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Chloroplast and nuclear evidence for multiple origins of polyploids and diploids of *Hedera* (Araliaceae) in the Mediterranean basin

Virginia Valcárcel, Omar Fiz, and Pablo Vargas*

Royal Botanic Garden of Madrid, Plaza de Murillo 2, CSIC, Plaza Murillo 2, 28014 Madrid, Spain

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Abstract

Chloroplast (*trnT–L*) and nuclear rDNA (ITS) sequence analyses of the Araliaceae provide strong molecular evidence for the monophyly of the genus *Hedera*. Phylogenetic reconstructions suggest multiple origins and an active polyploidization process not only in the formation of tetraploids ($2n = 96$), hexaploids ($2n = 144$), and octoploids ($2n = 192$), but also of diploids ($2n = 48$). A high basic chromosome number of $x = 24$, extensive polyphyly in widespread diploids, and terminal placement of *Hedera* in phylogenies of the Araliaceae reveal that extant diploid taxa may be, in fact, assemblages of ancestral polyploids from plants of $n = 12$. Four major lineages containing four types of chloroplast (chlorotypes I, II, III, and IV), which are defined by different *trnT–L* nucleotide substitutions and two large insertions (50- and 30-bp), provide evidence for evolutionary processes and historical biogeography in *Hedera*. We propose a scenario where an initial colonization in the Mediterranean basin by Asian ancestors (carrying the ancestral Araliaceae chlorotype I) is followed by differentiation into the four chlorotypes of the Mediterranean region, and then recolonization of Asia and northern Europe only by chlorotype III. The Macaronesian taxa (*Hedera azorica*, *Hedera maderensis* ssp. *maderensis*, and *Hedera canariensis*) appear to have originated from a single-colonization event to each archipelago with no further contact either with continental or insular species.

Keywords: Araliaceae; *Hedera*; Macaronesia; Polyphyly; Polyploidization; Pre-Holocene and postglacial recolonization

1. Introduction

Polyploidy appears to be the most significant mode of sympatric speciation (Turelli et al., 2001), although homoploid hybrid speciation is also feasible (Rieseberg and Carney, 1998). The importance of polyploidy as an active process is manifested by the estimated number of speciation events (2–4%, Otto and Whitton, 2000) and historical success of polyploids in different floras (Stebbins, 1971). As more information is obtained from different sources of evidence, the number of presumed polyploid lineages increases. In the 1970s, it was estimated that 30–35% of angiosperm species were polyploids (Stebbins, 1971); c. 52% in the 1980s (Grant, 1981); and c. 70% in the 1990s (Masterson, 1994). The threshold of $n = 12$ as the basic chromosome number above which plants were considered to be polyploids (Goldblatt, 1980; Grant, 1963; Stebbins, 1950) has also been evaluated using an angiosperm phylogenetic framework (Soltis and Soltis, 2000). Given the possibility of decrease of chromosome numbers from polyploid lineages through chromosomal reorganization (e.g., chromosomal fission, fusion, and dysploidy in certain groups; Baldwin and Wessa, 2000; Goldblatt, 1980; Jones, 1998; Rieseberg, 2001), polyploidy may be even more extensive. Consequently, very few lineages of extant angiosperm species may have been unaffected by polyploid events at some time in their evolution.

The genus *Hedera* constitutes a polyploid group of widely distributed perennial species, all with active vegetative reproduction, as is typical for successful polyploids (Grant, 1981; Stebbins, 1950). This genus

* Corresponding author. Fax: +91-4200157.
E-mail address: vargas@ma-rjb.csic.es (V. Valcárcel).

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includes approximately 12 species of ivies distributed in Eurasia, Macaronesia, and northern Africa. Taxonomy of ivies has been based on vegetative characters because few reproductive characters are informative. Chromosome counts and epidermal hair types have been recently used to distinguish new species (McAllister, 1981; McAllister and Rutherford, 1990; Rutherford et al., 1993) that were formerly considered subspecies, varieties or races in earlier classifications (Seeman, 1868; Tobler, 1912). Two species have long, white, stellate hairs on the pedicels, calyces, and foliage (*Hedera azorica* and *Hedera helix*); nine species are characterized by small, reddish scale hairs (*Hedera algeriensis*, *Hedera canariensis*, *Hedera colchica*, *Hedera cypria*, *Hedera maderensis*, *Hedera maroccana*, *Hedera nepalensis*, *Hedera pastuchovii*, and *Hedera rhombea*); and one species (*Hedera hibernica*) has white, appressed hairs, an intermediate trichome between the other two groups. In a previous paper (Vargas et al., 1999a), four ploidal levels were established in *Hedera*: diploid (*H. azorica*, *H. canariensis*, *H. helix*, *H. maroccana*, *H. nepalensis*, and *H. rhombea*), tetraploid (*H. algeriensis*, *H. hibernica*), hexaploid (*H. maderensis*, *H. cypria*, and *H. pastuchovii*) and octoploid (*H. colchica*). Nuclear rDNA ITS (Internal Transcribed Spacers) sequences, in concert with chromosome counts and morphological characters, allowed the following interpretations (Vargas et al., 1999a): (1) allopolyploids were detected by the occurrence of sites displaying ITS additive characters in three species (*H. hibernica*, *H. maderensis*, *H. pastuchovii*); (2) 604 of 609 ITS nucleotides were accurately reconstructed for one putative diploid ancestor of all the polyploid species, except for *H. algeriensis*; (3) the ITS phylogeny revealed two major lineages of primarily diploids and polyploids; and (4) two independent polyploidization processes on the western and eastern sides of the Mediterranean basin were detected.

Molecular markers also led to inferences of the nature of polyploidization, including auto-, allo-, or autoallopolyploid speciation (Grant, 1981; Sang et al., 1997; Wendel, 2000), monophyletic groups of polyploids within a genus (Vargas et al., 1999a), and number of polyploidization events involved in the formation of a single species (Soltis and Soltis, 2000). Comparative phylogenies between biparental (nuclear) and uniparental (chloroplast and mitochondrial) markers serve to identify reticulation and the mode of polyploidization in particular groups (Brochmann et al., 1996; Sang et al., 1997; Soltis and Kuzoff, 1995). As far as we know, there have been no comparative phylogenetic examination investigating the origin of those genera of the Araliaceae in which polyploidy has been detected: *Eleutherococcus*, *Aralia*, *Fatsia*, *Hedera*, *Polyscias*, and *Panax* (Fedorov, 1974; Goldblatt, 1981–1988; Goldblatt and Johnson, 1990–2000; Vargas et al., 1999a) (see Table 1 for *Hedera*).

Investigations of the generic relationships of the Araliaceae using chloroplast (Plunkett et al., 1997), nuclear (Wen et al., 2001), and both sources of sequences (Plunkett and Lowry, 2001) did not deduce a clear sister group to *Hedera*. However, a relatively well-defined *Hedera* group, formed by *Hedera*, *Kalopanax*, *Dendropanax*, *Eleutherococcus*, *Trevesia*, *Fatsia*, *Euaraliopsis*, *Brassaiopsis*, *Sinopanax*, *Oreopanax*, *Oplopanax*, *Gamblea*, and *Evodiopanax*, has been identified (Plunkett et al., 1997; Wen et al., 2001). In these studies, several genera of Araliaceae have been shown to be non-monophyletic (*Schefflera*, *Polyscias*, and *Aralia*), but a single origin of *Hedera* has not been tested to date.

In this paper, we compare both nrDNA (ITS) and chloroplast (intergenic spacers *trnT–L* and *trnL–F*) in the Araliaceae to explore the origin of the polyploid series (2×, 4×, 6×, and 8×) found in *Hedera* (McAllister, 1981; Vargas et al., 1999a). The specific aims of this study of *Hedera* are to (1) test its monophyly; (2) infer phylogenetic relationships among ivy species; (3) investigate the auto- vs. allopolyploid origin of ivies; (4) determine the number of polyploid events responsible for the formation of tetra-, hexa-, and octoploids; (5) identify the parentage of polyploids; (6) interpret the causes of the high basic chromosome number; (7) infer historical biogeographic patterns among ivy populations; and (8) evaluate the centers of diversification.

2. Materials and methods

2.1. Plant material and sample

We sampled from the population level (10–11 individuals of each three populations of *H. helix* ssp. *helix*) to the generic level in the Araliaceae. One-hundred four populations of the 12 species of *Hedera*, plus eight genera from the Araliaceae (*Aralia*, *Cussonia*, *Dendropanax*, *Dizygotheca*, *Fatsia*, *Polyscias*, *Pseudopanax*, and *Schefflera*) were used for the present study (Table 1). A pilot study using only four *Hedera* species was initially performed to search for the most variable chloroplast markers (*trnT–trnL*, *trnL–trnF*, *rplP*16, *matK*). As a result, a total of 41 *trnT*(UGU)–*trnL*(UAA) and 16 *trnL*(UAA)–*trnF*(GAA) intergenic-spacer sequences were obtained because these regions displayed the highest variation among the chloroplast sequences. Additionally, four new ITS sequences of *Hedera* were obtained (Table 1) and analyzed together with the ITS sequences of *Hedera* (26) and the Araliaceae (45) taken from previous studies (Mitchell and Wagstaff, 1997; Plunkett and Lowry, 2001; Vargas et al., 1999a; Wen et al., 2001) and deposited in GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/). Individuals and populations were selected to represent the geographical distribution of the species.

Table 1
Accessions used in the molecular study of the Araliaceae, including population numbers after taxa names of *Hedera*; natural distribution of taxa; locality of wild populations or material source; voucher and gametic chromosome number (inferred number in italics); molecular markers and GenBank Accession No. (in parenthesis); and *trnT–trnL* chlorotype and sequence size (in parenthesis)

