

The complex history of the genus *Ilex* L. (Aquifoliaceae): evidence from the comparison of plastid and nuclear DNA sequences and from fossil data

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Abstract. A plastid phylogeny of the genus *Ilex* based on three different loci (the *atpB-rbcL* spacer, *trnL-trnF* and *rbcL*) is compared with its nuclear phylogeny based on two different loci (the ribosomal ITS and the 5S RNA spacer). These two sets of molecular data are then compared to geographical and temporal data from the fossil record. The plastid phylogeny is strongly correlated with the geographic distribution of extant species. However, the nuclear phylogeny is strongly incongruent with the plastid phylogeny, suggesting frequent interlineage hybridizations. Moreover, the comparison of the ribosomal ITS tree and the 5S RNA spacer tree indicates also possible lineage sorting. Particularly interesting is the finding of two different *Ilex* lineages in the plastid American clade showing different biogeographic patterns in South America. One of them has a simple North American/South American biogeographical relationship. The other has complex biogeographical relationships, some species showing direct Asian/South American biogeographical relationships. During its history, the genus *Ilex* probably experienced frequent lineage sorting and interlineage hybridization with subsequent nuclear or cytoplasmic introgression, making the study of its history very complex.

Key words: Aquifoliaceae, plastid DNA (*atpB-rbcL* spacer, *rbcL* and *trnL-trnF*), fossil record,

phylogeography, *Ilex* L., molecular phylogeny, nuclear DNA (ITS and 5S RNA spacer).

Introduction

The cosmopolitan genus *Ilex* (Aquifoliaceae), comprising more than 400 tropical and temperate species, represents a particularly interesting lineage in the field of the phylogeography, even though its phylogeny is still not fully resolved. The last monograph of Loesener (1901, 1908, 1942) is not supported by more recent biosystematic studies (Baas 1973, 1975; Lobreau-Callen 1975, 1977; Loizeau and Spichiger 1992; Loizeau 1994), nor by chloroplast DNA sequence comparisons (Cuénoud et al. 2000). The main areas of extant diversification of *Ilex* are East Asia and South America. The genus is also well represented in South East Asia, Central America 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. A few species occur in Hawaii, Tahiti, the Caribbean, the Canary Islands, the Azores,

Madeira, New Caledonia and Fiji. The genus *Ilex* is suggested a typical Arcto-Tertiary lineage (Boufford and Spongberg 1983, Lavin and Luckow 1993), with a recent colonisation of South America from North America, late in the Tertiary (Burnham and Graham 1999). However, because of the occurrence of Cretaceous pollen of *Ilex* in South Australia and in other part of the world (Martin 1977), the lineage was claimed to be cosmopolitan in the late Tertiary (Cronquist 1988). A Gondwanan origin of the genus has been suggested, with subsequent migrations to Eurasia and North America and massive extinctions in Africa and in Australia (Raven and Axelrod 1974).

The recent analysis of two chloroplast DNA fragments (*rbcL* and the *atpB-rbcL* spacer, Cuénoud et al. 2000) in 116 *Ilex* species allowed several inferences: the common ancestor of the extant lineage *Ilex* is much younger (probably Eocene) than the age predicted from the fossil record (Turonian, Cretaceous) by Martin (1977). This could be explained either by the extinction of its basal branches, or because the Cretaceous records of *Ilex* are wrong, or because the lineage *Ilex* had a particularly slow rate of nucleotide substitution compared with that of its sister group *Helwingia* Willd./*Phyllonoma* Willd. ex Schultes. This last possibility was rejected by the relative rate test (Cuénoud et al. 2000). According to this work, other inferences can be drawn: Several Asian/North American disjunctions are observed, as well as a North American/South American biogeographical relationship. The plastid DNA tree substantially contradicts the systematics of Loesener (1942). *Nemopanthus* (now *Ilex mucronata*, a new combination used here, Powell et al. 2000), closely related to *Ilex amelanchier*, is nested in the genus *Ilex*, which is organized in four clades, each of them having geographical distribution peculiarities. One clade exclusively comprises Eurasian species (the Eurasian clade) and another one comprises exclusively American species (the

American clade). Two additional clades comprise species from North America and Asia together. One of them, strongly sustained, comprises *Ilex mucronata* (the Asian/North American clade I). The other one comprises, among evergreen species, all the deciduous species, except the tandem *Ilex mucronata* and *Ilex amelanchier* (the Asian/North American clade II). The phylogenetic position of *Ilex canariensis* is undefined. From these data it was not possible to deduce the hierarchy between these clades. A relationship between the Eurasian clade and the American clade was suggested but not supported.

Using chloroplast RFLPs, *trnL-trnF* sequencing and nuclear ITS sequencing, a recent study of Asian *Ilex* of the Bonin Island and of the Ryukyu Island (Setoguchi and Watanabe 2000) demonstrates that hybridization played a role in this region, leading to interspecific introgressions independently observed on both Islands. The role of interspecific hybridization in plant evolution is now well documented on the basis of DNA data (Rieseberg 1995, Rieseberg et al. 1996, Arnold 1997). Natural interspecific hybridization does occur in *Ilex*, and controlled interspecific crossing has also been successful (Galle 1997). This could explain the difficulties encountered by students of the genus. Another problem is the very large genetic distance between *Ilex* and its so far considered closest relatives *Helwingia* and *Phyllonoma* (APG 1998) used in the analysis as outgroup. In a phylogeographical perspective, this makes the rooting of the genus *Ilex* difficult.

In this work, adding one more cpDNA fragment (*trnL-trnF*), and the sequences of two nuclear regions (ITS and the 5S RNA spacer) to previous data (Cuénoud et al. 2000) allowed the rooting of the genus *Ilex*. This leads to a discussion about biogeographical patterns of the lineage and the characterization of two different lineages in South America. As a general survey on the difficult genus *Ilex*, this paper will provide a starting point for further studies.

Materials and methods

DNA sequencing

The amplification and sequencing of the *atpB-rbcL* spacer and of *rbcL* were previously described (Cuénoud et al. 2000). The *trnL-trnF* region was amplified and sequenced according to Taberlet et al. (1991), the ITS region according to White et al. (1990), and the 5S DNA spacer according to Cox et al. (1992). Because of sequence heterogeneity in ITS and in the 5S RNA spacer for a large number of individuals (12 specimens for ITS, and 2 specimens for the 5S DNA spacer), it was necessary to clone the amplified fragment. The PCR-script cloning kit (Stratagene) was used and 4 different clones were sequenced for these specimens to have a representation of the polymorphism. A strict consensus tree was produced from all the cloned ITS and all the directly sequenced ITS together. It indicates (data not shown) that, except for *I. dumosa* and *I. brevicuspis* (of which two divergent copies are presented in Fig. 2), all the clones from one species are monophyletic (only one copy is presented in Fig. 2. see Denduangboripant and Cronk 2000). Two specimens (*I. anomala* and *I. brevicuspis*) were found to have different copies of the 5S RNA spacer, which were monophyletic.

Specimens and DNA matrices

Two new matrices were build up with 49 representative *Ilex* specimens (47 species, see Table 1) exactly corresponding to the specimens used by Cuénoud et al. (2000). The plastid matrix contains the *atpB-rbcL* spacer, *rbcL* and *trnL-trnF*. The nuclear matrix contains the ribosomal internal transcribed spacer (ITS 1, 5.8S rDNA and ITS 2) and the 5S RNA spacer. The 5S RNA spacer (234 sites) of the outgroup *Helwingia* and *Phyllonoma*, being unalignable with *Ilex*, was coded with question marks, as well as *I. laurina* for which the 5S RNA spacer is not available. Alignments were performed manually and some regions that could not be aligned with confidence were excluded from the analyses. Gaps were scored as missing data and indels coded as 0 (absence) or 1 (presence). Matrices are available on the web (<http://www.cjb.unige.ch/recherche/bio-mol/aquifol/aquifol.html>).

