular data only. In this respect, the position of *I. arnhemensis* (Australia), *I sebertii* (New Caledonia) and *I. vitiensis* (Fiji) in the phylogenetic tree should be verified. However, from the morphological or anatomical data currently available, there is no indication of particular ancestral characters in these three species.

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