Molecular characterization of Hedera (Araliaceae) from Atlantic Iberian Peninsula

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Running head: *Hedera* in the Atlantic Iberian Peninsula

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Molecular characterization of *Hedera* (Araliaceae) from Atlantic Iberian Peninsula

Abstract

The Atlantic territories of western Europe and their surrounding areas are cohabited by two different species of ivies (*Hedera*), which are morphologically very similar, although they present different ploidy levels: *Hedera helix* (2x) and *Hedera hibernica* (4x). Concerning the northwest Atlantic Iberian territories and their surrounding areas, there are discrepancies regarding the identity of *Hedera* individuals at the specific level, since they have been identified as exclusively *H. hibernica,* but also as belonging to both species. In this context, we have aimed to determine whether the *Hedera* found in Atlantic Iberian Peninsula (Cantabrian Mountains territories and their surrounding areas) belong to *H. helix*, *H. hibernica* or to both. In order to achieve this, high-copy nuclear marker *Internal Transcribed Spacer* (*ITS*) and low-copy nuclear marker *Granule-bound starch synthase I* (*GBSSI*) were analyzed and compared to the results of *Hedera* samples from Central Europe and the Spanish Mediterranean Basin. Combined analyses of *ITS*-*GBSSI* datasets discriminate the species *Hedera helix* and *Hedera hibernica*, and our data suggest that *H. hibernica* is the only representative of *Hedera* in the Atlantic Iberian territories.

Keywords: Granule-bound starch synthase I, Iberian Peninsula, Internal Transcribed Spacer, ivy, nuclear markers.

Introduction

The number of ornamental plant species cultivated throughout the world remains unclear. Recent estimates have disclosed 85,000–99,000 species of ornamental plants worldwide (Orlikowska et al. 2018) that comprise, in some cases, most species of a whole family or genus (i.e. *Arecaceae* Berchtold & Presl., *Cactaceae* Juss., *Philodendron* Schott, *Rosa* L.). Although, the preservation of their genetic resources is essential for breeding and future development of ornamental plants, their excessive use might cause the loss and degradation of native plant habitat, as has happened to common ivies in America (Clarke et al. 2006; Clements et al. 2017). Moreover, ivy's uncontrolled growth within its natural distribution has been associated with shifts in the species composition of European temperate forests (Perring et al. 2020).

Hedera L. (commonly called ivy) is a monophyletic genus distributed throughout Asia, Europe and the North of Africa (Valcárcel 2008; Green et al. 2011), which in the last years has become popular as both an indoor and outdoor ornamental vining plant (Clements et al. 2017). The low variability of reproductive characters exhibited by its members, as well as the high variability of vegetative characters within the same species, has been regarded as the main source of taxonomical difficulties (Rutherford et al. 1993; Valcárcel 2008; Green et al. 2011). As a consequence, current taxonomy is based on a mixture of morphological, cytological and molecular characteristics (Valcárcel et al. 2017).

The Mediterranean Basin and Macaronesia harbour 10 out of the 13 currently recognized species, including all the observed ploidy levels (Ackerfield and Wen 2003). In this western edge of the *Hedera* distribution range, three species are endemic to the Macaronesian Islands (*Hedera maderensis* K.Koch ex A.Rutherf., *Hedera canariensis* Willd. and *Hedera azorica* Carrière), while other 4 can be found in the Iberian Peninsula (*Hedera helix* L., *Hedera hibernica* (G.Kirchn.) Bean, *Hedera iberica* (McAll.) Ackerf. & J.Wen and *Hedera maroccana* McAll.) (Ackerfield and Wen 2003; Green et al. 2011). However, according to *Flora Iberica*,

only 3 species of *Hedera* exist in the Iberian Peninsula: *H. helix* (including the subspecies *H. helix* subsp. *helix* and *H. helix* subsp. *rhizomatifera* McAll., *H. hibernica* and *H. maderensis* (specifically the considered subspecies *H. maderensis* subsp. *iberica*). These taxa differ from each other in their ploidy level and the morphology of their foliar trichomes (McAllister 1981; McAllister and Rutherford 1990; Green et al. 2011).

Uniparental and biparental molecular markers have been used as tools for taxonomical identification at the species level (e.g. Vargas et al. 1999; Grivet and Petit 2002; Clarke et al. 2006). However, incongruencies have been shown regarding the monophyletic character of the clades comprising the diploid and polyploidy species (Valcárcel et al. 2003; Valcárcel 2008). For instance, the strict consensus tree based on the chloroplastic marker *trnT-trnL* displayed a different topology from that obtained in previous studies based on the nuclear marker *Internal Transcribed Spacer* (*ITS*) (Vargas et al. 1999; Ackerfield and Wen 2003). The high-copy ribosomal marker *ITS* have proven to be useful in various studies in discerning *Hedera* species by a phylogenetic analysis as it allows to discriminate diploid and polyploidy species in different clades (e.g. Valcárcel et al. 2003; Valcárcel et al. 2014), while no chloroplastic haplotype exclusive to each species exists, generating topologies in which individuals from the same species are placed in different major clades (Valcárcel et al. 2003; Green et al. 2013).