Phylogenetic analysis

As in Cuénoud et al. (2000), the outgroup used to study the genus *Ilex* comprises *Helwingia* and *Phyllonoma*, so far the closest relatives of the genus *Ilex* (APG 1998). However, since this outgroup is still too distantly related to *Ilex* (Cuénoud et al. 2000), a simple outgroup sequence was constructed from only those positions that are invariant between *Helwingia* and *Phyllonoma*, all other positions being scored as question marks. This decreases the chance for spurious ingroup branches pairing with the outgroup (Smith 1994).

All the analyses were done using the heuristic search option of PAUP 4.0b3a (Swofford 2000). Trees were produced by heuristic searches with tree bisection reconnection (TBR) swapping. To avoid the production of only one island of solutions, 100 replications with RANDOM addition of taxa were carried out, saving no more than the first 100 most parsimonious trees.

All trees shown are strict consensus obtained after successive weighting according to the maximum RC values of characters. The clades observed in the starting equally weighted analyses are identified with a thick bar. The support of clades was estimated by 1000 bootstrap replications with TBR branch swapping, 10 trees being kept at each replicate.

Congruence assessments

The extent of incongruity between the plastid tree and the nuclear tree was evaluated using the Wilcoxon signed ranks test (WSR) according to Mason-Gamer and Kellogg (1996) using the consensus and the 70% bootstrap plastid and nuclear trees on both the plastid and the nuclear matrices, respectively.

The Kimura 2-parameter pairwise distance matrices of the plastid and nuclear sequences were calculated using PAUP. The resulting distance matrices were compared to determine whether both the nuclear and plastid matrices are correlated. A Mantel test was computed for this purpose, using F-stat programme (Goudet 1995).

Biogeography

Plastid (Fig. 1) and nuclear (Fig. 2) phylogenetic trees were analyzed with TASS (Nelson and

Table 1. List of *Ilex* specimens and species used in this work with their geographical distribution and the accession numbers of the DNA sequences. Voucher informations are published in Cuénoud et al. (2000)

Species	Sample No.	Distribution	<i>atpB-rbcL</i>	<i>rbcL</i>	<i>trnL-trnF</i>	ITS	5S spacer
<i>amelanchier</i> Curtis	100	USA	AF471593	X98722	AJ275349	AJ275340	AJ492893
<i>anomala</i> Hook. & Arn.	101	Hawaii	AF471594	X98723	AJ492567	AJ492652	AJ492876
<i>argentina</i> Lillo	139	South America	AF471592	AJ492699	AJ492570	AJ492655	AJ492878
<i>brasiliensis</i> (Spreng.) Loes.	102	South America	AF471600	X98735	AJ492575	AJ492661	AJ492883
<i>brevicuspis</i> Reiss.	105	South America	AF471603	X98719	AJ492576	AJ492662/3	AJ492884
<i>canariensis</i> Poir.	90	Canary Islands	AF471608	X98727	AJ275348	AJ275339	AJ492875
<i>cassine</i> L.	145	USA, Mexico, Bahamas, Cuba	AF471620	AJ492704	AJ492580	AJ492667	AJ492888
<i>collina</i> Alexander	81	East Asia	AF471615	AJ492716	AJ492592	AJ492679	AJ492904
<i>crenata</i> Thunb. ex Murray	14	East Asia	AF471618	L01928	AJ492590	AJ492677	AJ492902
<i>decidua</i> Walt.	73	USA	AF471626	X98724	AJ492593	AJ492680	AJ492905
<i>dumosa</i> Reiss.	103	South America	AF471630	X98725	AJ492571	AJ492656/7	AJ492879
<i>glabra</i> (L.) A. Gray	143	Canada, USA	AF471639	AJ492720	AJ275351	AJ275342	AJ492911
<i>goshiensis</i> Hayata	10	South America	AF471640	X98734	AJ492600	AJ492687	AJ492914
<i>guianensis</i> (Aubl.) Kuntze	H7	South America-Mexico	AF471642	AJ492706	AJ492582	AJ492669	AJ492890
<i>guianensis</i> (Aubl.) Kuntze	H307	South America-Mexico	AF471637	AJ492705	AJ492581	AJ492668	AJ492889
<i>hippocrateoides</i> Kunth	H8	South America	AF471644	AJ492724	AJ492572	AJ492658	AJ492880
<i>integerrima</i> (Vell.) Reiss.	106	South America	AF471649	X98726	AJ492577	AJ492664	AJ492885
<i>latifolia</i> Thunb. ex Murray	62	East Asia	AF471655	X98731	AJ492604	AJ492691	AJ492918
<i>laurina</i> Kunth	H1	South America	AF471660	AJ492730	AJ492566	AJ492651	–
<i>leucoclada</i> (Maxim.) Makino	159	East Asia	AF471656	AJ492728	AJ492603	AJ492690	AJ492917
<i>liebmannii</i> Stanley	71	Central America, Mexico	AF471659	AJ492700	AJ492573	AJ492659	AJ492881
<i>macrocarpa</i> Oliver	76	East Asia	AF471663	AJ492727	AJ492602	AJ492689	AJ492916
<i>macropoda</i> Miq.	91	East Asia	AF471662	AJ492726	AJ492601	AJ492688	AJ492915
<i>maximowicziana</i> Loes.	15	East Asia	AF471674	AJ492715	AJ492591	AJ492678	AJ492903
<i>micrococca</i> Maxim.	79	East Asia	AF471670	X98721	AJ492597	AJ492684	AJ492909
<i>microdonta</i> Reiss.	118	South America	AF471665	AJ492702	AJ492578	AJ492665	AJ492886
<i>mitis</i> (L.) Radlk.	63	Africa	AF471667	X98730	AJ275347	AJ275338	AJ492913
<i>mucronata</i> (L.) M. Powell & al.	(–)	Canada, USA	AF471672	X69747	AJ275345	AJ275336	AJ492892
<i>mutchagara</i> Makino	95	East Asia	AF471673	AJ492712	AJ492587	AJ492674	AJ492898
<i>oppositifolia</i> Merr.	134	Borneo	AF471680	AJ492719	AJ492598	AJ492685	AJ492910
<i>pedunculosa</i> Miq.	75	East Asia	AF471682	X98728	AJ275350	AJ275341	AJ492901