Taxon	Natural distribution	Locality/source	Voucher	Chromosome number (*n*)	Markers (GenBank Accession No.)	*trnT–trnL* chlorotype (size)
Aralia	North America, East Asia, and Malaysia			*12, c. 14, 24*		
A. californica S. Watson	Californian Floristic Region and Oregon	USA, California, San Francisco, Stinson Beach	P. Vargas 74PV01 (MA)	*24*	*trnT–trnL* (AF506114)	I (621 bp)
Cussonia	Tropical and Southern Africa and Madagascar			?		
C. thyrsiflora Thunb.	Cape Peninsula	South Africa, Hermanus	P. Vargas 511PV00 (MA)	?	*trnT–trnL* (AF506117)	I (624 bp)
Dendropanax	Tropical America, East Asia, and Malaysia			?		
D. arboreus (L.) Decne. & Planche	Tropical America	Ecuador, Orellana, Yasuní National Park	M.J. Macía et al. 857 (MA639461)	?	*trnT–trnL* (AF506119)	I (602 bp)
Dizygotheca	New Caledonia			?		
D. elegantissima Vig. & Guillam	New Caledonia	Cultivated	P. Vargas s.n. (MA)	?	*trnT–trnL* (AF506116)	I (601 bp)
Fatsia	East Asia			12, 24		
F. japonica (Thunb.) Decne. & Planche	East Asia	Cultivated	P. Vargas s.n. (MA)	*24*	*trnT–trnL* (AF506121) *trnL–trnF* (AF515528) *matK* (AF515548) ITS (AJ131215)	I (592 bp)
Hedera	Macaronesia, Northern Africa, Europe, and Asia			24, 48, 72, 96		
*H. algeriensis*1 Hibberd	Algeria and Tunisia	Algeria, Kabylie	H.A. McAllister 838H.A.M. (MA)	48	*trnT–trnL* (AF506093) *trnL–trnF* (AF515534) ITS (AJ131216)	II (643 bp)
*H. algeriensis*2		Cultivar "Gloire de Marengo"	H.A. McAllister and P. Vargas s.n. (MA)	*48*	*trnT–trnL* (AF506094) ITS (AJ131217)	II (643 bp)
*H. algeriensis*3		Cultivated	P. Vargas 410PV00 (MA)	*48*	*trnT–trnL*	II (c. 643 bp)
*H. algeriensis*4		Tunisia, Ain-Draham	J.A. Aldasoro 2890 (MA)	*48*	*trnT–trnL*	II (c. 643 bp)
*H. azorica*1 Carrière	Macaronesia Azores Islands	Portugal, Azores Islands, Pico	F. Brightman s.n. (LIV)	24	*trnT–trnL* (AF506106) *trnL–trnF* (AF515541) ITS (AJ131218)	IV (693 bp)
*H. azorica*2		Portugal, Azores Islands, São Miguel	Hilliers s.n. (LIV)	24	*trnT–trnL* (AF506107) ITS (AJ131219)	IV (693 bp)
*H. azorica*3		Portugal, Azores Islands, Faial	O. Fiz 229OF00 (MA)	*24*	*trnT–trnL*	IV (c. 693 bp)
*H. azorica*4		Portugal, Azores Islands, Pico	O. Fiz 238OF00 (MA)	*24*	*trnT–trnL*	IV (c. 693 bp)
*H. azorica*5		Portugal, Azores Islands, Faial	O. Fiz 226OF00 (MA)	*24*	*trnT–trnL*	IV (c. 693 bp)
*H. azorica*6		Portugal, Azores Islands, Terceira	V. Valcárcel s.n. (MA)	*24*	*trnT–trnL*	IV (c. 693 bp)
*H. canariensis*1 Willd.	Macaronesia Canary Islands	Spain, Canary Islands, Tenerife	H.A. McAllister 237H.A.M. (MA)	24	*trnT–trnL* (AF506085) *trnL–trnF* (AF515532) *rpl*16 (AF515544) ITS (AJ131220)	I (593 bp)

Table 1 (*continued*)

Taxon	Natural distribution	Locality/source	Voucher	Chromosome number (*n*)	Markers (GenBank Accession No.)	*trnT–trnL* chlorotype (size)
*H. canariensis*2		Spain, Canary Islands, La Palma	J. Francisco-Ortega and A. Santos s.n. (LIV)	*24*	ITS (AJ131221)	?
*H. canariensis*3		Spain, Canary Islands, La Gomera	A. Fernández s.n. (MA)	*24*	*trnT–trnL* (AF506086)	I (593 bp)
*H. canariensis*4		Spain, Canary Islands, La Palma	J.J. Santos s.n. (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
*H. canariensis*5		Spain, Canary Islands, Tenerife	C. Aedo 6684CA (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
*H. canariensis*6		Spain, Canary Islands, La Gomera	C. Aedo 6699CA (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
*H. canariensis*7		Spain, Canary Islands, Tenerife	V. Valcárcel 28VV00 (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
*H. colchica*1 K. Koch	Caucasus, Turkey	Georgia, Zagodeki	R. Lancaster L296 (LIV)	96	*trnT–trnL* (AF506104) *trnL–trnF* (AF515540) ITS (AJ131222)	III (673 bp)
*H. colchica*2		Georgia, T'elavi	R. Lancaster 269 (1979)	96	*trnT–trnL* (AF506105) ITS (AJ131223)	III (673 bp)
*H. colchica*3		Cultivated	O. Gómez s.n. (MA)	*96*	*trnT–trnL*	III (c. 673 bp)
*H. colchica*4		Turkey, Sinop	V. Valcárcel 188VV01 (MA)	*96*	*trnT–trnL*	III (c. 673 bp)
*H. colchica*5		Turkey, Trabzon	V. Valcárcel 371VV01 (MA)	*96*	*trnT–trnL*	III (c. 673 bp)
*H. colchica*6		Georgia, Krashodar	Latschaschvili s.n. (MA576348)	*96*	*trnT–trnL*	III (c. 673 bp)
*H. colchica*7		Turkey, Rize	S. Nisa 760SN (MA)	*96*	*trnT–trnL*	III (c. 673 bp)
*H. cypria*1 McAllister	Cyprus	Cyprus, Limasol	J. Edmonson s.n. (LIV)	72	*trnT–trnL* (AF506113) ITS (AJ131224)	IV (693 bp)
*H. cypria*2		Cyprus, Kakopetria	Mrs. Della, via R. Meilke s.n. (LIV)	*72*	*trnT–trnL* (AF506095) *trnL–trnF* (AF515535) *rpl*16 (AF515545) ITS (AJ131225)	II (643 bp)
*H. cypria*3		Cyprus, Ayia Napa	Iter Mediterraneum IV (Cyprus) 1230 (MA496034)	*72*	*trnT–trnL*	II (c. 643 bp)
H. helix L. ssp. *helix*1	Europe	Spain, Cádiz	V. Valcárcel et al. 101VV00 (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
H. helix L. ssp. *helix*2		Spain, Jaén	V. Valcárcel 170VV01 (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
H. helix L. ssp. *helix*3		Spain, Málaga	H.A. McAllister s.n. (MA)	*24*	*trnT–trnL* (AF506090) *matK* (AF515549)	I (593 bp)
H. helix L. ssp. *helix*4		Spain, Guadalajara	V. Valcárcel 3VV01 (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
H. helix L. ssp. *helix*5		Spain, Guadalajara; 10 individuals	V. Valcárcel 383VV01 (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
H. helix L. ssp. *helix*6		Spain, Burgos	P. Vargas 128PV01 (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*7		Spain, Burgos	P. Vargas 251PV00 (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*8		Spain, Huesca	P. Vargas et al. s.n. (MA)	*24*	*trnT–trnL* (AF506098) ITS (AF506077)	III (673 bp)
H. helix L. ssp. *helix*9		Spain, Huesca; 10 individuals	P. Vargas et al. 154PV01 (MA)	*24*	*trnT–trnL*	III (c. 673 bp)

H. helix L. ssp. *helix*10		Spain, Gerona	G. Nieto Feliner 4344GN (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*11		France, Bourg-en-Bresse	D. Grivet s.n. (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*12		France, Corsica	D. Grivet s.n. (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*13		Great Britain, Scotland, S. Uist	H.A. McAllister 570H.A.M. (MA)	24	*trnT–trnL* (AF506097) ITS (AF506078)	III (673 bp)
H. helix L. ssp. *helix*14		Great Britain, Scotland	H.A. McAllister s.n. (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*15		Denmark, Jernhatten, Mols.	K. Larsen and P. Pedersen 257 (MA186341)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*16		Germany, Wiesbaden; 11 individuals	P. Vargas 11PV99 and 169PV01 (MA)	*24*	*trnT–trnL* (AF506096)	III (673 bp)
H. helix L. ssp. *helix*17		Poland, Bochnia	M. Gostyńska-Lakuszewska and J. Mesjasz 648 (MA223476)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*18		Ukraine, Crimean Peninsula, Yalta	G. Proskuriakova and L. Kramarenko 12378 (MA 366981)	*24*	*trnT–trnL*	IV (c. 693 bp)
H. helix L. ssp. *helix*19		Turkey, Mugla	J.A. Compton s.n. (LIV)	*24*	*trnT–trnL* (AF506091) *trnL–trnF* (AF515536) ITS (AJ131226)	II (643 bp)
H. helix L. ssp. *helix*20		Turkey, Gümüşhane	A. Hernández AH1200 (MA)	*24*	*trnT–trnL*	II (c. 643 bp)
H. helix L. ssp. *helix*21		Turkey, Zonguldak	C. Aedo 6481CA (MA)	*24*	*trnT–trnL*	II (c. 643 bp)
H. helix L. ssp. *helix*22		Turkey, Zonguldak	C. Aedo 6519CA (MA)	*24*	*trnT–trnL*	II (c. 643 bp)
H. helix L. ssp. *helix*23		Turkey, Safranbolu	C. Aedo 6378CA (MA)	*24*	*trnT–trnL*	II (c. 643 bp)
H. helix L. ssp. *helix*24		Turkey, Safranbolu	C. Aedo 6347CA (MA)	*24*	*trnT–trnL*	II (c. 643 bp)
H. helix L. ssp. *helix*25		Bosnia, Mostar	Č.Šilič s.n. (MA350591)	*24*	*trnT–trnL*	IV (c. 693 bp)
H. helix L. ssp. *helix*26		Slovakia, Silica–Gombasek	A. Zertova 25934 (MA215977)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*27		Italy, Verona	P. Vargas 55PV97bis (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*28		Italy, Sicily	A. Hernández AH1002 (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*29		Italy, Naples	M.J. Macía et al. s.n. (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*30		Italy, Rome	R. Morales et al. s.n. (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*31		Switzerland, Murten	U. Trier s.n. (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*32		"Manda's Crested" Cultivar	Friex Nurseries LTD (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix L. ssp. *helix*33		"Parsley Crested" Cultivar	Friex Nurseries LTD (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
H. helix ssp. *rhizomatifera*1 McAllister	Southern Iberian Peninsula	Spain, Málaga	P. Vargas 5PV97 (MA)	*24*	*trnT–trnL* (AF506081) *trnL–trnF* (AF515529) *rpl*16 (AF515546) ITS (AJ131227)	I (593 bp)
H. helix ssp. *rhizomatifera*2		Spain, Granada	S.L. Jury s.n. (LIV)	24	ITS (AJ131228)	?
H. helix ssp. *rhizomatifera*3		Spain, Granada	H.A. McAllister 953H.A.M. (MA)	*24*	*trnT–trnL* (AF506082)	I (593 bp)
H. helix ssp. *rhizomatifera*4		Spain, Cádiz	P. Vargas 6PV99 (MA)	*24*	*trnT–trnL*	I (c. 593 bp)
H. helix ssp. *rhizomatifera*5		Spain, Alicante	H.A. McAllister 815H.A.M. (LIV)	*24*	*trnT–trnL*	IV (c. 693 bp)

Table 1 (*continued*)