Two highly morphologically similar species with overlapping leaf morphometric features, *H. helix* (2x) and *H. hibernica* (4x), co-habit the western Atlantic territories of Europe and their surrounding areas (Valcárcel 2008; Valcárcel and Vargas 2010; Clements et al. 2017). Lum and Maze (1989) based the differentiation of these two species on the foliar trichome morphology and the ploidy level nevertheless, some recent studies have remarked the problems of morphologic convergence of the trichomes in the overlapping areas of distribution such as the British Isles, France and Spain (Metcalfe 2005; Valcárcel 2008; Valcárcel et al. 2012). In this context, Valcárcel (2008) and Valcárcel et al. (2012) sustain that subtle differences between

the "intermediate" *H. helix* trichomes and the *H. hibernica* trichomes allow species assignation with high accuracy. The geographical distribution has also been taken into account during the identification, since niche segregation based on humidity has been reported (Valcárcel 2008; Valcárcel et al. 2012). Additionally, the observed low genetic diversity of this genus complicates identification of these two species (Vargas et al. 1999; Ackerfield and Wen 2003; Valcárcel et al. 2003). Although *H. helix* is a diploid species (2x) and *H. hibernica* is a tetraploid (4x), both share cpDNA haplotypes (Lum and Maze 1989; Vargas et al. 1999; Ackerfield and Wen 2003; Valcárcel et al. 2003).

To date, there is no consensus regarding the presence of *H. helix* and *H. hibernica* in the northwest regions of the Iberian Peninsula. For example, some studies (e.g. Valcárcel 2008; Valcárcel et al. 2012) have reported that both species grow in Asturias and surrounding areas, whereas Sahuquillo et al. (2001) has identified populations from Galicia and Asturias as *H. hibernica* based on ploidy and trichome morphology. These inconsistencies concerning the distribution of these two *Hedera* species could be explained by three main hypotheses: (1) misidentification of *H. helix* as compared to *H. hibernica*, (2) the presence of an overlapping area of their distribution ranges in the north of Spain and (3) inclusion of cultivated individuals as natural populations. In this context of morphologically similar species with low genetic diversity, we aim to determine whether *Hedera* individuals naturally found in the northwest Iberian Atlantic coast and their surrounding areas belong to *H. helix*, *H. hibernica* or to both of them by conducting a molecular analysis based on high-copy and low-copy nuclear markers.

Material and methods

Plant material

Twenty-two locations included within the distribution ranges of *H. helix* and *H. hibernica* in Spain and Europe are sampled in this study (Figure 1; Table 1). The northern Spanish samples

include individuals from the coastal locations of Asturias and the Cantabrian Mountains, for in this way each of these two species' distinct ecological preferences, that is, humid climates in the case of *H. helix* and hyperhumid climate for *H. hibernica*, will be represented (Lum and Maze 1989; Ackerfield and Wen 2002). Several leaves and vegetative branches from each individual were collected and kept in silica gel to be preserved for the molecular analysis. In addition, a visual analysis of the morphology of the foliar trichomes was conducted on young leaves of vegetative branches using a Stereomicroscope (Optika ST-30-2LR) and following the criteria of McAllister and Rutherford (1990), Valcárcel (2008) and Valcárcel and Vargas (2010). Trichome morphology has been widely used as taxon delimiting character in *Hedera*. Samples of *Hedera helix* present stellate-multiangulate trichome, while the trichomes of *H. hibernica* are stellate-rotate.

DNA extraction and amplification

Nuclear DNA of the 20 samples was extracted from leaf tissue using NucleoSpin® Plant II Columns (Macherey-Nagel) following the manufacturer's instructions. Extracted DNA was stored at -20º C. Two different nuclear genes were examined for each sample: the low-copy *Granule-Bound Starch Synthase I* (*GBSSI*) and the high-copy *Internal Transcribed Spacer* (*ITS*). Exon 10, two introns (intro 9 and intron 10) and partial sequences of exons 9 and 11 of *GBSSI* were amplified by means of primers GBSSI-1F and GBSSI-11R (Mitchell and Wen 2004). External primers, 17SE and 26SE (Sun et al. 1994), were used to amplify the ribosomal nuclear regions *ITS1*, *5.8S* and *ITS2*. PCR products were sequenced at the DNA Synthesis and Sequencing Facility Macrogen (Amsterdam, The Netherlands).

Phylogenetic analyses

Sequence data were manually edited in Geneious Prime v.2019 1.3 (Kearse et al. 2012). Bases

and ambiguities were coded following the International Union of Pure and Applied Chemistry (IUPAC). Double-peaks at certain sites were considered to be ambiguities when it was found in both reverse and forward amplicon sequences and in both amplicons the lower peak reached at least a third of height of the higher one. *ITS* and *GBSSI* sequences of *Hedera* from previous studies available at GenBank were included in our phylogenetic analysis (see Table S1). A species of Araliaceae family (*Fatsia japonica* (Thunb.) Decne. & Planch.), and closely related to *Hedera*, was used as outgroup (e.g. Green et al. 2011; Valcárcel et al. 2014; Valcárcel et al. 2017). A multiple sequence alignment was performed using MUSCLE (Edgar 2004) and the sequences were then trimmed using Geneious Prime. The evolutionary models were estimated by the Akaike Information Criterion (AIC) with the implementation of MrModeltest (Posada and Buckley 2004). The best fitting model for the *ITS* and *GBSSI* sequences were the General Time Reversible with gamma distribution (GTR+G) model (Tavaré 1986; Yang 1994) and the Hasegawa-Kishino-Yano (HKY) (Hasegawa 1985) respectively, while the GTR+G was estimated to be the best fitting for the combined sequences.