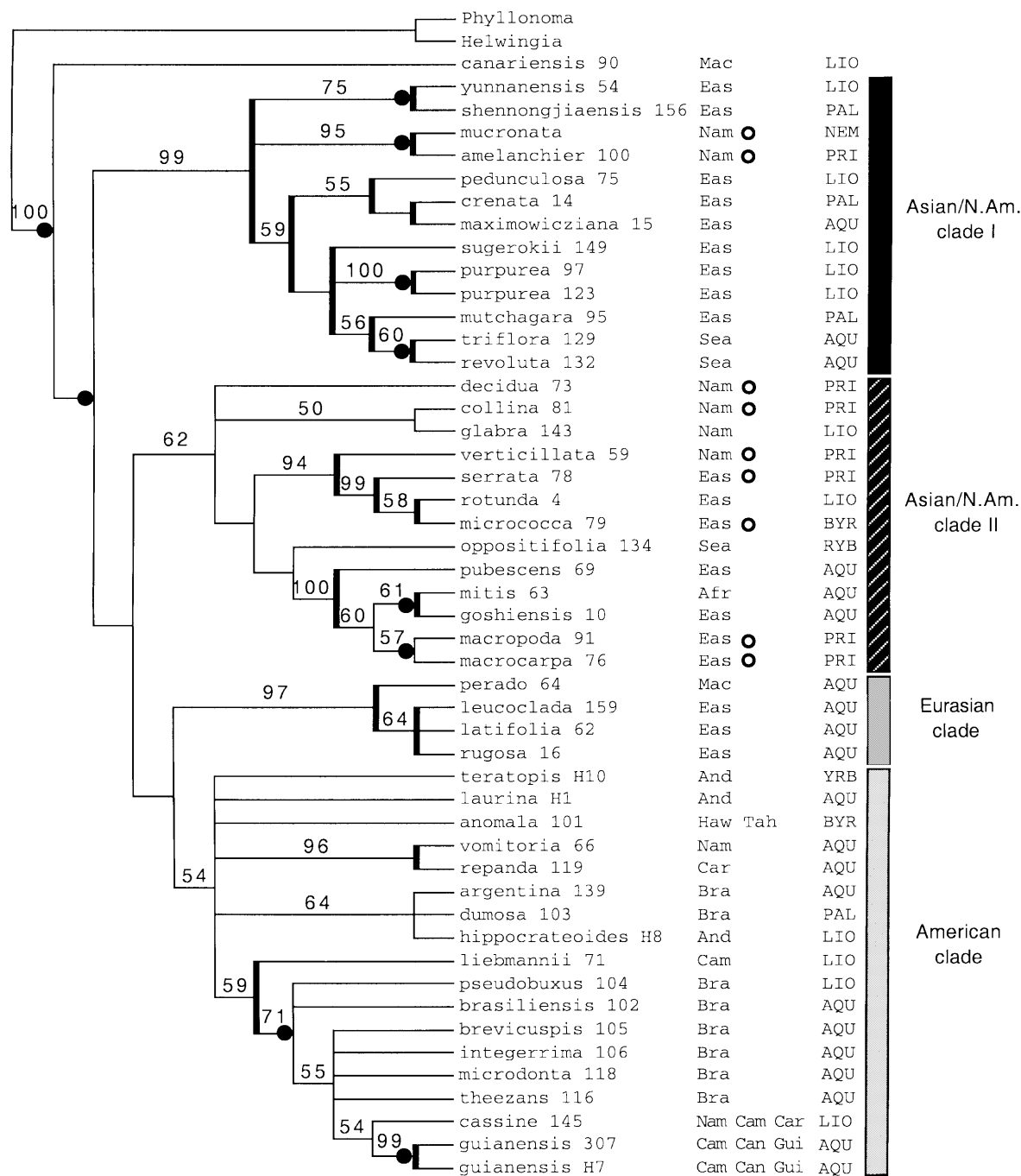
Table 1 (continued)

<i>perado</i> Ait. ssp. <i>perado</i>	64	Canaries	AF471687	X98729	AJ492606	AJ492693	AJ492920
<i>pseudobuxus</i> Reiss.	104	South America	AF471688	X98736	AJ492574	AJ492660	AJ492882
<i>pubescens</i> Hook. & Arn.	69	East Asia	AF471689	AJ492722	AJ492599	AJ492686	AJ492912
<i>purpurea</i> Hassk.	123	East Asia	AF471690	AJ492711	AJ492586	AJ492673	AJ492897
<i>purpurea</i> Hassk.	97	East Asia	AF471681	AJ492710	AJ492585	AJ492672	AJ492896
<i>repanda</i> Griseb.	119	Bhramas, Cuba	AF471694	AJ492697	AJ492569	AJ492654	AJ492821
<i>revoluta</i> Stapf	132	Borneo	AF471699	AJ492714	AJ492589	AJ492676	AJ492900
<i>rotunda</i> Thunb. ex Murray	4	East Asia	AF471695	X98720	AJ492596	AJ492683	AJ492908
<i>rugosa</i> F. Schmidt	16	East Asia	AF471698	X98733	AJ492605	AJ492692	AJ492919
<i>serrata</i> Thunb. ex Murray	78	East Asia	AF471704	AJ492718	AJ492595	AJ492682	AJ492907
<i>shenmongjiaensis</i> Dudley & Sun	156	East Asia	AF471701	AJ492707	AJ492583	AJ492670	AJ492894
<i>sugerokii</i> Maxim.	149	East Asia	AF471702	AJ492709	AJ492584	AJ492671	AJ492895
<i>teratopsis</i> (Loes.) Loes.	H10	South America	AF471707	AJ492695	AJ492564	AJ492649	AJ492874
<i>theezans</i> Mart. ex Reiss.	116	South America	AF471709	AJ492703	AJ492579	AJ492666	AJ492887
<i>triflora</i> Blume	129	South East Asia	AF471711	AJ492713	AJ492588	AJ492675	AJ492899
<i>verticillata</i> (L.) A. Gray	59	Canada, USA	AF471713	AJ492717	AJ492594	AJ492681	AJ492906
<i>vomitorea</i> Ait.	66	USA, Mexico	AF471715	M88583	AJ492568	AJ492653	AJ492877
<i>yunnanensis</i> Franch.	54	East Asia	AF471724	X98732	AJ275346	AJ275337	AJ492891

Ladiges 1995, 1996) in order to examine area relationships as in Cuénoud et al. (2000). The ancestral areas of origin were investigated from the plastid and the nuclear phylogenies with the method of Bremer (1992) as in Cuénoud et al. (2000).

Fossil data

The “Global Plotter” program (<http://ibs.uel.ac.uk/ibs/palaeo/datasys/plot>) was used to search and plot *Ilex* fossil data from the plant fossil database, according to the geological age of the records. *Ilex* fossil records were extracted using the



key words *Ilex*, *Ilexpollenites* and Aquifoliaceae from the “Genus Distribution” database (pass-word: ilex).

Results

The plastid DNA tree of Ilex

The strict consensus tree produced from the combined plastid data (the *atpB-rbcL* spacer, *rbcL* and the *trnL-trnF* region) is shown in Fig. 1. It comprises the 47 *Ilex* species (49 specimens) selected from the four previously defined groups on the *atpB-rbcL* spacer tree of 142 specimens (Cuénoud et al. 2000). The matrix comprises 3000 sites, with 238 variable characters, of which 119 are potentially informative. The 864 most parsimonious trees are 360 steps long, have a consistency index (CI) of 0.54, excluding uninformative sites, and a retention index (RI) of 0.77. Aside from the basal position of *I. canariensis*, the genus *Ilex* is organized into four clades having the same composition and characteristics that were previously described by Cuénoud et al. (2000) in a study based on 142 *Ilex* specimens. The addition of one more DNA fragment in the plastid matrix allows a better resolution of the internal branches and gives more support for the four clades. A well sustained branch (bootstrap 99%) defines the Asian/North American clade I comprising *I. mucronata* (formerly known as *Nemopanthus*). This clade is sister to all of the other *Ilex* species (except the basal *I. canariensis*), which are organised in three clades: the Asian/North