Taxon	Natural distribution	Locality/source	Voucher	Chromosome number (*n*)	Markers (GenBank Accession No.)	*trnT–trnL* chlorotype (size)
H. helix ssp. *rhizomatifera*6		Spain, Huelva	H.A. McAllister 16H.A.M. (MA)	24	*trnT–trnL*	I (c. 593 bp)
H. helix ssp. *rhizomatifera*7		Spain, Málaga	H.A. McAllister 950H.A.M. (MA)	24	*trnT–trnL*	I (c. 593 bp)
*H. hibernica*1 (Kirchn.) Bean	Atlantic Europe	Spain, Asturias	H.A. McAllister 937H.A.M. (MA)	*48*	*trnT–trnL* (AF506108) ITS (AJ131229)	IV (693 bp)
*H. hibernica*2		Portugal, Lindoso	H.A. McAllister 925H.A.M. (MA)	48	*trnT–trnL* (AF506109) *trnL–trnF* (AF515542) *rpl*16 (AF515547) *matK* (AF515550) ITS (AJ131230)	IV (693 bp)
*H. hibernica*3		Spain, Málaga	H.A. McAllister 949H.A.M. (MA)	48	*trnT–trnL* (AF506087) ITS (AJ131231)	I (593 bp)
*H. hibernica*4		Spain, Huelva	H.A. McAllister 545H.A.M. (MA)	48	*trnT–trnL* (AF506110) ITS (AF506079)	IV (693 bp)
*H. hibernica*5		Spain, Santander	P. Vargas 413PV00 (MA)	*48*	*trnT–trnL*	IV (c. 693 bp)
*H. hibernica*6		Spain, Murcia	C. Aedo 5934CA (MA)	*48*	*trnT–trnL*	IV (c. 693 bp)
*H. hibernica*7		Spain, Zaragoza	V. Valcárcel 25VV00 (MA)	*48*	*trnT–trnL*	IV (c. 693 bp)
*H. hibernica*8		France, Bordeaux	D. Grivet s.n. (MA)	*48*	*trnT–trnL*	III (c. 673 bp)
*H. hibernica*9		Great Britain, England, Channel Island, Sark	A. Rutherford s.n. (MA)	*48*	*trnT–trnL*	III (c. 673 bp)
*H. hibernica*10		United Kingdom, Scotland, Drummore	A. Rutherford s.n. (MA)	*48*	*trnT–trnL*	III (c. 673 bp)
*H. hibernica*11		"Hamilton" Cultivar	Friex Nurseries LTD (MA)	*48*	*trnT–trnL*	III (c. 673 bp)
*H. hibernica*12		"Deltoidea" Cultivar	Friex Nurseries LTD (MA)	*48*	*trnT–trnL*	III (c. 673 bp)
H. maderensis K. Koch *ex* Rutherford ssp. *maderensis*1	Macaronesia, Madeira	Portugal, Madeira, Funchal	H.A. McAllister 18H.A.M. (MA)	72	*trnT–trnL* (AF506111) *trnL–trnF* (AF515543) ITS (AJ131233)	IV (693 bp)
H. maderensis ssp. *maderensis*2		Portugal, Madeira, Das Queimadas Park	L.O. Franquinho s.n. (LIV)	*72*	*trnT–trnL* (AF506112) ITS (AJ131234)	IV (693 bp)
H. maderensis ssp. maderensis3		Portugal, Madeira, Achadas da Cruz	P. Vargas 325PV00 (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
H. maderensis ssp. *maderensis*4		Portugal, Madeira, Santana	M. Velayos 9817MV (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
H. maderensis ssp. *maderensis*5		Portugal, Madeira, Santana	M. Velayos 9818MV (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
H. maderensis ssp. *maderensis*6		Portugal, Madeira, Pousada das Vinháticos	M. Velayos 9744MV (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
H. maderensis ssp. *maderensis*7		Portugal, Madeira, Boca de Encumeada	C. Navarro 3130CN (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
H. maderensis ssp. *maderensis*8		Portugal, Madeira, Casa do Lombo do Mouro	C. Navarro 3394CN (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
H. maderensis ssp. *maderensis*9		Portugal, Madeira, Casa do Lombo do Mouro	C. Navarro 3395CN (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)

H. maderensis ssp. *maderensis*10		Portugal, Madeira, Faro	P. Vargas 108PV98tris (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
H. maderensis ssp. *maderensis*11		Portugal, Madeira, Cabo Girão	P. Vargas 241PV99 (MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
H. maderensis ssp. *iberica*1 McAllister	Southern Iberian Peninsula	Spain, Cádiz	H.A. McAllister 15H.A.M. (MA)	72	*trnT–trnL* (AF506088) *trnL–trnF* (AF515533) ITS (AJ131232)	I (593 bp)
H. maderensis ssp. *iberica*2		Spain, Cádiz	S.L. Jury and L.C. Jury 13127 (MA538148)	*72*	*trnT–trnL*	I (c. 593 bp)
H. maderensis ssp. *iberica*3		Portugal, Monchique Mountains	A. Segura Zubizarreta 2017 (MA350593)	*72*	*trnT–trnL* (AF506089)	I (593 bp)
*H. maroccana*1 McAllister	Morocco	Morocco, Tetuan	H.A. McAllister 868H.A.M. (LIV)	24	*trnT–trnL* (AF506083) *trnL–trnF* (AF515530) ITS (AJ131235)	I (593 bp)
*H. maroccana*2		Morocco, Marrakech	H.A. McAllister 861H.A.M. (MA)	*24*	*trnT–trnL* (AF506092) *trnL–trnF* (AF515531) ITS (AJ131236)	II (643 bp)
*H. maroccana*3		Morocco, Chefchaouen	P. Vargas 152PV00 (MA)	*24*	*trnT–trnL* (AF506084) ITS (AF506080)	I (593 bp)
*H. maroccana*4		Morocco, Marrakech	M. Guzmán s.n. (MA)	*24*	*trnT–trnL*	II (c. 643 bp)
*H. nepalensis*1 K. Koch	Himalaya, East Hindu-Kusha, and southwest China	India, Kashmir	H.A. McAllister 246H.A.M. (MA)	24	*trnT–trnL* (AF506102) ITS (AJ131237)	III (673 bp)
*H. nepalensis*2		Vietnam, Fan-si Pan	H.A. McAllister 895H.A.M. (LIV)	24	*trnT–trnL* (AF506101) *trnL–trnF* (AF515538) ITS (AJ131238)	III (673 bp)
*H. nepalensis*3		Cultivated	(MA)	*24*	*trnT–trnL*	III (c. 673 bp)
*H. nepalensis*4		Cultivated	Kr 2884 (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
*H. pastuchovii*1 Woronow	Caucasus, Iran	Iran, Elburz Mountains	H.A. McAllister 259H.A.M. (MA)	72	*trnT–trnL* (AF506103) *trnL–trnF* (AF515539) ITS (AJ131239)	III (673 bp)
*H. pastuchovii*2		Cultivated	(MA)	*72*	*trnT–trnL*	IV (c. 693 bp)
*H. rhombea*1 (Miq.) Bean	Japan, Korea	Cultivated RNG (418-79-05131BM4316)	(MA)	*24*	*trnT–trnL* (AF506100) ITS (AJ131240)	III (672 bp)
*H. rhombea*2		Korea, South Coast	S. Lee s.n. (LIV)	*24*	*trnT–trnL* (AF506099) *trnL–trnF* (AF515537) ITS (AJ131241)	III (673 bp)
*H. rhombea*3		Taiwan, Tangmingshan National Park	H.A. McAllister 869H.A.M. (MA)	*24*	*trnT–trnL*	III (c. 673 bp)
Polyscias	Tropical Old World and New Caledonia			?		
P. balfouriana L.H. Bailey	New Caledonia	Cultivated	P. Vargas s.n. (MA)	?	*trnT–trnL* (AF506118)	I (591 bp)
Pseudopanax	Tasmania, New Zealand, and Chile			24		
Pseudopanax sp.		Cultivated	P. Vargas 68PV01 (MA)	?	*trnT–trnL* (AF506115)	I (589 bp)
Schefflera	Tropical and Temperate World			12		
S. cf. arboricola Hayata	Hainan Islands, Taiwan	Cultivated	P. Vargas 317PV02 (MA)	?	*trnT–trnL* (AF506120)	I (628 bp)

Note. Herbarium acronyms following voucher numbers as in the Index Herbariorum Part I (http://www/nybg.org/bsci/ih).

2.2. PCR amplification and sequencing

Total genomic DNA was extracted from silica-dried material collected in the field and occasionally from herbarium specimens (MA). DNA extractions were performed in two ways: following the CTAB method as indicated in Vargas et al. (1999a) and using the DNeasy Plant Mini Kit (QIAGEN Laboratories, Germany), particularly for herbarium specimens. DNA was amplified using the PCR (Polymerase Chain Reaction) and the primers *trn*a and *trn*b for the spacer *trnT*(UGU)–*trnL*(UAA) (Taberlet et al., 1991; Fig. 1); *trn*e and *trn*f for the *trnL*(UAA)–*trnF*(GAA) spacer (Taberlet et al., 1991); F71 and R1661 for the *rpl* 16 intron (Jordan et al., 1996); *trnK*-3914F and *trnK*-2R for the *matK* intron (Johnson and Soltis, 1995); and the external 17SE and 26SE (Sun et al., 1994) for the ITS region (see Vargas et al., 1999a for the ITS sequencing procedure). PCR conditions were performed following Taberlet et al. (1991) for amplification of *trnT–trnL* and *trnL–trnF* spacers (50 °C annealing temperature, 2-min elongation time) and using a Perkin–Elmer (California) PCR System 9700 thermal cycler. Amplified products were cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California) following the protocols provided by the manufacturer. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, California) and run into polyacrylamide electrophoresis gels (7%) using a Perkin–Elmer/Applied Biosystems model 377 automated sequencer. The primers *trn*a, *trn*b, *trn*e, and *trn*f were used for cycle sequencing of the *trnT–trnL* and *trnL–trnF* spacers (Fig. 1) under the following conditions: 95 °C for 2 min followed by 25 cycles of 95 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Sequence data were placed in a contig file and edited using the program Seqed (Applied Biosystems). The limits of the *trnT–trnL* and *trnL–trnF* sequences were determined by comparison with *Vicia faba* (Bonnard et al., 1984) and many other asterid sequences available in GenBank. The ITS sequence limits were determined following Vargas et al. (1999a). IUPAC symbols were used to represent nucleotide ambiguities.

Large indels (≥30 bp) were found in the *trnT–L* spacer when aligning the 41 sequences, which encouraged us to design an internal primer to amplify fragments of different sizes (see Section 3). A new, reverse primer *trn*hiedra) was used for the amplification of region I (Figs. 1 and 2) of the *trnT–trnL* spacer in the Araliaceae, coupled with the forward primer *trn*a (Taberlet et al., 1991). The *trn*hiedra primer is a 20-nucleotide-long oligo (5′CTAGCGATATAGAATTTCGA 3′) designed to avoid palindromic sequences, and is complementary to a fragment situated between the regions I and II. The optimal PCR-amplification conditions were found when using total DNA, the reaction conditions indicated above and 35 cycles of 1 min at 94 °C, 1 min 30 s at 53 °C, and 4 min at 74 °C. Identification of sequence sizes of the amplicons was performed directly on 2% agarose gels (Fig. 2B). The ease of using these primer combinations allowed an increase of the *Hedera* sample to 104 populations (Table 1).

