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Quantitative morphology and species delimitation under the general lineage concept: Optimization for *Hedera* (Araliaceae)[1]

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- *Premise of the study*: The use of continuous morphological characters in taxonomy is traditionally contingent on the existence of discrete diagnostic characters. When plant species are the result of recent divergence and gene flow and/or hybridization occur, the use of continuous morphological characters may help in species identification and delimitation. Between nine and 15 species have been recognized in the last treatments of *Hedera*. The recent divergence of the species and the involvement of allopolyploidization as the main force in this process may have greatly impeded the establishment of clear limits and contributed to multiple taxonomic proposals.
- *Methods*: A multivariate statistical decision-making procedure was applied to 56 quantitative morphological characters and 602 specimens to identify and delimit *Hedera* species under the general lineage concept. Species' exclusive genetic ancestry was evaluated with the genealogical sorting index from the Bayesian inference trees of 30 *Hedera* ITS sequences.
- *Key results*: The decision-making procedure allowed recognizing 12 species and two groups (stellate and scale-like trichome groups) in *Hedera* and provided statistical support for making decisions about long-standing taxonomic controversies. Common ancestry was detected for the populations of three species even in the absence of the species monophyly.
- *Conclusions*: Quantitative variation supports discrete variation and provides statistical support for the taxa recognized in some recent proposals of *Hedera*. The need of explicit analysis of quantitative data are claimed to reduce taxonomic subjectivity and ease decision-making when qualitative data fail.

Key words: decision-making procedure; genealogical sorting index; general lineage concept; *Hedera*; multivariate statistics; quantitative morphology; species delimitation.

Species definition, characterization, and delimitation are difficult tasks that often lead to multiple final solutions and difficult decisions. Regardless of all the epistemological questions surrounding the "species debate", finding differences and establishing limits addresses biological problems derived from the steady process of evolution and the different mechanisms involved in speciation. In addition to the epistemological debate and the biological complexity of species characterization, improvements to the proper methodology for delimiting species have been systematically neglected. Some efforts have been made during the last decades to describe empirical tests of species boundaries and practical species delimitations that could contribute to more natural taxonomic treatments and more scientific procedures (Davis and Nixon, 1992; Doyle, 1995; Sites and Crandall, 1997; Brower, 1999; Stevens, 2000; Wiens and Servedio, 2000; Templeton, 2001; Wiens and Penkrot, 2002; Sites and Marshall, 2003, 2004; Tautz et al., 2003; Henderson, 2005a). In addition, most of these methods were developed for molecular data, but despite the recent explosion of molecular systematics, morphology remains the fundamental basis of most taxonomic studies (McDade, 1995; Stevens, 2000). Indeed, even recent initiatives such as DNA-barcoding need morphological taxonomic approaches for species delimitation (de Salle et al., 2005; but see the emerging field of phylogenetic nomenclature, e.g., de Queiroz, 2007b).

In practice, the empirical delimitation of species is based on the presence of at least one fixed or non-overlapping morphological character (Wiens and Servedio, 2000; Wiens, 2007). However questionable the theoretical justification for this criterion would be (Nixon and Wheeler, 1990), recent studies in taxonomy have revealed that as more specimens are examined, the variation among them turns out to be continuous (Bernardos, et al., 2005; Henderson, 2005b; Sun et al., 2006), and differences among species may be weak (Wiens and Servedio, 2000). In fact, a discrete distribution of biological variation is generally the result of restructuring an underlying quantitative continuum (Thiele, 1993). Therefore, differentiation between discrete and continuous characters is very often ineffective, and the treatment of discrete characters is sometimes problematic (Stevens, 1991; Wiens and Servedio, 2000). Besides, consideration only of characters, as defined by Davis and Nixon (1992), leads to the underestimation of species sampling errors (Wiens and Servedio, 2000) and may overlook parallel fixation.

Assuming that any morphological change has a genetic basis, we can arrive at the conclusion that a complete lack of gene flow is required for two species to be distinguished by one or more

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discrete fixed characters (characters as in Davis and Nixon, 1992) (Wiens, 2007). However, this statement can only be assumed if the anagenesis process has occurred in the evolutionary lineages of both the ancestral and the descendant species (Nixon and Wheeler, 1990). Soon after the evolutionary divergence of two independent lineages, a biased continuum of the morphological variation is expected before unequivocal morphological divergence is reached. Therefore, for groups that have diverged in recent times, the presence of characters, as defined by Davis and Nixon (1992), or even the detection of morphological discontinuities between independent evolving lineages seems unlikely. This situation can also be extended to nonrecently diverged groups with loss of hierarchy as a result of interlineage persistence of gene flow and/or active hybridization. In such scenarios, the use of qualitative characters or discrete quantitative ones for species identification and delimitation may be spurious.

The general lineage concept (GLC) of de Queiroz (1998, 2007a) defines a species as segments of separately evolving metapopulation lineages where contingent properties (e.g., intrinsic reproductive isolation, reciprocal monophyly, morphological divergence) are reached independently at different times along the evolutionary branch. In the cases of enough time for species divergence and lack of gene flow, the diagnostic capability of morphological variation, as described by Davis and Nixon (1992) in their implementation of the phylogenetic species concept of Nixon and Wheeler (1990), can be used as the most appropriate contingent property. However, this diagnostic capability may fail to detect independent evolutionary lineages in more complex scenarios, such as the ones described above, as neither trait nor character (Davis and Nixon, 1992) is expected to have occurred.