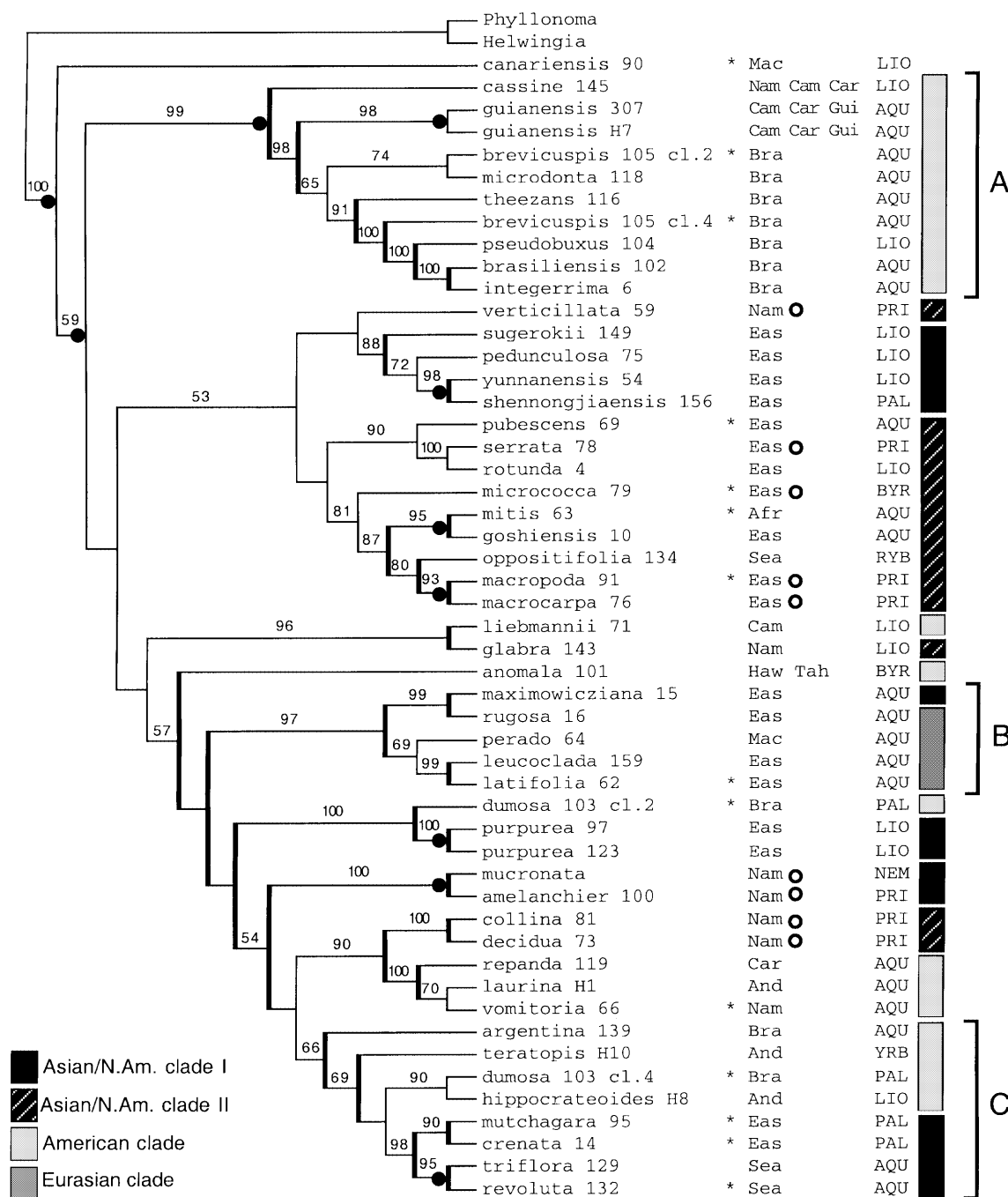
American clade II, comprising among evergreen species all the deciduous species, except the tandem *I. mucronata* and *I. amelanchier* from clade I (bootstrap 62%), the Eurasian clade (bootstrap 97%) and the American clade (bootstrap 54%). The Eurasian clade and the American clade are linked (but with poor support), as suggested previously (Cuénoud et al. 2000).

The nuclear DNA tree of Ilex

The strict consensus tree produced from the combined nuclear data (ITS and the 5S RNA spacer) is shown in Fig. 2. For two species (*I. dumosa* and *I. brevicuspis*) the ITS polymorphic sequences were not monophyletic and two divergent copies of ITS were included in the DNA matrix. The matrix comprises 991 sites, with 562 variable characters, of which 406 are potentially informative. The unique most parsimonious tree is 1433 steps long, has a CI of 0.49, excluding uninformative sites, and a RI of 0.65. As in the chloroplast tree, *I. canariensis* is still the most basal species of the genus (bootstrap 59%). However the internal topology of the nuclear tree is very different. Most of the plastid clades previously defined are polyphyletic. From the top of Fig. 2, one subclade of the previously defined American clade (clade A), comprising *I. pseudobuxus*, *I. brasiliensis*, *I. brevicuspis*, *I. integerrima*, *I. microdonta*, *I. theezans*, *I. cassine* and *I. guianensis* (bootstrap 71% in the plastid tree) is conserved in

Fig. 1. Plastid strict consensus of successively weighted most parsimonious trees and clade designation. Clades present in the equally weighted strict consensus tree are indicated by a thick bar. Bootstrap values are indicated above the branches. The deciduous species are indicated by empty dots. Shared clades between the plastid (Fig. 1) and the nuclear (Fig. 2) trees are indicated by filled dots. Abbreviations of the geographic distribution used in Figs. 1, 2 and 3 are alphabetically: *Afr* = Africa, *And* = Andes, *Bra* = Brazil, *Cam* = Central America, including South Mexico, *Can* = Canary Islands, *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. Abbreviations indicating the classification of Loesener (1942) used in Figs. 1 and 2 are: Genus *Nemopanthus* = *Nem*; Genus *Ilex*, subgenus *Rybonia* = *Ryb*; subgenus *Byronia* = *Byr*; subgenus *Yrbonia* = *Yrb*; subgenus *Prinus* = *Pri*; subgenus *Euilex*, series *Lioprinus* = *Lio*; series *Paltoria* = *Pal*; series *Aquifolium* = *Aqu*

I. yunnanensis and *I. shennongjiaensis*). The third large clade (at the bottom of Fig. 2) comprises species from the 4 previously defined chloroplast clades. In this clade, the Eurasian clade forms a clade (clade B) with *I. maximowicziana* from the Asian/North



American clade I. A group of South American species (*I. argentina*, *I. teratopis*, *I. dumosa* (clone 4) and *I. hippocrateoides*) makes a clade (clade C) with species of the Asian/North American clade I (*I. mutchagara*, *I. crenata*, *I. triflora* and *I. revoluta*). Most of the terminal clades of the nuclear tree are relatively well supported, but the hierarchy between these clades, suggested by the strict consensus, is not supported statistically.

In order to determine whether the nuclear tree and the plastid tree are significantly incongruent, the WSR test was performed on character steps using one or the other alternative tree topology. Table 2 indicates that both topologies are statistically and exclusively different. On the other hand, the Mantel test indicates that the correlation between the Kimura 2-parameter pairwise distance matrices of the plastid and nuclear sequences is significantly high (correlation 0.780, standard deviation 0.097, $P = 0.005$).

Because of the sequence polymorphism observed in several individuals, lineage sorting