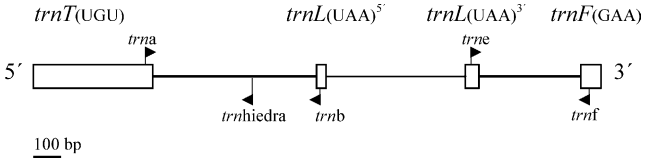

Fig. 1. Structure and directions of primers used to amplify *trnT–L* and *trnL–F* spacers. Proportional positions depicted for type I of chloroplast (chlrotype I) of *Hedera* (Taberlet et al., 1991). The internal primer *trn*hiedra was therein designed for amplification of a partial fragment of the *trnT–L* spacer.

Predicted secondary structures of the *trnT–L* and *trnL–F* spacers (Bonnard et al., 1984) and associated free energy values were investigated with the minimum-free energy algorithm (Zuker, 1989) to assess mechanisms of non-coding sequence evolution (Kelchner, 2000). Fold predictions were made at the M. Zuker web server (http://bioinfo.math.rpi.edu/mfold/dna/form1.cgi) and using the MFOLD program (version 3.0). Foldings were conducted at 37 °C using a search within 5% of the thermodynamic optimality setting (Mayol and Roselló, 2001). Base changes were identified in all the DNA secondary structures.

2.3. Sequence analyses

Four different sequence matrices were used to perform the phylogenetic analyses: ITS matrix of the Araliaceae, *trnT–L* matrix of the Araliaceae, a matrix of *Hedera* combining ITS and *trnT–L* sequences plus *Fatsia*, and a matrix of *Hedera* combining *trnT–L* and *trnL–F* sequences of *Hedera* plus *Fatsia*. The four alignments were obtained using the program Clustal X 1.62b (http://evolution.genetics.washington.edu/phylip/software.etc1.html) with further manual adjustments. The ITS matrix contains sequences from 26 previously published accessions of *Hedera* (Vargas et al., 1999a) plus four new ones selected for the present study (*H. helix* ssp. *helix*8 and 13; *H. hibernica*4; *H. maroccana*3), all 45 available sequences of the Araliaceae from GenBank (Mitchell and Wagstaff, 1997; Plunkett et al., 1997; Wen et al., 2001) and 10 from the Apiaceae, plus one of *Pittosporum* as the outgroup. The second matrix included 41 *trnT–trnL* sequences of the Araliaceae obtained in this study. The third matrix included one sequence of the *trnT–L* and *trnL–F* spacers per each taxon of *Hedera* (14), except two of *H. maroccana*, plus one of *Fatsia* (Table 1). Phylogenetic analyses were conducted using Fitch parsimony (as implemented in PAUP*, Swofford, 1999) with equal weighting of all

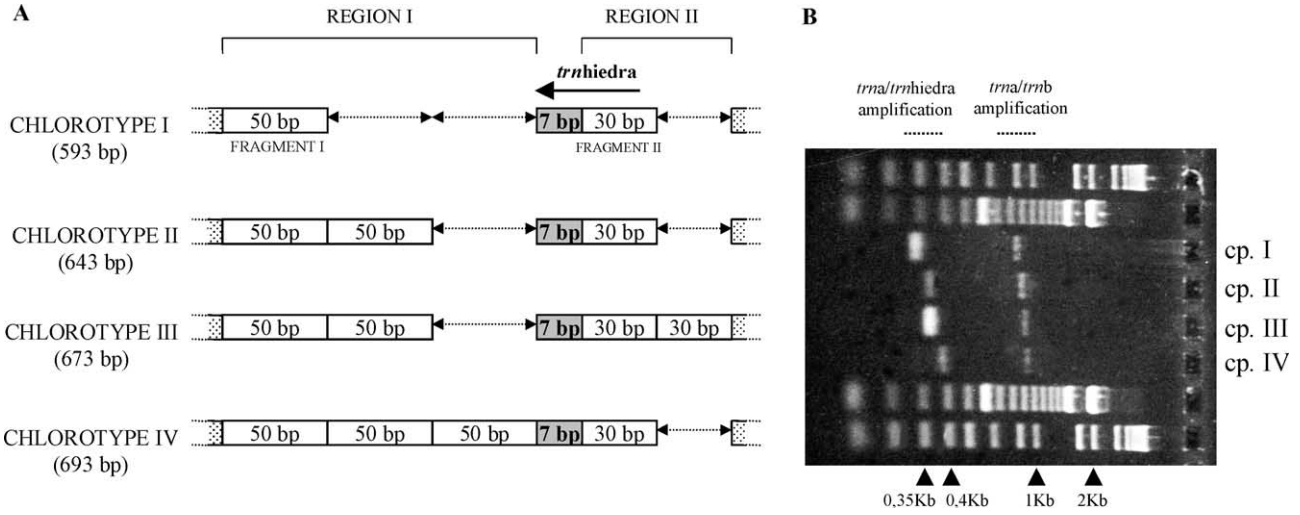

Fig. 2. Types of chloroplast (chlorotypes) of *Hedera* determined from different sizes of the *trnT–L* sequences. (A) Schematic representation of the four chlorotypes (cp. I, II, III, and IV), as aligned in the *trnT–L* sequence matrix. Region I has one, two, and three copies of a 50-bp fragment (fragment I); region II with one and two copies of a 30-bp fragment (fragment II). Relative position of the sites limiting the two regions and the new, reverse primer *trn*hiedra are indicated; (B) agarose gel resolution of the four chlorotypes from eight PCR amplicons when using primers *trna*/*trn*b (larger bands), and *trna*/*trn*hiedra (smaller bands) were loaded in four lanes: cp. I in *H. maderensis* spp. *iberica*1; cp. II in *H. helix* ssp. *helix*19; cp. III in *H. colchica*1; and cp. IV in *H. hibernica*2.

characters and of transitions:transversions. The effect of incorporating a transition:transversion bias in *Hedera* was also examined. Heuristic searches were replicated 100 times with random taxon-addition sequences, Tree Bisection-Reconnection (TBR) branch swapping and with the options MULPARS and STEEPEST DESCENT in effect. Relative support for clades identified by parsimony analysis was assessed by both "fast" bootstrapping (100,000 resamplings of each data set) and "full" bootstrapping (100 resamplings each) (see Mort et al., 2000 for discussion) using the heuristic search strategy as indicated above. In addition, phylogenetic reconstructions of *trnT–trnL* sequences were also performed in PAUP* using the Kimura-2-parameter distance model (Kimura, 1980) and the Neighbor-Joining method (Saitou and Nei, 1987). Indels in the *trnT–trnL* spacer were coded as appended characters following the logic of Kelchner (2000) and Simmons and Ochoterena (2000). As a result, four parsimony-informative indels were coded (two affecting the genera of the Araliaceae and two the regions I and II of *Hedera*) only as four appended characters with two character states, except for that of the region I with three.

3. Results

3.1. ITS sequence variation

The addition of four new ITS sequences to the previous 610-position matrix of *Hedera* (available from the authors) did not affect the basic features already published in Vargas et al. (1999a): all sequences have 609 bp except for the two samples of *H. algeriensis* and *H. maroccana*3 (608 bp); the ITS matrix has 72 variable positions and 27 parsimony-informative characters. Pairwise Kimura-2-parameter distances ranged from 0 (between many accession pairs from the same taxon) to 3% (between *H. maroccana*2 and the two accessions of *H. colchica*). Moreover, features of ITS sequences (length, pairwise variation) of *Hedera* are in the range of those found throughout the Araliaceae (Mitchell and Wagstaff, 1997; Plunkett et al., 1997; Wen et al., 2001).

3.2. Chloroplast sequence variation

The pilot study of four *Hedera* species rendered a low number of nucleotide substitutions/parsimony-informative characters: 3/0 in the *rpl*16 intron; 3/0 in the *matK* intron; 3/1 in the *trnL–F* spacer; and 3/1 in the *trnT–L* spacer. Consequently, an extended sample of *trnT–L* (41) and *trnL–F* (14) sequences for the 12 species of *Hedera* was obtained. The resulting *trnT–L* matrix consisted of 33 accessions of *Hedera* plus eight of the major groups of Araliaceae: *Aralia*, *Cussonia*, *Dendropanax*, *Dizygotheca*, *Fatsia*, *Polyscias*, *Pseudopanax*, and *Schefflera*. The number of variable/parsimony-informative characters was of 58/16 across the genera of the Araliaceae and 14/6 among the species of the *Hedera*. The number of transitions:transversions (24:33) was not further considered because these values do not appear to be sufficiently biased, a finding consistent with *trnL* intron regions from other angiosperms (Bakker et al., 2000) and may therefore not be biased in the Araliaceae.

The longest *trnT–L* sequence was found in *Hedera* (693 bp) and the shortest in *Pseudopanax* (499 bp). The highest pairwise Kimura-2-parameter distance was between *Fatsia japonica* and *Polyscias balfouriana* (3.3%) and 0.86% in *Hedera* (remarkably between *H. helix* ssp. *helix* populations 13 and 3), being 0 between some accession pairwise comparisons of 10 *Hedera* species. A single nucleotide substitution in *trnL–F* sequences separates *H. maderensis* ssp. *iberica*, *H. canariensis*, *H. helix* ssp. *rhizomatifera*, and *H. maroccana*1 (but not *H. maroccana*2) from the rest of *Hedera* and *Fatsia* accessions.

3.3. Chlorotypes

Within *Hedera*, chloroplast sequences of the *trnT–L* spacer varies greatly in size (593–693 bp), not only among species but also within single species (Table 1). The explanation for this major length variation in *Hedera* (including 100-bp maximum difference between accessions) was apparent after aligning the matrix. Two major indels of 50 and 30 bp were present in different numbers of copies. These indels define two regions (regions I and II) separated by a conserved sequence of 7 bp (Fig. 2A). We found three motifs of the 50 bp repeat (region I) and two motifs of the 30 bp repeat (region II), allowing us to distinguish four distinct types of chloroplast (chlorotypes) in the Araliaceae based on size differences of the *trnT–L* sequences (hereafter, cp. I, cp. II, cp. III, and cp. IV) (Fig. 2). To our knowledge, both indels are the largest found in the *trnL* intron and spacers from any small genus of angiosperms (Bakker et al., 2000; Kelchner, 2000). Seventeen subchlorotypes were also identified, based on nucleotide substitutions found in one or more accessions. *Hedera* populations displayed four chlorotypes, while the other eight genera sampled from the Araliaceae had only one (cp. I). To test the phylogenetic significance of the four major indels, we examined *trnT–L* and *trnL–F* sequence substitutions and the secondary structures of *trnT–L* spacer foldings. The four chlorotypes displayed different putative single-stranded secondary structures at 37 °C: cp. I (5, $\Delta G: -47.7$ to -50.1); cp. II (7, $\Delta G: -50.2$ to -52.7); cp. III (8, $\Delta G: -51.2$ to -53.8); and cp. IV (9, $\Delta G: -52.3$ to -55.0). Careful examination of loops, steam-loops, and nucleotide domains failed to reveal any hot spots for mutation, compensatory mutations or evident mutational triggers (Bonnard et al., 1984; Kelchner, 2000). Accordingly, we are not able to point to slipped-strand misparing or intramolecular recombination as factors accounting for the origin of these remarkable sequence repeats. Secondary structure formation does not appear to affect significantly the evolution of this non-coding chloroplast spacer (e.g., compensatory mutations) in view of phylogenetic reconstructions based on nucleotide substitutions and the four major indels (Bakker et al., 2000; Simmons and Ochoterena, 2000).