The phylogenetic analyses were conducted by two character-based methods: Maximum likelihood (ML) and Bayesian Inference (BI) methods. In both cases, the data of the two different markers were treated first separately and then combined in a single analysis with a bipartition. The ML tree was inferred in the IQ-TREE software online service (Nguyen et al. 2015; Trifinopoulos et al. 2016). The starting tree was a Neighbor Joining (NJ) tree and Nearest-Neighbor Interchange (NNI) was used as full-tree rearrangement operation. Node support was estimated by 10,000 bootstrap (BS) replications and partitioned analysis (Minh et al. 2013; Chernomor et al. 2016; Hoang et al. 2018). Finally, BI phylogenetic analysis was performed in MrBayes v3.2.7a (Ronquist et al. 2012). This analysis consisted in two simultaneous analyses, each of 6 Monte Carlo Markov Chains (MCMC) with 5 heated and 1 cold chain, for 3,000,000 generations, sampling trees every 100 generations. The burn-in fraction was determined using

Tracer v1.7.1 (Rambaut et al. 2018). Posterior probability (PP) of the branches of the obtained consensus tree was the statistical method of inference used. Finally, the relationships among sequences of the samples, *Hedera hibernica* and *H. helix* were inferred using the NeighborNet algorithm implemented in SplitsTree v.4.16.1 (CBOL Plant Working Group et al. 2006), applying uncorrected distances. Bootstrap support for internal splits was calculated with 1,000 replicates. Fit values ranging from 0 to 100% indicate how well the information contained in the data was graphically represented.

Results

The characteristics of *ITS* and *GBSSI* based on *Hedera hibernica* and *H. helix* are summarized in Table 2. The length of the 56 combined *ITS-GBSSI* sequences alignment was 1124 base pairs (bp) and presented 131 variable sites of which only 48 were parsimony informative sites. Consensus tree derived from combined analysis of *ITS* and *GBSSI* estimated using ML and BI analyses of concatenate regions are well-resolved (Figure 2). The topology of the *ITS-GBSSI* analyses shows two distinct and well-defined clades. First, the diploid clade (100 % posterior probability on the BI analysis (PP-BI); 89 % bootstrap in the ML analysis (BS-ML)) including all diploid species (except for *H. algeriensis*-4x) where *H. azorica*, *H. helix* and *H. maroccana* form a well-defined group (100 % PP-BI; 92 % BS-ML). Secondly, the polyploidy clade (99 % PP-BI; 89 % BS-ML) comprised by the monophyletic Asian species, which consisted of sequences of *H. colchica*, *H. cypria* and *H. pastuchovii* (100 % PP-BI; 93 % BS-ML), along with a sister clade exclusive to *H. maderiensis* (99 % PP-BI; 98 % BS-ML) and another monophyletic clade for *H. hibernica* (95 % PP-BI; 83 % BS-ML). Similar results were obtained when analyzing the datasets separately (data not shown). In the case of the *ITS* phylogeny, this marker mostly contributed to the location of the sample within the diploid clade or polyploid clade, whereas *GBSSI* seems to enable the discrimination of *H. hibernica* and *H. maderensis*

within the polyploid clade.

The phylogenetic network analysis of *ITS* and *GBSSI* can be deduced from the splits NeighborNet graph in Figure S1. In the case of *ITS*, the two species *Hedera hibernica* and *H. helix* can be clearly distinguished. However, the same is not true of the *GBSSI* marker where only the HED20 sample shows some differentiation but that is due to the presence of ambiguities in 5 nucleotides of its sequence.

Discussion

The species *Hedera helix* and *H. hibernica* have been traditionally identified based on trichome morphology (e.g. Sahuquillo et al. 2001; Valcárcel 2008). However, this method is not always easy to apply, especially when the species display similar phenotypes (Valcárcel 2008; Valcárcel et al. 2012), being the *Hedera* tetraploids of Sicily an extreme case (Mcallister and Rutherford 1990; Fridlender and Pech 2019). In this context, some recent studies have remarked on the problem of morphologic convergence of the trichomes of these two species when their areas of distribution overlap in regions such as the British Isles, France and Spain (Metcalfe 2005; Valcárcel 2008; Valcárcel et al. 2012).

Members of the polyploidy clade are considered to be autopolyploid and allopolyploid by various sources (Vargas et al. 1999, Valcárcel et al. 2003, et al. Green 2013). The origin of polyploidy *H. hibernica* has been controversial, since Vargas et al. (1999) and Valcárcel et al. (2003) considered an allopolyploidization generated by the hybridization of the ancestors of the diploids *H. helix* and a polyploidy, possibly *H. maroccana*; whereas Green et al. (2013) suggested that an autopolyploid origin, with *H. helix* being the ancestral species. The morphological data, ploidy levels and the incongruence between the nrDNA and cpDNA have been associated with homoplasia, reticulated evolution, rapid diversification and lineage sorting (Ackfield and Wen 2003; Valcárcel et al. 2003). According to Green et al. (2011), hybridization

between *H. helix* and *H. hibernica* is infrequent, although some triploid hybrid cultivars have been described (e.g. *H. helix*×*H. hibernica* 'Woerneri', *H. helix*×*H. hibernica* 'Negro') and detected in the USA (Green et al. 2013), Portugal and Hungary (Marshall et al. 2017).