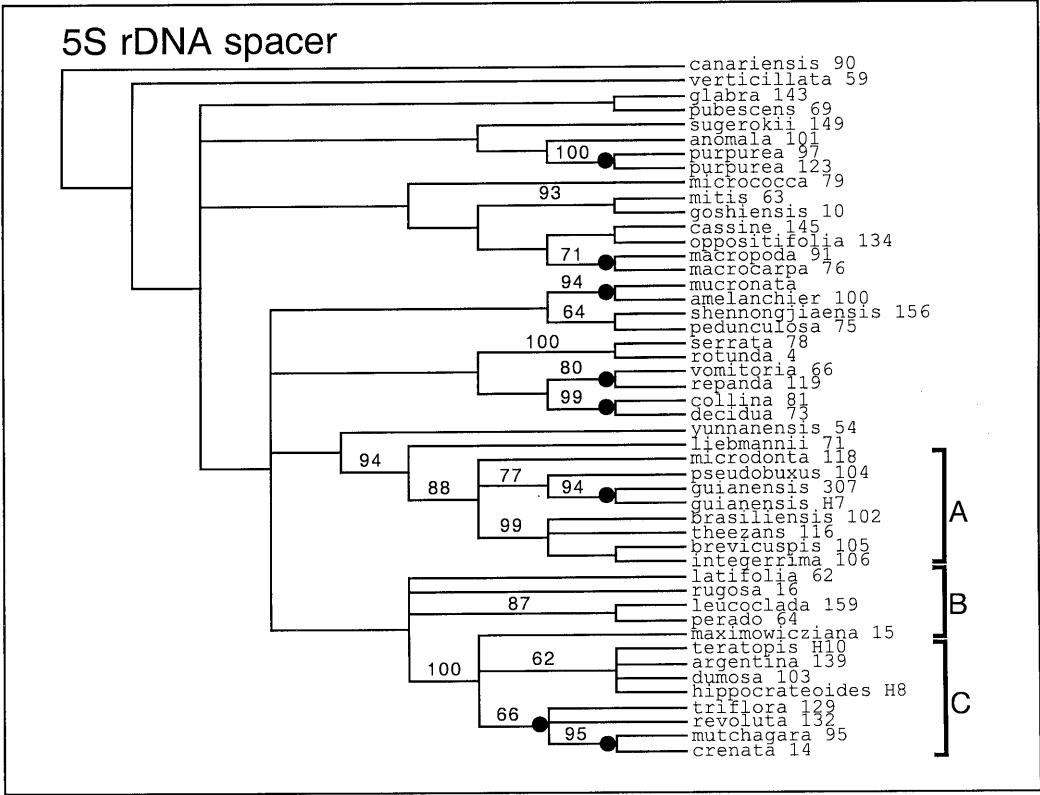
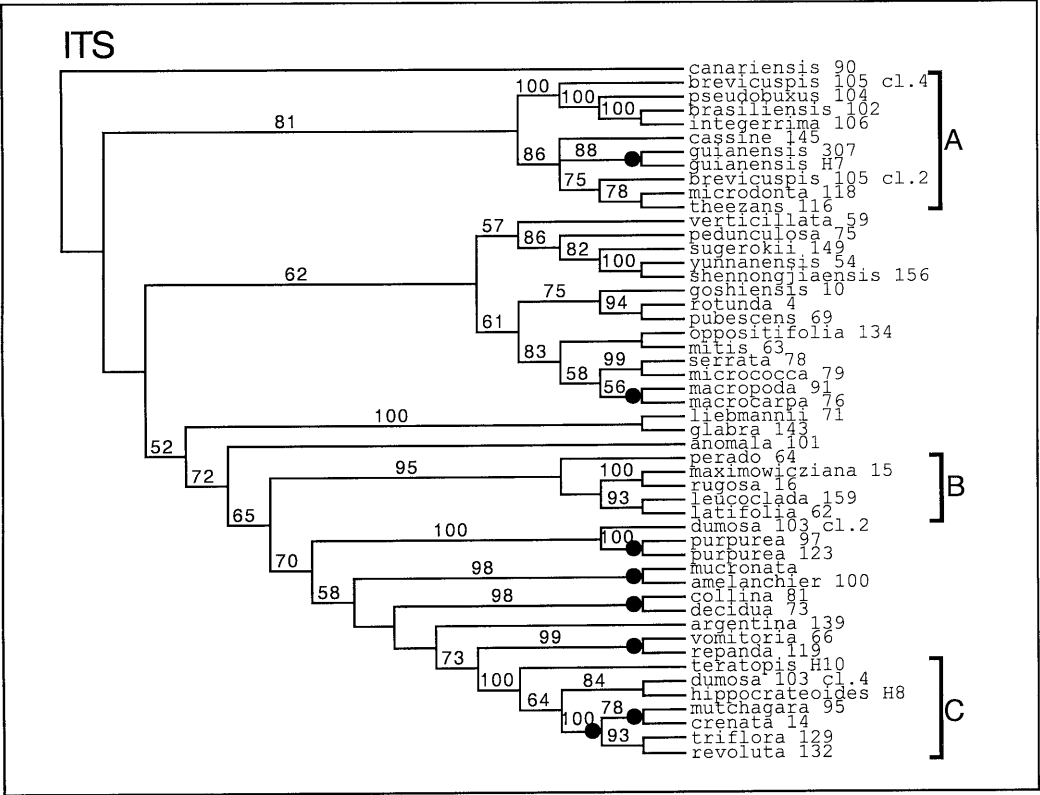
is expected to occur and to influence the topology of the nuclear tree (Wendel and Doyle 1998). In order to evaluate whether this process is involved in *Ilex*, the ITS tree and the 5S RNA spacer tree are compared. Since in higher eukaryote genomes, the 5S gene clusters are not linked to the rDNA clusters, the chance to observe a similar process of sorting, leading to the same tree topology for both clusters, is low. Figure 3 shows the strict ITS and 5S RNA spacer consensus trees for species in which both sequences are available (see materials and methods). Because the ITS and 5S RNA spacer matrices comprise 757 and 234 sites respectively, the combined ITS/5S spacer tree is very close to the ITS tree. Both trees show marked incongruences, suggesting frequent processes of lineage sorting for variants of both nuclear loci. However several clades have similar composition. The clade A of Fig. 2 [*I. cassine*, *I. guianensis*, *I. brevicuspis* (clone 2), *I. microdonta*, *I. theezans*, *I. brevicuspis* (clone 4), *I. pseudobuxus*, *I. brasiliensis* and *I. integerrima*] is found with minor exceptions in both the ITS and 5S spacer trees of Fig. 3. This is also the

Table 2. Wilcoxon signed ranks test results of the comparison of the plastid and nuclear trees on both nuclear and plastid DNA matrices, respectively

Data matrix	Constraint	No. steps			P
		Gain	Loss	Net	
Nuclear	Plastid strict consensus	891	6	885	<0.005
	Plastid 70% bootstrap	603	2	601	<0.005
Plastid	Nuclear strict consensus	107	2	105	<0.005
	Nuclear 70% bootstrap	74	6	68	<0.005



Fig. 2. Nuclear strict consensus of successively weighted most parsimonious trees and correspondence with the plastid clades. The shaded boxes indicates species or groups of species of the plastid Asian/North American clades I and II, of the plastid American clade and of the plastid Eurasian clade, respectively (see Fig. 1). The thick bars indicate branches observed with unweighted characters. Bootstrap values are indicated above the branches. The deciduous species are indicated by empty dots. Shared clades between the plastid tree (Fig. 1) and between the nuclear tree (Fig. 2) are indicated by filled dots. Stars indicate species (in fact individuals) with different copies of ITS sequences. When the different ITS copies are not monophyletic for one specimen, as in *I. dumosa* and *I. brevicuspis*, two divergent copies are included in the matrix. Abbreviations: as in Fig. 1



case for the clade C [*I. argentina*, *I. teratopis*, *I. dumosa* (clone4), *I. hippocrateoides*, *I. mutchagara*, *I. crenata*, *I. triflora* and *I. revoluta*]. In the 5S RNA spacer tree, the clade C also comprises *I. maximowicziana*, *I. rugosa*, *I. perado*, *I. leucoclada* and *I. latifolia*, forming a separate clade (clade B) in the ITS tree (Fig. 3) and in the nuclear tree (Fig. 2). Thus, these regions of the nuclear tree (clades A, B and C) do not show strong evidence of lineage sorting for one or the other nuclear marker, and the biogeographical discussion based on the differences observed between the plastid and nuclear phylogenies will be based exclusively on these clades.