Chlorotypes I (c. 593 bp) and II (c. 643 bp) are easily identified by visual inspection of bands in agarose gels (Fig. 2B) by using the *trnT–L* primers *trn*a and *trn*b. To distinguish the similar-sized amplicons of chlorotypes III (673 bp) and IV (693 bp), the reverse primer *trn*hiedra (Fig. 2A) permits resolution in agarose gels of two shorter fragments of c. 336 bp (in chlorotype III) and c. 386 bp (in chlorotype IV) by excluding region II (Fig. 2A). The inferred size of each band was confirmed in several accessions by sequencing the resulting fragments of the two *trnT–L* amplicons with primers *trn*a/*trn*b and *trn*a/*trn*hiedra. The sample was then increased to 71 additional *Hedera* populations to identify their chlorotypes by resolving the PCR-amplification bands in agarose gels. As a result, the following four chlorotypes were found in *Hedera* taxa: *H. algeriensis* (cp. II); *H. azorica* (cp. IV); *H. canariensis* (cp. I); *H. colchica* (cp. III); *H. cypria* (cp. II, IV); *H. helix* ssp. *helix* (cp. I, II, III, and IV); *H. helix* ssp. *rhizomatifera* (cp. I, IV); *H. hibernica* (cp. I, III, and IV); *H. maderensis* ssp. *maderensis* (cp. IV); *H. maderensis* ssp. *iberica* (cp. I); *H. maroccana* (cp. I, II); *H. nepalensis* (cp. III); *H. pastuchovii* (cp. III, IV); *H. rhombea* (cp. III). This taxonomic distribution of *trnT–L* chlorotypes indicates that not only three polyploid species have multiple chlorotypes (*H. hibernica*, *H. cypria*, *H. pastuchovii*), but also do some diploid taxa (*H. helix* ssp. *helix*, *H. helix* ssp. *rhizomatifera*, and *H. maroccana*). In contrast, a single chlorotype was found in the other diploids (*H. azorica*, *H. canariensis*, *H. nepalensis*, and *H. rhombea*) and the remaining polyploids (*H. algeriensis*, *H. colchica*, *H. maderensis* ssp. *maderensis*, and *H. maderensis* ssp. *iberica*).

3.4. Phylogenetic analyses

The parsimony analyses of 45 ITS sequences from the Araliaceae, ten representatives of the Apiaceae plus 30 of *Hedera*, and using *Pittosporum dalli* as the outgroup, retrieved 115,800 minimum-length trees of 1,471 steps on a single island with a consistency index (CI) of 0.51 and a retention index (RI) of 0.74 (Fig. 3). The strict consensus tree depicted a mostly resolved topology, although few clades are strongly supported, as was previously reported for the major clades of the Araliaceae using ITS data (Plunkett and Lowry, 2001; Wen et al., 2001). At basal branches, *Hydrocotyle* is sister to the Araliaceae (66% bootstrap), the Araliaceae genera are shown to be monophyletic (63% bootstrap), and weakly supported clades do not define major groups of the Araliaceae. A robust clade (100% bootstrap) including all ITS sequences of *Hedera* supports the monophyly of this genus. Within *Hedera*, the resolution of two major clades of mostly diploid species (82% bootstrap) and polyploid species (52% bootstrap) is consistent with that

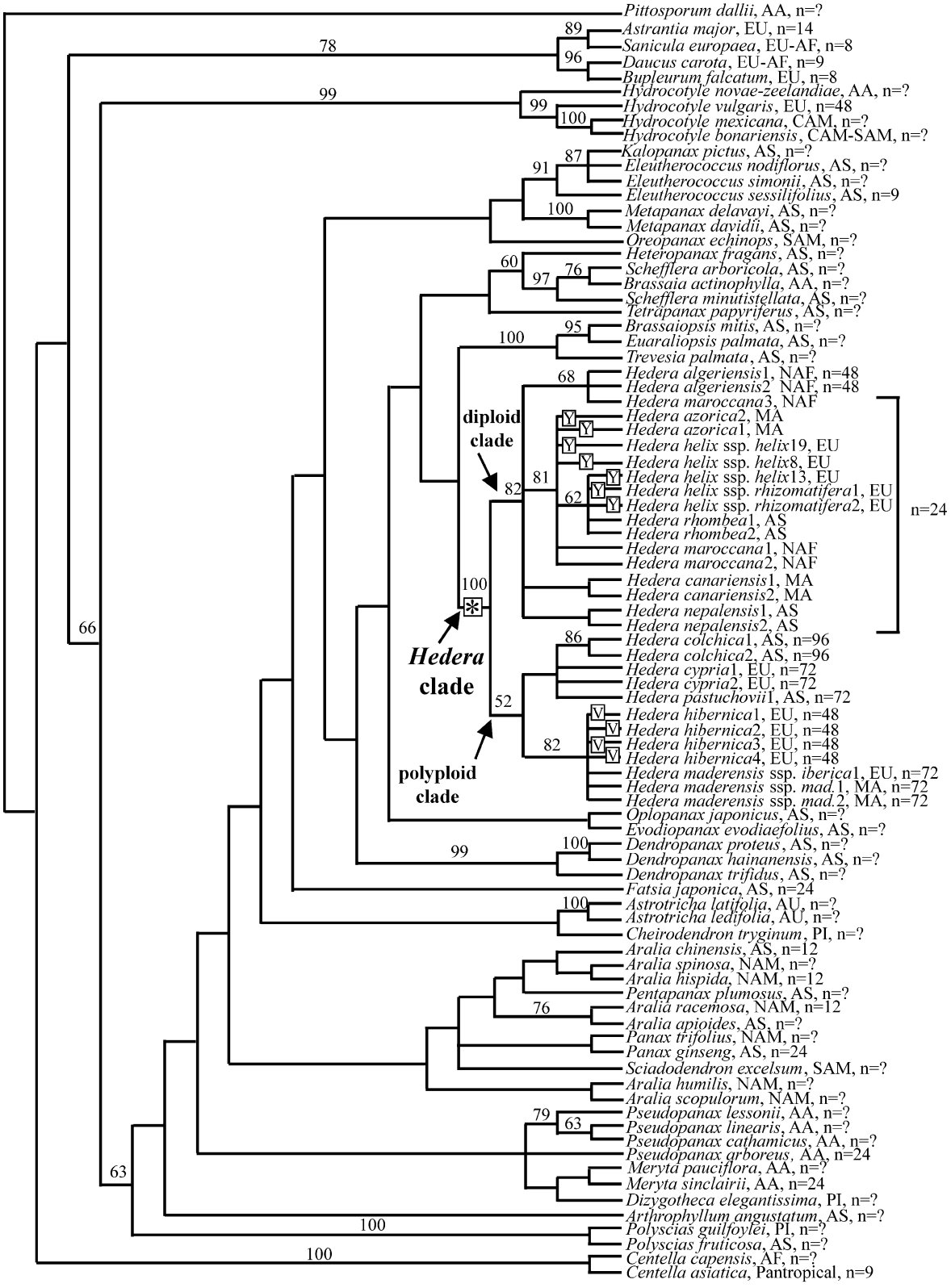

Fig. 3. Strict consensus tree of 115,800 most parsimonious trees of 1471 steps from the analysis of the 85 ITS sequences of the Araliaceae and Apiaceae (CI: 0.51; RI: 0.74). *Pittosporum dallii* served as the outgroup. Bootstrap values above 50% are shown above branches. Numbers after species names refer to population numbers as in Table 1, followed by distributional abbreviations (AA, Australasia; AF, Africa; AS, Asia; AU, Australia; CAM, Central America; EU, Europe; MA, Macaronesia; NAF, northern Africa; NAM, North America; PI, Pacific Islands; SAM, South America) and chromosome numbers, indicating a question mark (?) for cases of insufficient data.

of Vargas et al. (1999a), as is the terminal resolution of particular subclades, even though we expected lower resolution because of the potential impact of the addition of 45 ITS sequences from the Araliaceae.

Phylogenetic analysis of *trnT–L* sequences of *Hedera* plus eight genera of the Araliaceae using *Cussonia thyrsiflora* as the outgroup yielded 30 most-parsimonious trees of 67 steps (CI: 0.93; RI: 0.92) from a single island. The strict consensus tree (not shown) depicted all *Hedera* sequences in a single group (78% bootstrap) with two major clades. One major clade contained the 10 sequences with chlorotype I (53% bootstrap): two populations of four taxa (*H. helix* ssp. *rhizomatifera*, *H. canariensis*, *H. maderensis* ssp. *iberica*, and *H. maroccana*) plus one population of *H. helix* ssp. *helix* and one of *H. hibernica*. The other major clade consisted of the rest of the 23 sequences with chlorotypes II, III, and IV (<50% bootstrap). When coding the four parsimony-informative indels mentioned above, 114 trees of 73 steps (CI: 0.92; RI: 0.94), on one island, were obtained. The resulting strict consensus tree (Fig. 4) yielded higher support for the monophyly of *Hedera* (89% bootstrap) and the same two major clades, but with higher bootstrap values: clade 1 (64% bootstrap) of accessions with chlorotype I; and clade 2 (76% bootstrap) of those with chlorotypes II, III, and IV. Within clade 2, a subclade including all accessions with chlorotype III (63% bootstrap) is identified, related with haplotypes found by Grivet and Petit (pers. commun.) using different chloroplast markers on the same populations (11 and 12 of *H. helix* ssp. *helix*; 8 of *H. hibernica*) (Table 1). Three additional subclades were identified including two populations of *H. maroccana* (1, 3) plus *H. helix* ssp. *helix*3 (65% bootstrap), the two populations of *H. algeriensis* (82%), all chlorotype III sequences (three populations of *H. helix* ssp. *helix*, the two of *H. rhombea*, the two of *H. nepalensis*, the two of *H. colchica* and *H. pastuchovii*1) (63% bootstrap). The last five populations form a weakly supported subgroup (52% bootstrap). The tree resolution and support values largely agree with the topology and branch length of the Neighbor-Joining tree (results not shown). The analysis of the third matrix, which combines ITS plus *trnT–L* sequences of *Hedera* from the same taxa and individuals, displayed a tree similar to that of the ITS sequence analysis (results not shown). The higher number of parsimony-informative characters among ITS sequences (27) than that among *trnT–L* sequences (6) has a major impact in the resulting topology.