The northwest Iberian Atlantic coast territories have raised controversy regarding the presence of these species, since the morphological analysis of trichomes led Valcárcel (2008) and Valcárcel et al. (2012) to report the presence of both species, whereas Sahuquillo et al. (2001) and Green et al. (2011) reported exclusively the presence of *H. hibernica*. In those Spanish "overlapping areas", the trichomes of *H. helix* have been reported to shift from the typical morphology to a rotated, sessile, centrally fused one, which may lead to a confusion with the typical *H. hibernica* trichomes, due to the effect of the environmental conditions and the morphological variability of *H. helix* (Mcallister and Rutherford 1990; Ackerfield and Wen 2002; Valcárcel 2008; Valcárcel et al. 2012). Given this ambiguity, in the present work we proceeded to use molecular markers aiming to delimit both taxa.

The nuclear ribosomal DNA (nrDNA) *ITS* has been widely used in plant molecular systematics and has been selected as the formal barcode marker (CBOL Plant Working Group et al. 2006). Phylogenetic analysis based on *ITS* have been tested successfully in various studies related with *Hedera*, differentiating species according to their ploidy level (e.g. Valcárcel et al. 2003; Valcárcel et al. 2014). Our molecular analysis based on this marker generated the same topology as in previous studies, differentiating diploid and polyploid clades. On the other hand, low-copy *GBSSI* has been successfully used in previous studies to clarify the phylogenetic relationship in groups such as *Rosaceae* (Evans et al. 2000), *Bromus* L. (Fortune et al. 2008) and even *Araliaceae* (Mitchell and Wen 2004). Green et al. (2011) used this marker in *Hedera* in order to clarify the complex phylogenetic relationship within this group and obtained a polytomy. The topology based on the *GBSSI* phylogenetic analysis (see Figure S1) was unresolved due to the few parsimony information found in our data set.

Therefore, molecular analysis based only on *GBSSI* cannot discriminate *Hedera* at species level.

Our combined dataset presented fewer sequences than independently analysed markers. Nonetheless, the number of parsimonious sites was enough to allow the discrimination of groups within the classic diploid and polyploid clades. Therefore, combined *ITS-GBSSI* phylogenetic analysis generated an exclusive *H. hibernica* clade, which contained all our northwest coastal Iberian samples. This result gives further support to the hypothesis of the occurrence of *H. hibernica* in the northwest coastal Iberian regions as reported by Sahuquillo et al. (2001). Nevertheless, these data contradict the proposed co-ocurrence of *H. hibernica* and *H. helix* suggested by Valcárcel (2008) and Valcárcel et al. (2012) and Green et al. (2013). Finally, our study suggests that there is no co-occurrence of these two species in those territories, and that the northwest Iberian territories and their neighbouring areas should be excluded from the distribution range of *H. helix*.

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Table 1. Code, populations and GenBank accessions for DNA sequences of *Hedera's* samples analysed in the present study.

Code	Population	Coordinates	GenBank accession	
			ITS	*GBSSI*
HED1	Tuiza de Abajo (Asturias, Spain)	43º 01' 05.95" N 5º 54' 35.03" W	MT276669	MT478175
HED2	La Cruz (Asturias, Spain)	43º 01' 22.21" N 5º 51' 37.64" W	MT276670	MT478176
HED3	Gillón (Asturias, Spain)	43º 01' 26.70" N 6º 32' 56.54" W	MT276671	MT478177
HED4	Larna (Asturias, Spain)	43º 04' 04.87" N 6º 36' 55.37" W	MT276672	MT478178
HED5	Caso (Asturias, Spain)	43º 07' 08.24" N 5º 18' 48.31" W	MT276673	MT478179
HED6	Gijón (Asturias, Spain)	43º 34' 02.91" N 5º 42' 21.61" W	MT276674	MT478180
HED7	Niembru (Asturias, Spain)	43º 26' 07.49" N 4º 50' 37.75" W	MT276675	MT478181
HED8	Niembru (Asturias, Spain)	43º 26' 16.23" N 4º 50' 55.61" W	MT276676	MT478182
HED9	Cazamular (Asturias, Spain)	43º 31' 50.57" N 5º 34' 28.70" W	MT276677	MT478183
HED10	Nogueira (Asturias, Spain)	43º 20' 59.62" N 7º 05' 41.26" W	MT276678	MT478184
HED11	Nogueira (Asturias, Spain)	43º 20' 59.62" N 7º 05' 29.24" W	MT276679	MT478185
HED12	Oviedo (cult., Asturias, Spain)	43º 21' 22.57" N 5º 52' 20.80" W	MT276680	MT478186
HED13	Parc Natural de Font Roja (Alicante, Spain)	38º 39' 41.20" N 0º 32' 58.20" W	MT276681	MT478187
HED14	Parc Natural de Font Roja (Alicante, Spain)	38º 39' 41.24" N 0º 32' 58.22" W	MT276682	MT478188
HED15	Cueva (Burgos, Spain)	43º 02' 13.49" N 3º 40' 03.10" W	MT276683	MT478189
HED16	Rinas de Hórtola (Valencia, Spain)	39º 22' 0.26" N 1º 08' 00.20" W	MT276685	MT478190
HED17	Valle d'Aosta (Italy)	45° 42' 16.85" N 7° 8' 52.50" E	MT276686	MT478192
HED18	Valle d'Aosta (Italy)	45° 42' 16.75" N 7° 8' 52.59" E	MT276687	MT478193
HED19	Jardin botaniques de la ville de Genève (Geneva, Switzerland)	46° 13' 39.42" N 6° 8' 48.55" E	MT276688	MT478194
HED20	HolyRood (Edinburgh, Scotland)	55° 56' 49.25" N 3° 10' 24.87" O	MT276689	MT478195

Table 2. Summary of the characteristics of the *Hedera* sequences of *ITS*, *GBSSI* and the combination of both (*ITS-GBSSI*) analyzed in this study. Differences for *Hedera hibernica* and *Hedera helix* sequences are highlighted.