The *Ilex* fossil record

The plant fossil database (<http://ibs.uel.ac.uk/ibs>) contains thousands of *Ilex* records. The fossil record of the genus appears to be more complete than for most angiosperms. This is probably because *Ilex* pollen grains are claimed very distinctive (Martin 1977, but see the discussion). Probably for the same reason, *Ilex* represents one of the most ancient lineages in the angiosperm fossil record (see Muller 1981). The plant fossil database of *Ilex* (M.C. Boulter) should be considered as a preliminary start point and entries should be re-examined, because many of them do not stand up modern standards (see the discussion). Thus, this database should be used with caution. Nevertheless, plotting the *Ilex* fossil records (<http://ibs.uel.ac.uk/ibs>, data not shown here) shows at least that the genus is widespread across North America, Europe and Asia throughout the Tertiary. The only exceptions to this are a few records in South America (Table 4) and in Australia (reviewed by Martin 1977). Similarly, a general northern distribution is observed for the Cretaceous records.

Biogeography

The phylogenies produced by the plastid matrix (Fig. 1) and by the nuclear matrix (Fig. 2) were analysed with TASS software (Nelson and Ladiges 1995) in order to investigate relationships between areas. Results are shown in Fig. 4. From the plastid phylogeny, several Asian/North American relationships are observed. South-East Asia is related to East Asia, while Africa is related to Asia. Hawaii is related to Americas. No direct relationship is observed between Asia and South America. The nuclear phylogeny indicates the same relationships between East Asia, South-East Asia and Africa. Hawaii is still related to the Americas. Most importantly, and in disagreement with the plastid phylogeny, is the finding of direct South American and Asian relationships (excluding North America) in both the last nuclear TASS subtrees of Fig. 4. The area cladogram resulting from the plastid subtrees is partially resolved, but not the area cladogram resulting from the nuclear subtrees.

Ancestral area analyses (Bremer 1992) on both the plastid and the nuclear trees are shown in Table 3. The plastid phylogeny indicates clearly that East Asia has the highest coefficient and is probably the ancestral area of the extant *Ilex* plastids. On the other hand, the nuclear phylogeny does not discriminate between any ancestral area, North America and Asia having similar coefficients (respectively 1.00 and 0.99) close to the coefficient of South America (0.79).

Discussion

Phylogenetics of *Ilex*

Based on morphological and anatomical data, the study of general evolutionary trends in *Ilex* is rather difficult. Since the last monograph on

Fig. 3. Comparison of the ITS and of the 5S RNA spacer consensus trees. Clades of identical composition in both trees are indicated by filled dots, and groups of similar composition are bracketed. Bootstrap values are indicated above the branches

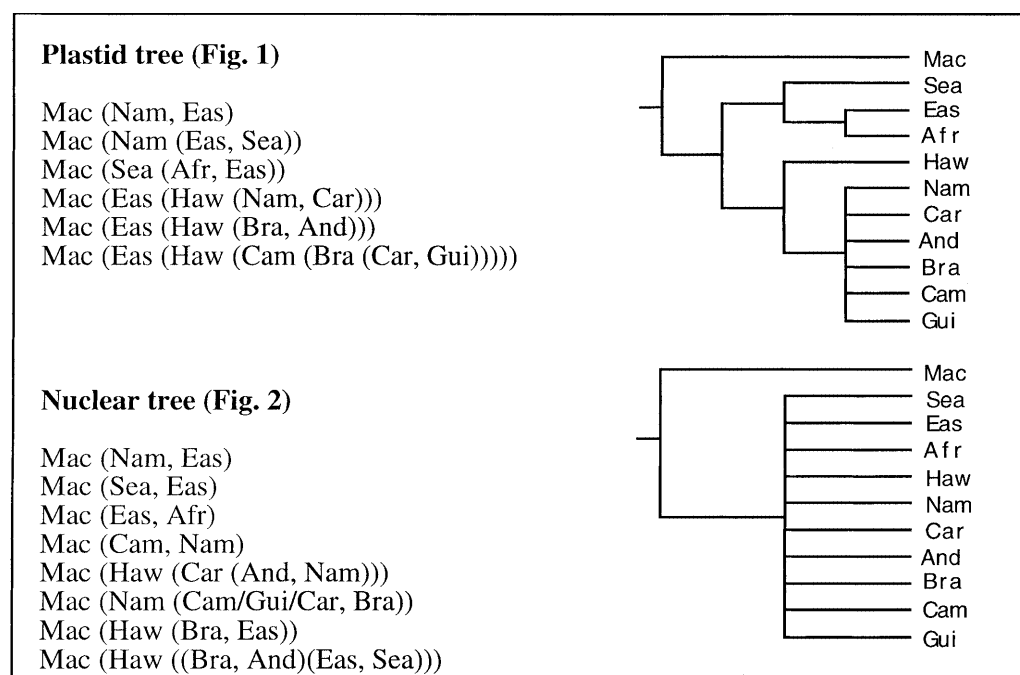


Fig. 4. Subtrees isolated by TASS from the plastid (see Fig. 1) and nuclear (see Fig. 2) trees, and the corresponding area cladograms. Cam/Gui/Car mean that the information is given by a species for which the distribution overlaps the indicated areas. Abbreviations: as in Fig. 1

the genus *Ilex* (Loesener 1901, 1908, 1942), several botanists have tried to elucidate these trends from morphological and biogeographical considerations (Hu 1967, Martin 1977), leaf anatomy (Baas 1975), wood anatomy (Baas 1973), pollen morphology (Lobreau-Callen 1975, 1977) and inflorescence structure (Loizeau and Spichiger 1992, Loizeau 1994). All, without exception, found disagreements when their data sets were compared with Loesener's work, and except Hu's study based on Asiatic *Ilex* (Hu 1949, 1950), any of them was able to propose a new classification based on their own data. The plastid and the nuclear phylogenies described here are incongruent. Moreover neither are the plastid or the nuclear relationships similar to the morphological relationships proposed by Loesener (see Figs. 1 and 2). This is understandable since the plastid phylogeny is based on maternal inheritance, and consequently evidence of potential speciation caused by hybridization and introgression could have been lost through

Table 3. Bremer coefficients (gain/loss, G/L) for the different areas applied to the plastid tree and the nuclear tree, respectively

	Plastid tree (Fig. 1)	Nuclear tree (Fig. 2)
Asia	1.00	0.99
East Asia	1.00	0.91
South-East Asia	0.17	0.17
North and Central America	0.44	1.00
North America	0.35	0.91
Central America	0.13	0.86
South America	0.57	0.79
Guiana	0.06	0.71
Brasil	0.73	0.44
Andes	0.43	0.30
Hawaii/Tahiti	0.11	0.29
Macaronesia	0.50	0.41
Caribbean Islands	0.13	0.39
Europe	0.20	0.19
Africa	0.10	0.19

chloroplast capture. On the contrary the nuclear phylogeny, based on biparental inheritance, should be closer to morphological relationships. This is however not the case.

A discussion of external evidence to support both the plastid and nuclear phylogenies is necessary. The phylogeny produced by the three chloroplast sequences is strongly congruent with the geographical distribution of the extant *Ilex* and the well known East Asian/North American relationship (Boufford and Spongberg 1983, Tiffney 1985). If this result does not prove helpful for the classification of *Ilex*, it provides strong external evidence for the likelihood of the plastid tree. Indeed, given the tree of Fig. 1, the probability to have, by chance, 18 of the 23 American species in the American clade (comprising 19 species) is 0.0017. Moreover, the chance to have any of the 22 Asian species in this clade is much far less (<0.0000). Thus, it can be considered that the plastid phylogeny resulting from maternal inheritance, and due to seed dispersal through continents, represents a valuable tool for phylogeographical investigations of the genus *Ilex*.