The single parsimony-informative substitution found in *trnL–F* sequences discriminates consistently the accessions having chlorotype I (adenine) from those with chlorotypes II, III, and IV (guanine). Congruence between the *trnT–L* phylogeny and the *trnL–F* parsimony-informative substitution also supports that multiplication of the two large motifs of 30 and 50-bp to form the four chlorotypes is not homoplasious. Non-homoplasious indels have also been documented in other angiosperms with large length mutations (Mes and Hart, 1994; van Ham et al., 1994). The analysis of the fourth matrix combining *trnT–L* and *trnL–F* sequences, and using identical phylogenetic conditions, displays the same resolution as Fig. 4, but higher support for the two major clades of *Hedera* (results not shown).

There is no clear affinity between taxon identification and either monophyletic groups of populations in the *trnT–L* tree (Fig. 4) or chlorotypes. First, only the two accessions of *H. algeriensis* form a monophyletic group. In addition, all accessions of some taxa are placed in only one of the two major clades, whereas those of others are found in both clades. Clade 1 contains all the accessions of three taxa (*H. helix* ssp. *rhizomatifera*, *H. canariensis*, and *H. maderensis* ssp. *iberica*), and clade 2 is formed by all accessions of seven taxa (*H. azorica*, *H. algeriensis*, *H. colchica*, *H. cypria*, *H. maderensis* ssp. *maderensis*, *H. nepalensis*, and *H. rhombea*). In contrast, accessions of *H. helix* ssp. *helix*, *H. hibernica*, and *H. maroccana* are in both clades. Second, some taxa contain only one chlorotype: seven genera of the Araliaceae plus *H. helix* ssp. *rhizomatifera*, *H. canariensis*, and *H. maderensis* ssp. *iberica* with cp. I; *H. algeriensis* with cp. II; *H. rhombea*, *H. nepalensis*, *H. pastuchovii*, and *H. colchica* with cp. III; *H. azorica*, *H. maderensis* ssp. *maderensis* with cp. IV. While four taxa contain more than one chlorotype: *H. cypria* (cp. II, IV); *H. hibernica* (cp. I, IV); *H. maroccana* (cp. I, II); and *H. helix* ssp. *helix* (cp. I, II and III). The increase of the sample with 71 additional populations (Table 1) yielded additional information because we found one additional chlorotype in four taxa: *H. helix* spp. helix (cp. IV); *H. hibernica* (cp. III); *Hedera helix* ssp. *rhizomatifera* (cp. IV); and *H. pastuchovii* (cp. IV).

3.5. Chlorotype geographic distribution

Chlorotype distribution of the extended sample (104 populations) from Eurasia, northern Africa, and Macaronesia is shown in Fig. 5. Three Macaronesian archipelagos harbor two *Hedera* chlorotypes: chlorotype IV in *H. azorica* (six populations from three Azores islands) and in the Madeiran *H. maderensis* ssp. *maderensis* (11 populations); and chlorotype I in three Canarian islands of *H. canariensis* (six populations). On the continent, we found only chlorotype III in Asia (*H. colchica*, *H. nepalensis*, *H. pastuchovii*, and *H. rhombea*), chlorotype III in Central Europe (*H. helix* ssp. *helix* and *H. hibernica*), and the highest chlorotype diversity (four chlorotypes) in the Mediterranean region. Chlorotype I is found only in the western Mediterranean, particularly in the Iberian Peninsula, Morocco, and the Canary Islands. The diploid *H. helix* ssp. *helix* displayed the highest number of chlorotypes (four) with a geographic distribution in which Central European populations have only chlorotype III, whereas the Mediterranean populations have the four

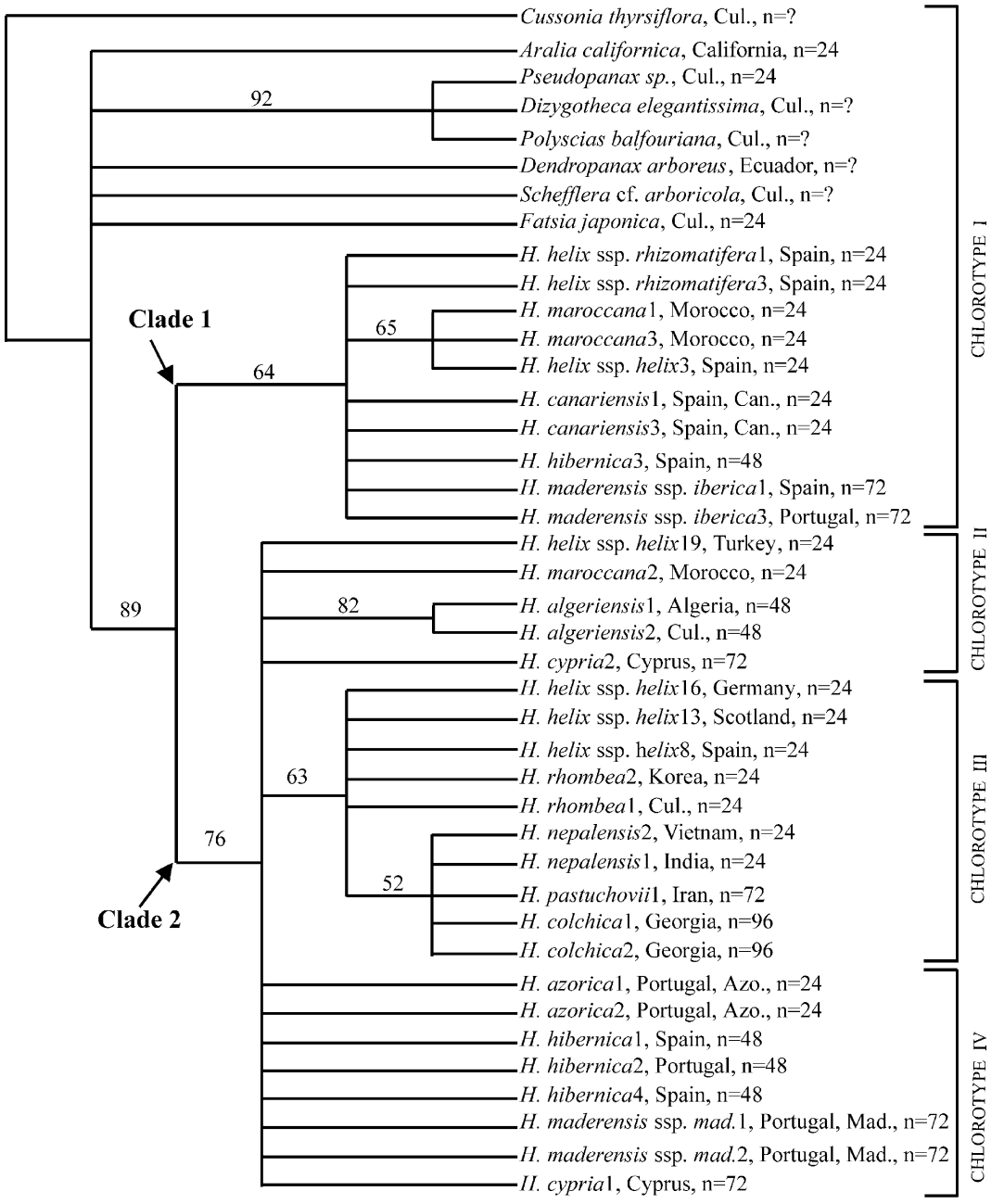

Fig. 4. Strict consensus tree of 114 trees of 73 steps (CI: 0.92; RI: 0.94) from the analysis of 41 *trnT–L* spacer sequences plus recoding of four indels, four affecting two sequences of the Araliaceae genera and two for the regions I and II of *trnT–L* (see text and Fig. 2). *Cussonia thyrsiflora* served as the outgroup. Bootstrap values above 50% are shown above branches. Numbers after species names refer to population numbers as in Table 1 and Fig. 3, followed by country and region of origin, and gametic chromosome number. Abbreviations: Azo., the Azores; Can., the Canary Islands; Cul., cultivated and cultivars; Mad., Madeira; mad, maderensis. Accessions grouped by the four Araliaceae chlorotypes (cp. I, II, III, and IV).

chlorotypes. Chlorotype polymorphism among individuals of two populations of *H. helix* susbp. *helix* from the Iberian Peninsula was tested—where the highest chlorotype differentiation was detected (Fig. 5)—and in one population from Germany. A single chlorotype is shared by the 10 individuals from the center (cp. I) and the 10 individuals from the northeast (cp. III) of the Iberian Peninsula, as well as the 11 individuals from Germany (cp. III).

4. Discussion

4.1. Monophyly of Hedera

Both the ITS and *trnT–L* phylogenies strongly support the monophyly of the extant species of *Hedera* (100 and 89% bootstrap, respectively). Although genetic compatibility of artificial intergeneric hybrids (*Fatshedera* = *Hedera* × *Fatsia*) (Maberley, 1997) has been dem-