		ITS	*GBSSI*	Combined (*ITS-GBSSI*)
Hedera				
	Number of taxa	11	11	11
	Number of sequences	75	106	46
	Range of length of sequences (pb)	550-616	499-504	1054-1114
	Alignment length (pb)	628	504	1124
	(C+G) %	58.5	40.8	50.5
	Conserved sites	432	459	1038
	Parsimomious-informartive sites	149	23	47
H. helix vs ***H. hibernica***				
	Number of sequences	34	44	28
	Conserved sites	576	475	1064
	Variable sites	33	29	49
	Parsimomious-informartive sites	24	16	33

Note: outgroup (*Fatsia japonica*) was not included. Sites are considered to be variable when there are at least two types different bases. When variable sites present two of their nucleotide types with at least a frequency of two, they are considered Parsimony-informative sites

Figure 1. A) Distribution area of *Hedera helix* and *Hedera hibernica* in the Europe. B) Sampling locations (blue stars) in the northwest Iberian Atlantic coast territories. Map data: Europe ; north of the Iberian Peninsula: (Flora Iberica et al. 2020), Software: QGIS 3.8 Zanzíbar (Open Source Geospatial Foundation Project, 2020). Population codes (1-20) are given in Table 1.

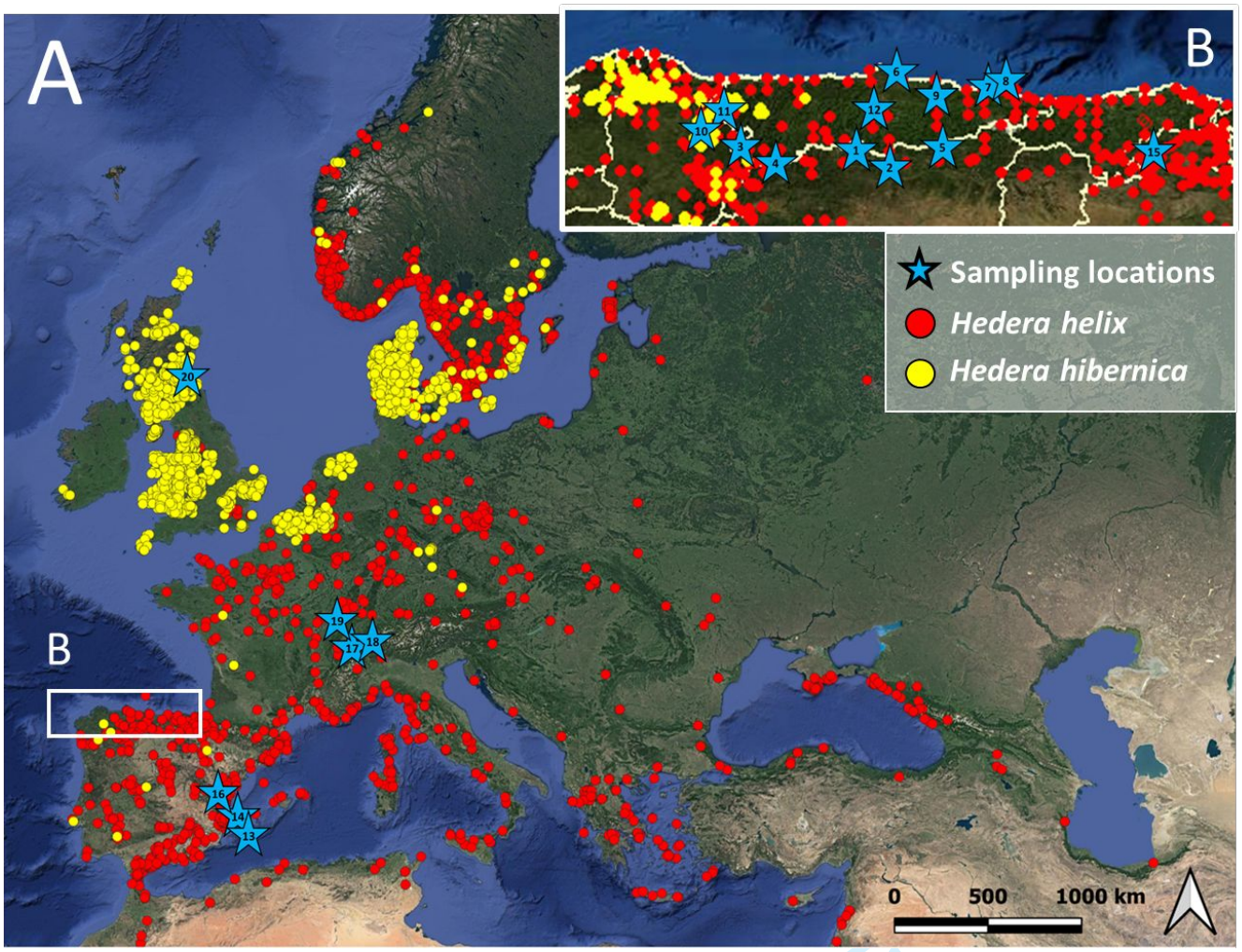
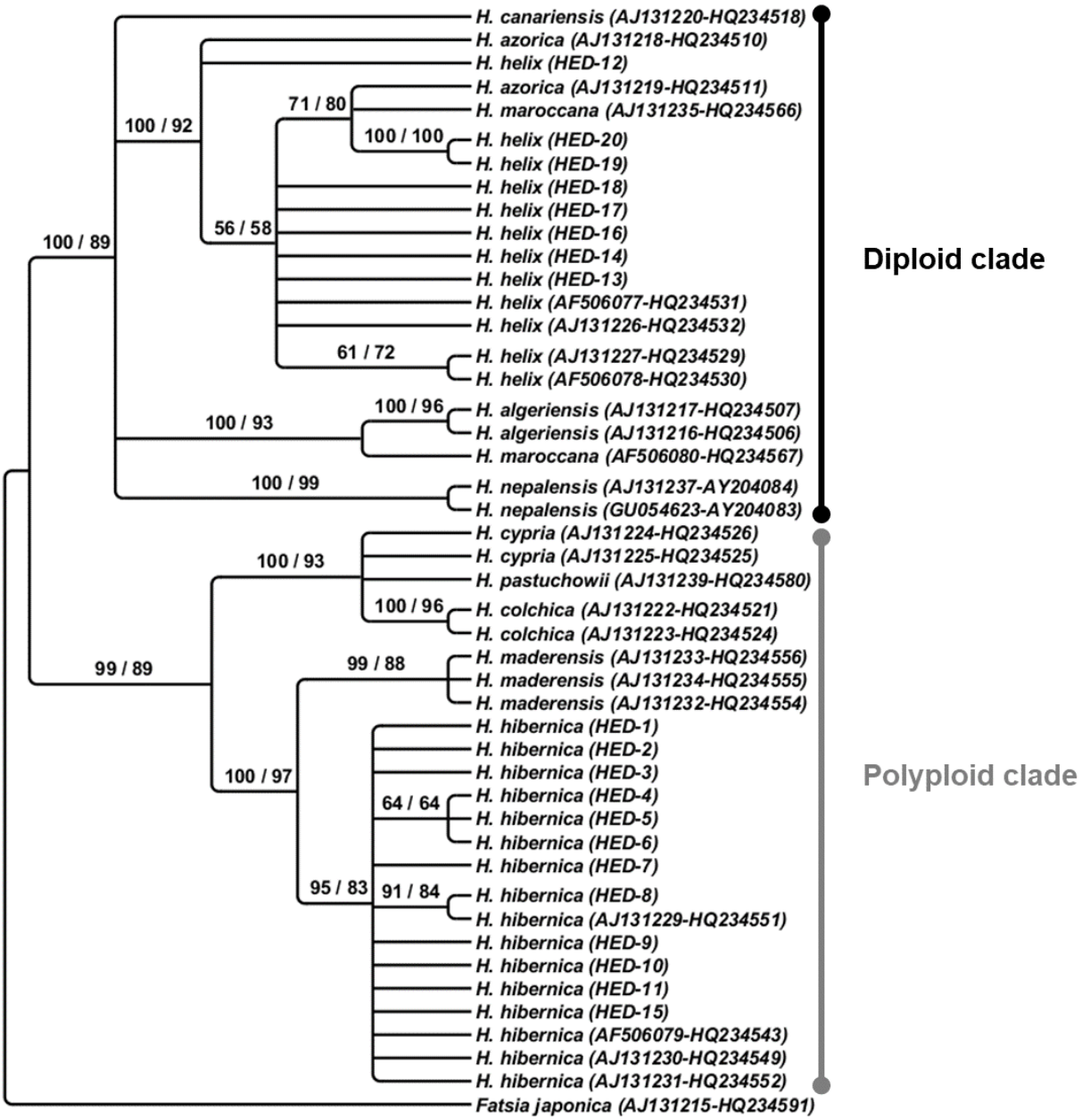