The phylogenetic tree produced from combined nuclear ITS and 5S RNA spacer sequences is congruent neither with the plastid phylogeny, nor with morphological data, nor with geographical distribution of extant species. Several subclades are shared by the plastid and the nuclear trees: *I. canariensis* at the base of the tree; the clade comprising *I. pseudobuxus*, *I. brasiliensis*, *I. integerrima*, *I. microdonta*, *I. theezans*, *I. cassine* and *I. guianensis*; *I. yunnanensis* and *I. shennongjiaensis*; *I. mitis* and *I. goshiensis*; *I. macropoda* and *I. macrocarpa*; *I. mucronata* and *I. amelanchier*; *I. triflora* and *I. revoluta* (see Figs. 1 and 2). This explains the statistical correlation found between the plastid and nuclear distance matrices. This partial congruence argues in favour of important events that have similarly shaped both phylogenetic trees. Indeed a perfect congruence would be expected between the plastid and the nuclear tree only under strong circumstances such as complete absence of hybridization, ancient speciation events and

no lineage sorting, among others. This is obviously not the case here.

A major problem with the nuclear data, is that individuals of 12 species studied (indicated in Fig. 2) have multiple copies of their ITS sequences. Even if the cloned sequences are monophyletic for most of the species, it cannot be ruled out that the sampling used here is insufficient to describe all the intraspecific variability. Paralogous sequences, as those observed in *I. dumosa* and *I. brevicuspis*, could be more widespread, and lineage sorting for variants of one or the other locus could complicate the construction of a nuclear phylogenetic tree. The comparison of the ITS and the 5S RNA spacer data (representing unlinked genomic repeat clusters) shows however that, independently of the possibility of lineage sorting, several clades of importance for the following discussion are nearly conserved (Fig. 3). Another argument sustains the nuclear tree of Fig. 2: two different specimens of *I. guianensis* and two different specimens of *I. purpurea* have the same position in the nuclear tree. The clade B (*I. maximowicziana*, *I. rugosa*, *I. perado*, *I. leucoclada* and *I. latifolia*), observed in the nuclear tree, but not in the plastid tree, is in total agreement with the ITS tree found for Asian species recently studied by Setogushi and Watanabe (2000). If the intraspecific variability of ITS was so substantial, the chance to obtain identical relationships using different specimens of the same species would be low.

In summary, even if the nuclear phylogeny needs further studies because of variable paralogous sequences found in some individuals, the data presented here could be used for preliminary phylogeographical investigations. The plastid tree indicates three different maternal lineages with Asian/North American relationships: (1) the Asian/North American clade I, (2) the Asian/North American clade II, and (3) the relation observed between the American clade and the Eurasian clade. Thus, three different maternal lineages share similar geographical distributions, and consequently interlineage hybridization could not be ruled

out. If *Ilex* species experienced interlineage hybridization, it is not surprising to observe disagreements between the plastid and nuclear phylogenies. Interspecific hybridization probably played an important role in the speciation of *Ilex*, making phylogenetic studies very complex (see Baas 1978, Setogushi and Watanabe 2000). The fact that morphological and anatomical data are not congruent with molecular phylogenetic classification of *Ilex* could be explained by the fact that interspecific and interlineage hybridizations and introgression as well as lineage sorting resulted in the disjunction or homogenization of morphological and anatomical characters (Baas 1978).

The fossil record of Ilex

The strong and repeated North-American/Asian relationships observed in the plastid phylogeny of *Ilex* suggest a perfect example of Arcto-Tertiary lineage (Boufford and Spongberg 1983, Lavin and Luckow 1993, Boulter and Fisher 1994, Guo and Ricklefs 2000), with a recent colonization of South America from North America (Burnham and Graham 1999). A search in the plant fossil database (<http://biodiversity.org.uk>) for Aquifoliaceae, *Ilex* and *Ilexpollenites* indicates also that a very large majority of Tertiary records are distributed in the northern hemisphere.

Within the palaeobotany literature too, early identifications of the leaves in the Americas by Berry (1921) and in Europe by Engelhardt (1895) have been used by prominent botanists such as Raven and Axelrod (1974)

and Cronquist (1988) in reviewing Aquifoliaceae. But more recently, great doubt has been poured on these identification of leaf fossils. There is no work from cuticle studies, no scanning electron microscopy and no realization that the leaf form of *Ilex* is very similar to other fossilized taxa. Also, the wood structure is not unique.

This means that the only reliable megafossil organ that is preserved from *Ilex* is the fruitstone, of which a large card database of well-identified specimens is maintained in Berlin by Dieter Mai (Knobloch and Mai 1986; Mai 1970, 1995). There are thousands of specimens in the database, and most of them are from Europe. North America is represented by specimens identified from the Brandon Lignite by Tiffney (1977). In addition, fossil flowers of *Ilex* are recorded in the Baltic amber (Conwentz 1886). From an exhaustive review of the literature, and enquiry of the contemporary experts (Dieter Mai, Bruce Tiffney, Mary Dettmann and David Greenwood), reliable published records of *Ilex* megafossils are absent from southern hemisphere pre-Pliocene sediments.

So we are left with the pollen. Their geographical distribution has been compiled by Kedves (1988) showing that pollen of *Ilex* has been recorded from all continents through the Tertiary. Cretaceous records occur only in the northern hemisphere, and a few doubtful Tertiary records occur in South America and Africa. His map shows that *Ilex* is widespread across North America, Europe and Asia throughout the Tertiary. This observation is

Table 4. Records of *Ilex* pollen found in South America

Sole de Porta	1960	Colombia	Pliocene
Sole de Porta	1961	Colombia	Oligocene
van der Hammen and Wijmstra	1964	British Guiana	Eocene
van der Hammen	1966	Colombia	Pliocene
Penny	1969	Colombia	Miocene
Graham	1976	Caribbean	Oligocene
Graham	1976	Mexico	Miocene
Graham	1987	Panama	Miocene
Graham	1987	Costa Rica	Miocene

clear from our Tertiary pollen database comprising more than 900 pollen citations in the published literature from around the world. The only exceptions to this are in South America (Table 4) and in Australia. The pollen record in Australia was reviewed by Martin (1977) who began: “The pollen of *Ilex* is very distinctive and fossil specimens can be related to it with certainty”. The Australian records begin in the Turonian, about 95 million years ago, predating the first appearance of *Nothofagus* Blume, and extend in increasing diversity up to the Miocene, where they suddenly stop. But no pictures of these very early fossil pollens could be found, as for other Cretaceous *Ilex* pollen reported in other continents. The evidence left does not stand up to modern standards and a re-evaluation of these specimens is requested.

In conclusion, the Tertiary record is mainly of the northern hemisphere but a few Tertiary records found in the southern hemisphere in South America (from Eocene to Pliocene, see Table 4) and in South Australia (from Eocene to Oligocene, see Table 2 of Martin, 1977) should be taken into consideration. As calculated by Cuénoud et al. (2000), molecular data do not sustain a Cretaceous origin of *Ilex* but a more recent origin. This is also confirmed from a tentative calibration of the angiosperm tree based on *rbcL*, *atpB* and 18S rDNA (Wikström et al. 2001). However, these results cannot be used to claim that the Cretaceous origin of *Ilex* is wrong. Cretaceous branches of the lineage *Ilex* could be extinct, making the age of the common ancestor of extant species younger.

Phylogeography of *Ilex*

In both the plastid and nuclear trees, *I. canariensis* (endemic in the Canary Islands and Madeira) is the most basal taxon of the extant species. Island isolation and possible accidental long branch attraction to the outgroup might be the reason for its basal position. However there are several examples to the contrary: *I. perado* also in the Canary Islands

is undoubtedly nested in the plastid Eurasian clade, and *I. anomala* endemic of Hawaii and Tahiti is undoubtedly related to the plastid American clade. It is well documented that a part of the flora of the Canary Islands is of ancient origin (Bramwell and Bramwell 1974). The geological age of the western Canarian islands is recent (Quaternary); however, the age of the eastern islands Fuerteventura and Lanzarote is still in debate. This varies from the early Miocene (Schmincke 1979) to the Senonian (Boutin 1994), the question being if these islands were emerged above sea level before the volcanic eruptions of the Miocene, or not. The Canarian endemic flora is related to the Arcto-Tertiary paleoflora (Sunding 1979). Indeed some specimens from Tertiary deposits in the Rhône valley (Bramwell 1976) and *Ilex lotschii* from the Lusatian Miocene brown-coal (Mai 1970) have been assigned to *I. canariensis*. So far, there is no reason to reject the position of *I. canariensis* at the base of the phylogenetic tree of the extant species, especially when this is independently observed in both the plastid and the nuclear trees.