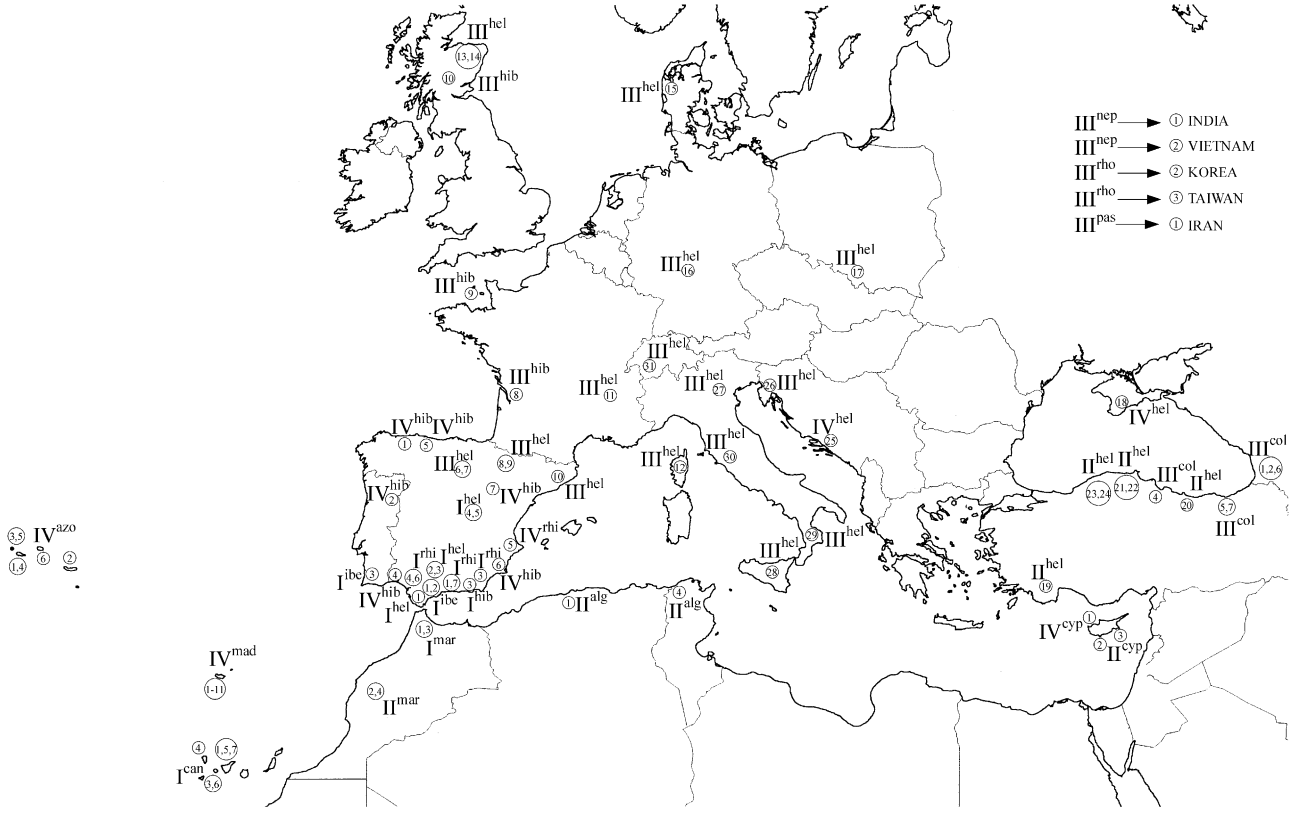

Fig. 5. Distributional map of the four Araliaceae chlorotypes (I, II, III, and IV) identified in *Hedera* (see text and Fig. 2). Species identification and circled population number as in Table 1. Abbreviations: alg, *H. algeriensis*; azo, *H. azorica*; can, *H. canariensis*; col, *H. colchica* ; cyp, *H. cypria*; hel, *H. helix* ssp. *helix*; hib, *H. hibernica*; ibe, *H. maderensis* ssp. *iberica*; mad, *H. maderensis* ssp. *maderensis*; mar, *H. maroccana* ; nep, *H. nepalensis*; pas, *H. pastuchovii*; rhi, *H. helix* ssp. *rhizomatifera*; rho, *H. rhombea*.

onstrated, there is no molecular evidence for a reticulate origin of *Hedera*. Naturalness of this genus has not been questioned since its description by Linnaeus (1753), due to stable morphological characters throughout the species, such as paniculate inflorescences, free pedicels, polygamous flowers, valvate aestivation, 5-locular ovaries, and ruminate endosperm (Eyde and Tseng, 1971). In contrast, phylogenetic analysis of chloroplast and nuclear sequences indicates that some genera of the Araliaceae are not monophyletic: *Pseudopanax* (Mitchell and Wagstaff, 1997); *Polyscias* (Plunkett et al., 2001); *Aralia*, *Eleutherococcus*, *Schefflera* (Wen et al., 2001).

4.2. Discordant phylogenies and allopolyploidization

An ongoing process of auto- and allopolyploidization of ivies may have occurred in the Mediterranean basin from $2n = 24$ (Vargas et al., 1999a). Phylogenetic reconstruction of uniparental-inherited *trnT–L* and *trnL–F* sequences suggest multiple origins not only for three polyploids (*H. cypria*, *H. hibernica*, and *H. pastuchovii*), but also for three diploid taxa (*H. helix* ssp. *helix*, *H. helix* ssp. *rhizomatifera*, and *H. maroccana*) (Table 1; Fig. 4). Fundamental discordance between the ITS (Fig. 3) and *trnT–L* (Fig. 4) phylogenies and morphological characters may be due to the independent origin of similar characters (homoplasy), lineage sorting or reticulation (Avise, 1994; Doyle, 1992; Neigel and Avise, 1986). Independent acquisition of two basic types of hairs through gene mutation at least four times in the ITS phylogeny (Fig. 3) or, alternatively, three times in the *trnT–L* phylogeny would be necessary to reconcile the two phylogenies, and seems like a most unparsimonious solution. Lineage sorting, which is extinction of ancestral gene polymorphisms through stochastic processes, is unlikely because it would imply random inheritance of not only some polymorphic ITS and *trnT–L* copies, but also genes involved in hair morphology (Wendel, 2000). In contrast, discordance between uniparental (*trnT–L*) and biparental (ITS) phylogenies, intermediate hair characters, microsatellite chromosome patterns of the tetraploid *H. hibernica* (Jacobsen, 1954), and the ITS nucleotide additivity in three species, all suggest active reticulation in *Hedera*. Nucleotide additivity has only been found in ITS sequences of the polyploids *H. maderensis*, *H. cypria*, and *H. hibernica*, which suggests that hybridization is related to increment of chromosome complements, i. e. increment of ploidal level. Alternative nucleotides at the polymorphic sites were detected among the extant diploids (Vargas et al., 1999a) in

agreement with hybrid speciation. Although recent hybridization of polyploids at the same ploidal level (homoploid hybridization) is not fully ruled out (Rieseberg and Carney, 1998), most reticulation observed in *Hedera* may be the result of increment of ploidal level and ancient chloroplast capture (allopolyploidization) (Rieseberg and Soltis, 1991; Soltis and Soltis, 2000).

We postulate successive doubling of two distinct genomes (allopolyploidization) as the major evolutionary processes in the formation of lineages of *H. colchica*, *H. cypria*, *H. hibernica*, *H. maderensis*, and *H. pastuchovii*, but likely autopolyploidy in *H. algeriensis*. The bulk of evidence and the need to invoke fewer assumptions support allopolyploidization over autopolyploidization in the first five species because (1) additive nucleotides at 10 ITS positions indicate hybridization; (2) a single ploidal level is observed within each species, unlike cases of autopolyploidy in which some populations may contain transitional stages of diploidy and triploidy (Ramsey and Schemske, 1998); (3) lack of evidence of extant hybridization at the diploid level within *Hedera* populations suggests that homoploid hybridization is not of major influence (Rieseberg and Carney, 1998); and (4) one pair of satellite chromosomes in the tetraploid *H. hibernica* seems to be the result of inheritance from two dissimilar diploid progenitors (Jacobsen, 1954; Vargas et al., 1999a). Autopolyploidization after homoploid hybridization or hybridization after polyploidization is not completely ruled out as a mechanism to both increase the number of complements from $n = 24$ to $n = 96$ (*H. colchica*) and include nucleotide polymorphisms (Ferguson and Sang, 2001; Sang et al., 1997). Single- or low-copy nuclear genes may shed light on this issue in future investigations. Consideration of putative parents involved in the formation of *H. hibernica*, *H. maderensis*, and *H. pastuchovii* has been discussed elsewhere (Vargas et al., 1999a). A case of autopolyploidization is suggested for *H. algeriensis* (Vargas et al., 1999a) based on the relatively high number of ITS (3) and *trnT–L* (2) autapomorphies, a lack of nucleotide polymorphism, two pairs of satellite chromosomes (Jacobsen, 1954), and an absence of intermediate morphological characters. Our ITS (Fig. 3) sequence reconstruction, together with data from epidermal hair types and northern African chlorotype distribution (Fig. 5), is congruent with an autopolyploid origin of *H. algeriensis* from a common ancestor with certain populations of *H. maroccana*. Incongruence in the placement of the population 3 of *H. maroccana* between the ITS and *trnT–L* phylogenies is, however, intriguing.

4.3. Ancestral polyploidy in Hedera diploids

Polyploidization appears to have been an active process since the formation of *Hedera* in the Tertiary (Dilcher and Dolph, 1970; Muller, 1981; Rim, 1994). Its basic chromosome number ($x = 24$) is high among the angiosperms (Goldblatt, 1980; Grant, 1981), as well as in the Araliaceae ($x = 12$). Two chromosome numbers, $n = 12$ and $n = 24$, are found in some Araliaceae (Fedorov, 1974; Goldblatt, 1981–1988; Goldblatt and Johnson, 1990–2000). Accordingly, it is reasonable to suggest a secondary ($x = 24$) and a primary basic chromosome numbers ($x = 12$) (Jacobsen, 1954; Vargas et al., 1999a), which is fully in agreement with the threshold of $n = 12$ for the basic chromosome number of genera of angiosperms (Goldblatt, 1980; Grant, 1963; Stebbins, 1950). Presence of both chromosome numbers within unrelated genera (*Aralia*, *Fatsia*, *Nothopanax*, and *Panax*) and in a single species of *Schefflera* (*S. actinophylla*) indicates independent polyploidization processes. Chromosome counts for many species in the Araliaceae are lacking, and future cytotaxonomical investigations are highly recommended to fill in caryological gaps and infer primitive vs. derived chromosome numbers in phylogenetic reconstructions (Fig. 3). Increase in the number of chromosome complements through intergeneric genome allopolyploidization between extant genera of the Araliaceae in the formation of *Hedera* is unlikely. Neither nuclear (Fig. 3) nor chloroplast (Fig. 4) markers of the extant ivies is significantly closely related to those of other Araliaceae.

Molecular evidence for recurrent formation of two ivy diploids (*H. helix* and *H. maroccana*) is suggested by the ITS (Fig. 3) and *trnT–L* (Fig. 4) phylogenies, coupled with high diversity of chlorotypes (Fig. 5). In northern Africa, the populations of the Moroccan ivy (*H. maroccana*) distributed in the Rif and the Atlas mountains have at least two ancient origins as inferred from the two chlorotypes found (cp. I, II) and the ITS and *trnT–L* lineages. Occurrence of the ancestral chlorotype I, the derived chlorotype II, and relatively high genetic differentiation in ITS (0.18–1.4%, Kimura-2-parameter) and *trnT–L* (0.5%, Kimura-2-parameter) sequences in the Moroccan ivy suggests that two lineages are involved in the formation of this putative ancestral polyploid from plants of $n = 12$. The four *trnT–L* chlorotypes (cp. I, II, III, and IV) in 33 populations of *H. helix* ssp. *helix* (Table 1), which are distributed in three groups of the *trnT–L* phylogeny (Fig. 4), may also indicate multiple origins of this taxon. Recurrent reticulation in this putative ancestral polyploid may not have occurred recently, as given the absence of ITS nucleotide polymorphisms (Vargas et al., 1999a), a single pair of chromosome microsatellites (Jacobsen, 1954), the occurrence of the ancestral chlorotype I, and relatively high genetic differentiation among *trnT–L* sequences (0–0.87%, Kimura-2-parameter) and among bands from the fingerprinting marker ISSR (Inter-Simple Sequence Repeats, unpublished data). In contrast, the two Macaronesian (*H. azorica* and *H. canariensis*) and Asian (*H. nepalensis* and *H. rhombea*) diploid species are shown to

be monophyletic. In the future, a comprehensive sampling strategy should be designed to investigate a single origin of these diploids.

Therefore, we envision various allopolyploidization events in the formation of some ploidal levels of the polyploid series of *Hedera* ($2n = 24$, 48, 72, 96) from $n = 12$ as a long-term speciation process in *Hedera*, not only in the formation of the most recent polyploids (neoploidy) (Goldblatt, 1980), but also in extant derived diploids (paleopolyploids) ($n = 24$) (Eckardt, 2001). ITS sequence examination of nucleotide additivity reveals that a diploid ancestor, not found in nature, may have conferred four additive nucleotides and 11 nucleotide characters exclusively to the species of the polyploid clade (Fig. 3) (Vargas et al., 1999a). Extinction of derived diploid ancestors ($n = 24$) involved in the formation of the most recent polyploid species indicates active polyploidization and extinction episodes since the formation of the genus.