Figure 2. Consensus phylogenetic tree of BI analysis based on concatenated ITS-GBSSI sequences of *Hedera* (HED) in the Atlantic Iberian Peninsula. The numbers over the branches correspond to BI posterior probability values and ML bootstrap values, respectively.



Molecular characterization of *Hedera* (Araliaceae) from Atlantic Iberian Peninsula

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Figure S1. NeighbourNet network for *ITS* and *GBSSI* sequences of *Hedera* (HED) in the Atlantic Iberian Peninsula. The least squares fit index for the split network has a value (%) of 72.86 (*ITS*) and 98.11 (*GBSSI*). Numbers along branches are bootstrap values from 1,000 replicates. Population codes are given in Table 1.

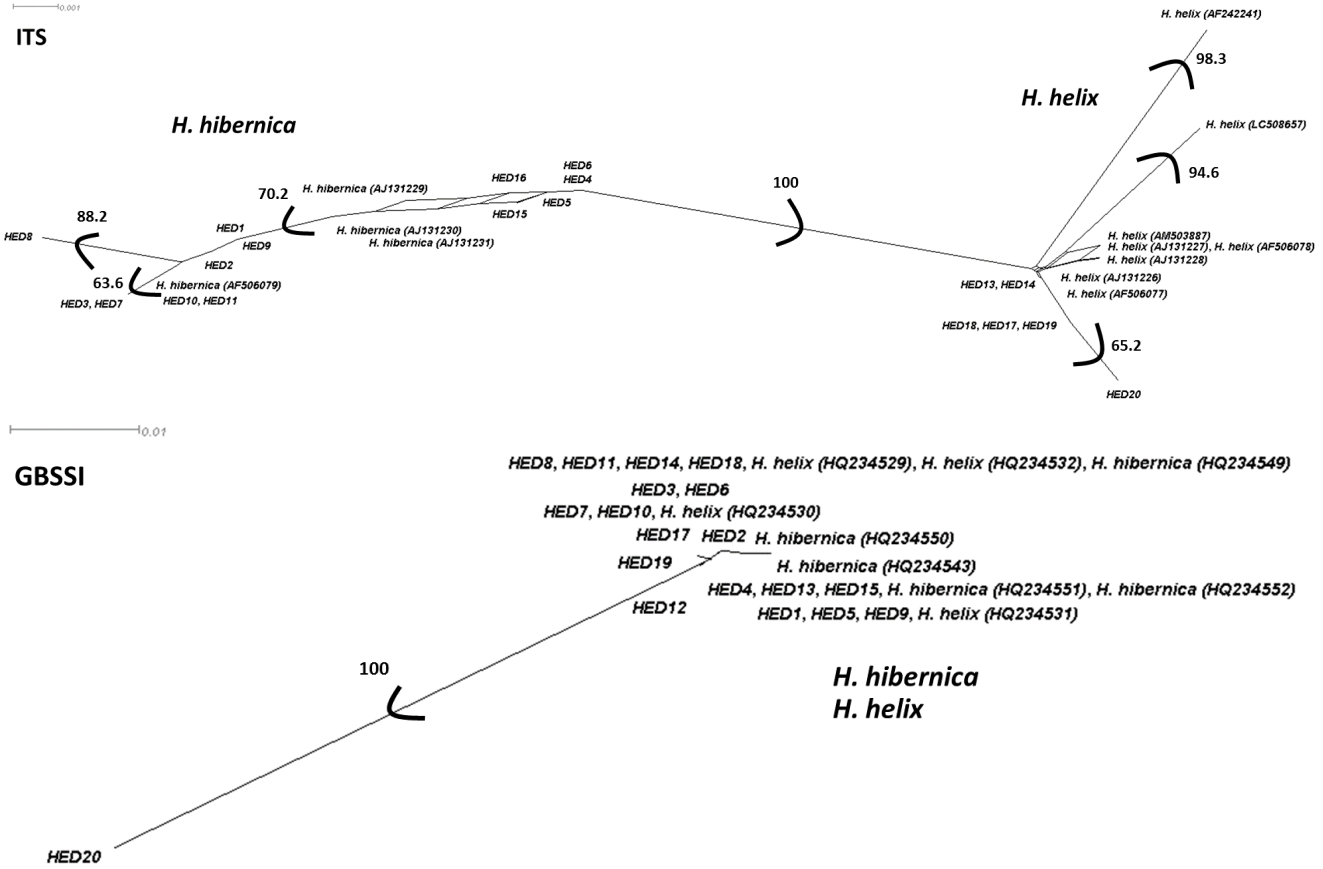

Table S1.

List of *Hedera* and *Fatsia* taxa from Vargas et al. (1999), Valcárcel et al. (2003), Green et al. (2011) and Mitchell and Wen (2004) used in the phylogenetic analysis. The associated data has been gathered from these previous studies, corresponding the order of the displayed data to the following information: taxon name, collection site, collector, voucher and GenBank accession numbers.

ITS: ***Fatsia japonica*** (Thunb.) Decne. and Planch., East Asia by P. Vargas (MA), AJ131215. ***Hedera algeriensis*** Hibberd; Kabylie (Algeria), by H. A. McAllister (838H.A.M), AJ131216. ***Hedera algeriensis*** Hibberd; Cultivar "Gloire de Marengo, by P. Vargas, AJ131217. ***Hedera azorica*** Carrière; Pico, Azores Islands (Portugal), by F. Brightman, AJ131218. ***Hedera azorica*** Carrière; São Miguel, Azores Islands (Portugal), by Hilliers, AJ131219. ***Hedera canariensis*** Willd., Tenerife, Canary Islands (Spain) by H. A. McAllister (237H.A.M.), AJ131220. ***Hedera colchica (***K. Koch) K. Koch, Zagodeki (Georgia) by R. Lancaster (L296 (LIV)), AJ131222. ***Hedera colchica (***K. Koch) K. Koch, T'elavi (Georgia) by R. Lancaster (269 (1979)), AJ131223. ***Hedera cypria*** McAll., Limasol (Cyprus) by J. Edmonson, AJ131224. ***Hedera