The main purpose of this study was a discussion on the origin and on the route of differentiation of the lineage *Ilex*. Several evidences are in favour of an Arcto-Tertiary ancestry of the extant species, with a southerly migration to South America late in the Tertiary (Burnham and Graham 1999): (1) the geographical distribution of extant *Ilex*, (2) the richness of the Arcto-Tertiary fossil record, (3) the age of the common ancestor of extant *Ilex* (mid-Tertiary) calculated from cpDNA data (Cuénoud et al. 2000), (4) the basal position of *I. canariensis* an endemic of the Canary Islands and Madeira which are not suspected of Gondwanan origin, (5) the repeated Asian/North American relationships observed in the plastid data, and (6) the maternal phylogeny that indicates a relations between North America to South America and no direct Gondwanan relationship between South America and Australasia (Fig. 4).

On the other hand, without taking into account the claimed Gondwanan origin of *Ilex*

(supported by some fossil records, but rejected for the extant species on which this work is based), the nuclear phylogeny does not sustain a recent colonization of South America for all the South American species studied here, as the plastid phylogeny does. Indeed, in South America two different groups of are present: the lineage comprising *I. pseudobuxus*, *I. brasiliensis*, *I. brevicuspis*, *I. integerrima*, *I. microdonta*, *I. theezans*, *I. cassine* and *I. guianensis* which from a pure plastid and nuclear clade, and the rest of the South American species comprising *I. hippocrateoides*, *I. dumosa*, *I. teratopis*, *I. argentina*, *I. vomitoria*, *I. laurina*, *I. repanda* and *I. liebmanni*. The nuclear phylogeny indicates that some species of this last group have direct relations with species from North America, but that some others have direct relations with species from East Asia. This is illustrated when looking at the different nuclear TASS subtrees showing a circular relationship between East Asia and North America, between North America and South America and between South America and East Asia. Both the first relationships are also observed in the plastid phylogeny, and are probable. How supported are the direct relationships between South America and East Asia in the nuclear tree? They are observed in both of the last two TASS subtrees of Fig. 4. One of them results from the clade comprising *I. dumosa* (clone 2) and *I. purpurea* (bootstrap 100%), the other results from the clade comprising *I. revoluta*, *I. triflora*, *I. crenata*, *I. mutchagara*, *I. hippocrateoides*, *I. dumosa* (clone 4), *I. teratopis* and *I. argentina* (bootstrap 66%). Although the support of this last clade is not very high, two very characteristic indels in the 5S RNA spacer are shared by all these species (data not shown), giving a high likelihood for the existence of this clade. Moreover this clade is observed with minor exceptions in both the ITS tree and in the 5S RNA spacer tree, separately. Therefore the circular geographical relationship observed in the nuclear tree results from a species tree and not from a gene tree, and consequently is highly probable.

Thus at least the species *I. hippocrateoides*, *I. dumosa*, *I. teratopis* and *I. argentina*, having direct relations with East Asia, form a specific lineage in South America, different from the South American clade *I. pseudobuxus*, *I. brasiliensis*, *I. brevicuspis*, *I. integerrima*, *I. microdonta*, *I. theezans*, *I. cassine* and *I. guianensis*, which has no direct relation with East Asia, except via North America. Thus, the main finding of this study is that the South American *Ilex* comprise two lineages. In agreement with Burnham and Graham (1999), it is highly probable that one of them (represented in this work by *I. pseudobuxus*, *I. brasiliensis*, *I. brevicuspis*, *I. integerrima*, *I. microdonta*, *I. theezans*, *I. cassine* and *I. guianensis*) has recently colonized South America from North America when a landbridge was established between both continents. The period of colonization of South America by the other lineage (represented here by *I. hippocrateoides*, *I. dumosa*, *I. teratopis* and *I. argentina*) is so far difficult to define. Mid-Tertiary pollen found in south America (Table 4) would indicate a possible more ancient colonization. However, an explanation of the unusual post-Cretaceous direct relationship between species of South America and species of East Asia is to be found.

Perspectives

The following directions need to be investigated in order to understand the complete history of the genus *Ilex*:

(1) The first question is related to a possible Cretaceous origin of the genus. As discussed previously, it is not unlikely that the lineage is in fact more ancient than the Tertiary, if it is assumed that the basal Cretaceous branches are extinct. This eventuality fits with the enormous evolutionary gap observed between Aquifoliaceae and *Helwingia*/*Phyllonoma* (Cuénoud et al. 2000). A re-evaluation of the *Ilex*-like Cretaceous pollen should be undertaken.

(2) Another question is the post-Cretaceous relationships observed between South American and Asian species. One possibility is that

the Asian/south American clade C of Fig. 2 (*I. revoluta*, *I. triflora*, *I. crenata*, *I. mutchag-ara*, *I. hippocrateoides*, *I. dumosa* (clone 4), *I. teratopis* and *I. argentina*) is the result of migration from Asia via North America with a subsequent extinction of the North American ancestor, as postulated for members of Papaveraceae (Blattner and Kadereit 1995). Another possibility of long distance migration across the Pacific Ocean during the mid-Tertiary should be considered. Such Tertiary migrations have been postulated for *Abrotanella* Cass. (Asteraceae, Swenson and Bremer 1997), and *Ilex*, known for seed dispersion by birds (Van der Pijl 1972), is a candidate.

(3) The fact that both South American lineages have similar plastid genomes (Fig. 1) and different nuclear genomes (Fig. 2) implies nuclear or cytoplasmic introgression in one or the other lineage. One possibility is that anciently established South American species have captured the plastid of founder populations of the more recent northern arriving species. Such processes are demonstrated in several species and result from pollen competition between species having different population sizes (Rieseberg 1995, Rieseberg et al. 1996).

(4) Although probably subject to intralinear hybridization, the three maternal lineages observed (Fig. 1) are the result of seed propagation and thus represent a useful picture for biogeographical studies. As these three lineages have the same eastern Asian and North American distribution (one of them having reached South America from North America), it would be interesting to correlate these patterns with time, geography, climate and evolution (Tiffney 1985). Have these three lineages occurred simultaneously or stepwise? The study of particularities of the three lineages, such as ecology and habit, should answer this question. The fact that one of the lineage comprises most of the deciduous species of *Ilex* support this theory.

(5) South America is probably the crux of the question. In this work, the geographic distribution of extant *Ilex* in America is subdivided in five region: North America

(excluding tropical South Mexico), Central America (including tropical South-Mexico), Caribbean Islands, Andes, Brazil and Guiana. TASS sub-trees (Fig. 4) indicate complex relationships between these areas, as complex as those observed for angiosperms in general (Burnham and Graham 1999). The addition of more South American species to the data and a precise study of their biogeographical relationships should resolve several fundamental question about migrations into the Americas.

In conclusion, the understanding of the complex distribution and phylogeny of extant *Ilex* needs further investigation. Hybridization and lineage sorting played a role in the convoluted history of *Ilex*, as well as the superposition of vicariance (the Asian/North American Tertiary relationship) and dispersal events (the Quaternary South American colonization from North America, and possibly Tertiary long-distance migrations into South America). Hybridization and the superposition of several biogeographic patterns create noise that makes the perception of general biogeographic patterns difficult, but interesting in the general perspective of angiosperm phylogeography.

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