4.4. Cytological causes of polyploidization

The two major routes of polyploid formation are somatic and gametic doubling as intermediate stages to polyploid stabilization (Ramsey and Schemske, 1998). Gametic non-reduction appears to be the predominant mechanism of polyploid formation in angiosperms through either one-step or triploid-bridge stages (Bretagnole and Thompson, 1995; Harlan and de Wet, 1975). Alternatively, somatic doubling in meristem tissue (endopolyploidy) has been observed in plants with particular shoots and tumors (D'Amato, 1952, 1964; Lewis, 1980). Shifts in genome size within a single individual of *H. helix* ssp. *helix* from the juvenile to the adult phase have been detected in some studies (Domoney and Timmis, 1980; Maire and Brown, 1993; Schäffner and Nagl, 1979), but not in others (Köning et al., 1987; Obermayer and Greilhuber, 2000). Although there is no consensus about which form of chromatine multiplies in vegetative cells (heterochromatin vs. euchromatin), the tendency for genome augmentation to occur, coupled with active vegetative reproduction, may have favored somatic doubling in *H. algeriensis* (Rogers and Bonnett, 1989). Gametic doubling is likely in the formation of the other polyploids.

4.5. Centers of origin and diversification

The center of diversity of the Araliaceae has been placed in southern Asia, Australia, and New Caledonia because the highest number (30 of 47) and archaic genera (*Osmoxylon* and *Schefflera*) are distributed in this region (Bernardi, 1971; Brummitt, 1992; Mabberley, 1997; Takhtajan, 1997). This hypothesis is in agreement with biogeographic patterns based on nuclear and chloroplast phylogenies, which reveal a basal-most placement of genera distributed in southern Asia, Australia, and New Caledonia (Fig. 3) (Plunkett et al., 1997; Plunkett and Lowry, 2001; Wen et al., 2001). The center of origin for ivies may have been Asia because the phylogenetic reconstruction of the ITS phylogeny (Fig. 3) shows *Hedera* as a derived genus within the Araliaceae and closely related to other Asian genera. Occurrence of a macrofossil record of *Hedera* from the Oligocene in Asia (Rim, 1994) supports this concept. However, biphyletic topologies in both phylogenies do prevent from inferring whether the ancestral lineage of extant ivies is an Asian, northern-African, European or Macaronesian diploid. The occurrence of the chlorotype I in the eight genera chosen as representatives of the major lineages of the Araliaceae, but not in the Asian species of *Hedera*, leaves open another interesting possibility. Chlorotype I is found exclusively in ivies from the western Mediterranean basin, where the Iberian Peninsula and northern Africa harbor the highest chloroplast diversity (cp. I, II, III, and IV) (Fig. 5), a result also found by Grivet and Petit (pers. commun.). This molecular differentiation, coupled with ancient pollen fossil records from the upper Miocene in Iberia (Muller, 1981), suggests a secondary center of diversity in the western Mediterranean basin, during which time the ivies may have become extinct in Asia after their migration westward. Further colonization by *Hedera* in Asia is suggested by the only chlorotype (cp. III) found there and the terminal placement of the diploid Asian ivies in the *trnT–L* phylogeny (Fig. 4). A larger sample of Asian populations and results from different markers are needed to test this hypothesis of secondary colonization from the Mediterranean basin back to Asia.

4.6. Colonization history in Macaronesia

The three taxa (*H. azorica*, $2n = 2x$; *H. maderensis* ssp. *maderensis*, $2n = 6x$; *H. canariensis*, $2n = 2x$) endemic to the three Macaronesian archipelagos (the Azores, Madeira, and the Canary Islands) are placed into three different clades in the nuclear and chloroplast phylogenies (Figs. 3 and 4). A single dispersal and establishment (with no further contact either with continental or insular species) is suggested by the molecular uniformity of chlorotypes distributed on the islands of each archipelago: six populations from the Azores (cp. IV); 11 from Madeira (cp. IV); and six from the Canary Islands (cp. I) (Fig. 5). Results from J. Francisco-Ortega and collaborators, in conjunction with those from other authors, indicate that many genera of Macaronesian angiosperms originated from single introduction events (see review by Baldwin et al., 1998; Vargas, 2003). Fourteen natural groups have succeeded and radiated in Macaronesia from fourteen independent dispersal events: *Sinapidendron* (Warwick and Black, 1993); *Chamaecytisus* (Badr et al., 1994); Crassulaceae (Mes et al., 1996); *Echium* (Böhle et al.,

1996); *Sonchus* (Kim et al., 1996); *Argyranthemum* (Francisco-Ortega et al., 1997); *Isoplexis* (Carvalho and Culham, 1998); *Saxifraga* (Vargas et al., 1999b); *Cheirolophus* (Susanna et al., 1999); *Crambe* (Francisco-Ortega et al., 1999); *Pericallis* (Panero et al., 1999); *Bencomia* alliance (Helfgott et al., 2000); *Sideritis* (Barber et al., 2000); *Gonospermum* (Francisco-Ortega et al., 2001a). Two introductions with further radiation have been detected in three groups of Asteraceae: the *Asteriscus* alliance (Francisco-Ortega et al., 2001b), *Tolpis* (Park et al., 2001), and *Pulicaria* (Francisco-Ortega et al., 2001b). Interestingly, four genera consisting of two Macaronesian species each and characterized by endozoochorus fruits or fleshy cones (*Ilex*, Cuénod et al., 2000; *Hedera*, Vargas et al., 1999a; *Olea*, Hess et al., 2000; *Juniperus*, Martínez and Vargas, 2002) have colonized Macaronesia after at least two introductions each with no further specific differentiation. Fleshy fruits may be one of the most successful means of diaspore movement in long-distance dispersal to Macaronesia, as ancestor-reconstruction inferences based on molecular data suggest for Hawaii (c. 33% of the angiosperm flora of Hawaii originated from fleshy-fruited ancestors; Vargas and Baldwin, unpublished data). Active dispersal of those fleshy-fruited species in potential source areas on adjacent continents has already been documented (Guitián, 1987; Peterken and Lloyd, 1967; Rey and Alcántara, 2000). The Macaronesian colonization by hollies, olives, strawberry trees (Hileman et al., 2001), elders (Eriksson and Donoghue, 1997) and junipers displays a pattern in which an early introduction (*Ilex* in the Azores; *Arbutus* in the Canary Islands; *Sambucus* in Madeira; *Olea* in Madeira; *Juniperus* in the Azores) occurred in certain archipelagos followed, in some cases, by secondary introductions in different archipelagos (*Ilex* in the Canary Islands; *Olea* in the Canary Islands; *Juniperus* in the Canary Islands). Colonization of Macaronesian archipelagos by *Hedera* after successive dispersal events of drupaceous fruits is also demonstrated in the present study. The ITS and *trnT–L* phylogenies suggest that an ancient lineage of ivies colonized the Canary Islands via a single colonization of a diploid ancestor carrying chlorotype I (Valcárcel and Vargas, 2002) and reddish scale trichomes, characteristics presently found in northern African populations of *H. maroccana*. The Azorean (*H. azorica*) and Madeiran (*H. maderensis* ssp. *maderensis*) ivies are the result of independent introductions of two lineages carrying the same derived chlorotype IV but different ploidal levels, the former from a diploid ancestor and the latter after polyploidization.

4.7. Postglacial recolonization in Europe

Biogeographic reconstruction of Holocene plant distributions in Europe is favored by complex geographic barriers, the dramatic last ice age (maximum at 21–18,000 ago) (Frenzel et al., 1992), and the documentation of a continuous fossil pollen record over the last 13,000 years (Huntley and Birks, 1983). Glacial and postglacial episodes followed by particular recolonization modes are the most important factors to account for the extant genetic structure of animal and plant populations (Hewitt, 2000). Ivies are considered to be reliable indicators of warm periods in ancient (Oligocene, Rim, 1994) and recent (Holocene, Iversen, 1944) times across temperate Eurasia. The surviving threshold of *Hedera* at −1.5 °C as average for the coldest month (Iversen, 1944), coupled with the absence of pollen record for ivies in the Middle Weichselian (Holocene) in Britain (Godwin, 1975), leads us to postulate a decline and extinction of ivies between 20,000 and 10,000 years ago in northern Europe. The ice sheet, permafrost line, and low temperatures during the last glacial maximum in the Weischelian (Frenzel, 1992) may have determined the distribution of *Hedera* not only in the Eurosiberian floristic region, but also in Mediterranean areas. Documented glacial refugia for European biota suggest postglacial episodes in which genetic diversity contracted in three Mediterranean refugia: the Iberian, Italian, and Balkan peninsulas (Bennett, 1991; Taberlet et al., 1998). Our molecular data are congruent with this scenario, in which high levels of *Hedera* cpDNA diversity in particular areas of the Mediterranean basin may have served as refugia for recolonization (Fig. 5). Occurrence of a single chlorotype (cp. III) in northern Europe is interesting because it parallels the homogeneous distribution of haplotypes found in the common beech (Demesure et al., 1996), rather than a patchy pattern of cpDNA genetic variation as described for white oaks (*Quercus* spp.) (Dumolin-Lapègue et al., 1997; Petit et al., 1997). Assuming total extinction of *Hedera* during the last ice age in northern Europe and considering the extant chlorotype distribution, chlorotype III has survived in Mediterranean source areas and recolonized northward in recent times.

The geographic distribution of chlorotypes shown in Fig. 5 is not fully determined due to the low number of samples of *H. hibernica* and *H. helix* from northern Europe. However, some conclusions can be drawn from the 31 distant populations of *H. helix* ssp. *helix* analyzed because they encompass most of the geographic distribution of this European ivy in the last 6000 years (Huntley and Birks, 1983). The potential of *Hedera* for long-distance dispersal is manifested by multiple introductions to Macaronesia. However, we have not found clear molecular evidence to distinguish between the two major modes of post-glacial dispersal in northern Europe: neighborhood diffusion and long-distance dispersal (Berg, 1983; Shigesada et al., 1995). Rapid postglacial expansion of *Hedera* populations over the past 10,000 years has been documented in northern Europe (Godwin, 1975) from pollen records, which may

be a consequence of stratified diffusion (Hengeveld, 1999) at a pace similar to that of forests (2000 m/y, Bennett, 1991). Accordingly, we hypothesize that the presence of a single chlorotype in northern Europe may be the result of neighborhood recolonization of local founder populations containing chlorotype III from some Mediterranean refugia. This hypothesis is supported by results from Grivet and Petit (pers. commun.), who detected in northern Europe an assemblage of subchlorotypes related to our chlorotype III, which are found in neighboring refugia. Increased sampling of populations and cytoplasmic markers (already in progress), coupled with ecological studies, are needed to gain a more comprehensive phylogeographic understanding of chlorotype distribution and habitat preferences of ivies.

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