cypria*** McAll., Kakopetria (Cyprus) by Mrs. Della via R. Meilke, AJ131225. ***Hedera helix*** L., Cultivated in Fort Collins, Colorado (U.S.A.) by J. Wen 2481 (CS), AF242241. ***Hedera helix*** L., Huesca (Spain), P. Vargas, AF506077. ***Hedera helix*** L., S. Uist, Scotland, (UK) by H.A. McAllister (570H.A.M. (MA)), AF506078. ***Hedera helix*** L., AM503887. ***Hedera helix*** L., Mugla (Turkey) by J.A. Compton, AJ131226. ***Hedera helix*** L., Málaga (Spain) by P. Vargas (5PV97 (MA)), AJ131227. ***Hedera helix*** L., Granada (Spain) by S.L. Jury, AJ131228**.** ***Hedera helix*** L., Tang-e Bostan, Kamfiruz, Fars (Iran) by H. Moradkhani 803299 GKUH, LC508657**.** ***Hedera hibernica*** (G. Kirchn.) Bean (1914) 609, Kirchn (1864) 419, AF506079. ***Hedera hibernica*** (Kirchn.) Bean, Huelva (Spain) by H.A. McAllister (545H.A.M. (MA)), AF506079. ***Hedera hibernica*** (Kirchn.) Bean, Asturias (Spain) by H.A. McAllister (937H.A.M. (MA)), AJ131229. ***Hedera hibernica*** (Kirchn.) Bean, Lindoso (Portugal) by H.A. McAllister (925H.A.M. (MA)), AJ131230. ***Hedera hibernica*** (Kirchn.) Bean, Málaga (Spain) by H.A. McAllister (949H.A.M. (MA)), AJ131231.***Hedera iberica*** (McAll.) Ackerf. & J Wen, Cádiz (Spain) by H.A. McAllister (15H.A.M. (MA)), AJ131232. ***Hedera maderensis*** K.Koch ex Rutherf., Funchal, Madeira (Portugal) by H.A. McAllister (18H.A.M. (MA)), AJ131233. ***Hedera maderensis*** K.Koch ex Rutherf., Das Queimadas Park, Madeira (Portugal) by L.O. Franquinho, AJ131234. ***Hedera maroccana*** McAll., Chefchaouen (Morocco) by P. Vargas (152PV00 (MA)), AF506080. ***Hedera maroccana*** McAll., Tetuán (Morocco) by H.A. McAllister (868H.A.M. (LIV)), AJ131235. ***Hedera nepalensis*** K. Koch**,** (Tobler) Handel-Mazzetti (1933) 693, Kashmir (India) by H.A. McAllister (246H.A.M. (MA)), AJ131237. ***Hedera nepalensis*** K. Koch, Hunan (China) by Xinning, J. Wen 9278 (US), GU054623. ***Hedera pastuchovii*** G. Woronow (1932) 108, s (Iran) by H.A. McAllister (259H.A.M. (MA)), AJ131239.

GBSSI: ***Fatsia japonica*** (Thunb.) Decne. and Planch.; cultivated in Washington (U.S.A) by University of Washington campus arboretum, *T. Ramsey 374849* (WTU), HQ234591. ***Hedera algeriensis*** Hibberd, Kabyle Mountains (Algeria), by J. Whitehead, AIS 88-188; *T. Ramsey 374854* (WTU), HQ234506-HQ234507. ***Hedera azorica*** Carrière, São Miguel, Azores (Portugal), anonymous, AIS 82-259 ('São Miguel'); *T. Ramsey 374857* (WTU), HQ234511. ***Hedera canariensis*** Willd., La Mercedes, Tenerife, Canary Islands (Spain) by Glasgow Naturalist Expedition, AIS 94-052; *T. Ramsey 374858* (WTU), HQ234518. ***Hedera colchica*** K. Koch 'My Heart', cultivated in Longwood Gardens, Pennsylvania (U.S.A) by Longwood Gardens, AIS 94-058 ('My Heart'); *T. Ramsey 374859* (WTU), HQ234521 and HQ234524. ***Hedera cypria*** McAll., Troodos Mountains (Cyprus), anonymous, AIS 03-079; *T. Ramsey 374860* (WTU), HQ234525 and HQ234526. ***Hedera helix*** L. subsp. ***helix*** 'Emerald Gem'; cultivated in New Jersey (U. S. A.) by the American Ivy Society; AIS 87-139 ('Emerald Gem'); T. Ramsey 374861 (WTU), HQ234529 and HQ234530. ***Hedera helix*** L. (1753) 202 subsp. ***helix*** 'Baltica', cultivated in the American Ivy Society, New Jersey (U. S. A) by the American Ivy Society, AIS 83-063; *T. Ramsey 374862* (WTU), HQ234531 and HQ234532 ***Hedera hibernica*** (G. Kirchn.) Bean, Clydesbank, Scotland (UK) by O. Kernaghan, AIS 06-023; *T. Ramsey 374869* (WTU), HQ234549, HQ234550, HQ234551 and HQ234552. ***Hedera hibernica*** (G. Kirchn.) Bean, naturalized population in King Co., Washington (U.S.A) by A. Green, RL L94; *T. Ramsey 374868* (WTU), HQ234543. ***Hedera maderensis*** K. Koch ex Rutherf., Funchal, Madeira (Portugal) by D. McClintock, AIS 91-097; *T. Ramsey 374872* (WTU), HQ234554-HQ234556. ***Hedera maroccana***

McAll., Middle Atlas Mountains (Morocco) by International Dendrological, AIS 88-008; *T. Ramsey 374874* (WTU), HQ234562-HQ234567. ***Hedera maroccana*** McAll., naturalized population in Andalucía (Spain) by H. McAllister, *T. Ramsey 374873* (WTU), HQ234557-HQ234561. ***Hedera nepalensis*** K. Koch, Yunnan, Lao Cai (Vietnam) by Li, Li 13862 (F), AY204083. ***Hedera nepalensis*** K. Koch, Nepal by Wen, Wen 4933 (CS), AY204084. ***Hedera nepalensis*** K. Koch, [**=*Hedera nepalensis*** K. koch **var. *sinensis*** Rehder], cultivated in the American Ivy Society, New Jersey (U. S. A) by the American Ivy Society, AIS 88-259; *T. Ramsey 374875* (WTU), HQ234578-HQ234583. ***Hedera pastuchovii*** G. Woronow, cultivated in New Jersey (U. S. A.) by the American Ivy Society, AIS 88-264; T. Ramsey 374878 (WTU), HQ234580.