Hedera L. (Araliaceae) is a genus of evergreen climbers native to Europe, north Africa, and south Asia with a complex evolutionary history (Vargas et al., 1999; Grivet and Petit, 2002; Ackerfield and Wen, 2003; Valcárcel et al., 2003a). Despite the old origin of the genus inferred from fossils (Rim, 1994), the low level of molecular divergence detected in the extant species of ivies indicates that they are likely to be the result of recent speciation processes (Ackerfield and Wen, 2003). The existence of ancient and recent allopolyploidization processes (Vargas et al., 1999; Valcárcel et al., 2003a), together with the great long-distance dispersal and colonization capabilities of ivies, may have promoted rapid reproductive isolation that, in combination with short-term speciation processes, resulted in a complex distribution pattern of diversity (Valcárcel, 2008). As a result, the genus is well established both morphologically (Seeman, 1868; Eyde and Tseng, 1971) and molecularly (Valcárcel et al., 2003a), but the delimitation of species and recognition of taxa are still being discussed. Between nine and 15 species have been recognized in the last treatments (Fig. 1; Pojarkova, 1951; McAllister, 1982, 1988; McAllister and Rutherford, 1983, 1990; Rutherford, 1984, 1989; Rutherford et al., 1993; Rose, 1996; Ackerfield, 2001; Ackerfield and Wen, 2002). In addition to the particular evolutionary and biological complexity of *Hedera*, three methodological causes of the current taxonomic discrepancy can be identified: geographically restricted approaches (but see Hibberd, 1872, Ackerfield and Wen, 2002), insufficient sample size, and subjective methodology. These methodological aspects, common in plant monographs, deserve special attention to offer more robust, natural taxonomic proposals.

The main objective of this study was to provide a rigorous multivariate statistical procedure to analyze quantitative morphological variation to circumscribe *Hedera* specimens into diagnosable species. For this purpose, we performed a worldwide comprehensive morphometric study of the genus by screening quantitative macro- and micromorphological characters. For delimiting and testing the species hypotheses, quantitative data sets were analyzed with a rigorous statistical decision-making procedure inspired by the population aggregation analysis method (PAA; Davis and Nixon, 1992) and a method for herbarium taxonomy (PSC-PAA; Henderson, 2005a). The general lineage concept (de Queiroz, 1998, 2007a) was assumed using morphological diagnostic capability as the main operational criterion for identifying, delimiting and evaluating species. Strong geographical discontinuities and different inferred ploidy levels were also used as additional subcriteria for species recognition because they limit gene flow via geographical or reproductive isolation and may indicate ongoing speciation (Gavrilets, 2003). The power of morphological quantitative variation to detect independently evolving lineages was molecularly evaluated by using the genealogical sorting index (Cummings et al., 2008).

The specific goals of this study were as follows: (1) to evaluate the statistical robustness of the taxa recognized by the last treatments, particularly that of McAllister and Rutherford (McAllister, 1982, 1988; Rutherford, 1984, 1989; McAllister and Rutherford, 1983, 1990; Rutherford et al., 1993; hereafter called McAllister and Rutherford's proposal), (2) to establish an explicit criterion for identifying and delimiting species within *Hedera*, (3) to provide quantitative morphological characters for the diagnosis of taxa, (4) to evaluate the ability of quantitative morphology to detect common ancestry based on molecular phylogenetics, and therefore, (5) to evaluate its usefulness as an indicator of speciation in *Hedera*.

MATERIALS AND METHODS

Case study: Hedera L. (Araliaceae)—Like many other climbing plants, ivies present heteroblasty due to two different growth phases, the juvenile and the adult phases. Heteroblasty in *Hedera* is especially apparent in leaf morphology; the leaves are generally lobate in the juvenile phase but entire in the adult phase. Between nine (Rose, 1996) and 15 species (Pojarkova, 1951) are generally recognized within *Hedera*, and these are clustered in two groups according to trichome characteristics (Table 1). The taxonomic use of trichomes is generally restricted to infrageneric subdivisions, while species diagnosis rests on the size and shape of leaves from the juvenile and adult phases as well as the number and shape of lobes in leaves of the juvenile phase, with occasional support from reproductive features (McAllister and Rutherford, 1990; Rutherford et al., 1993; Ackerfield, 2001; Ackerfield and Wen, 2002). The taxonomic controversy of *Hedera* can be summarized using three main critical axes: (1) the *H. helix* group, (2) the western Mediterranean taxa, and (3) the Asian taxa (Fig. 1). Particularly, a different number of species (four in Pojarkova, 1951; one in McAllister and Rutherford, 1983, 1990) and infraspecific taxa (two in Rose, 1996; three in Ackerfield and Wen, 2002) have been recognized as related to *H. helix* (Fig. 1); three of the western Mediterranean taxa (*H. algeriensis*, *H. canariensis*, *H. maderensis*) are affected by taxonomic conflicts derived from their indistinct morphological limits (Rose, 1996; Ackerfield, 2001; Ackerfield and Wen, 2002; Fig. 1); from three (McAllister and Rutherford's criterion) to six (Pojarkova, 1951) species are generally recognized in Asia (Fig. 1).

Hypothesis tested—The initial hypothesis evaluated in the present study is that the data sets include 12 groups of specimens ("hypothesis" as in Henderson, 2005a). This hypothesis was based on the 12 species of *Hedera* proposed by McAllister and Rutherford (Fig. 1). These authors based their species recognition on discrete macro- and micromorphological characters as well as on chromosomal counts and geographical distribution (Table 1).

Taxon sampling—The specimens included in the analyses were loaned by 40 herbaria (ANK, BG, BJFC, BM, BP, BR, BREM, COI, E, G, GOET, HBG, HUB, JACA, KEW, KWNU, LD, M, MA, MANCH, MAK, MPU, NMW,

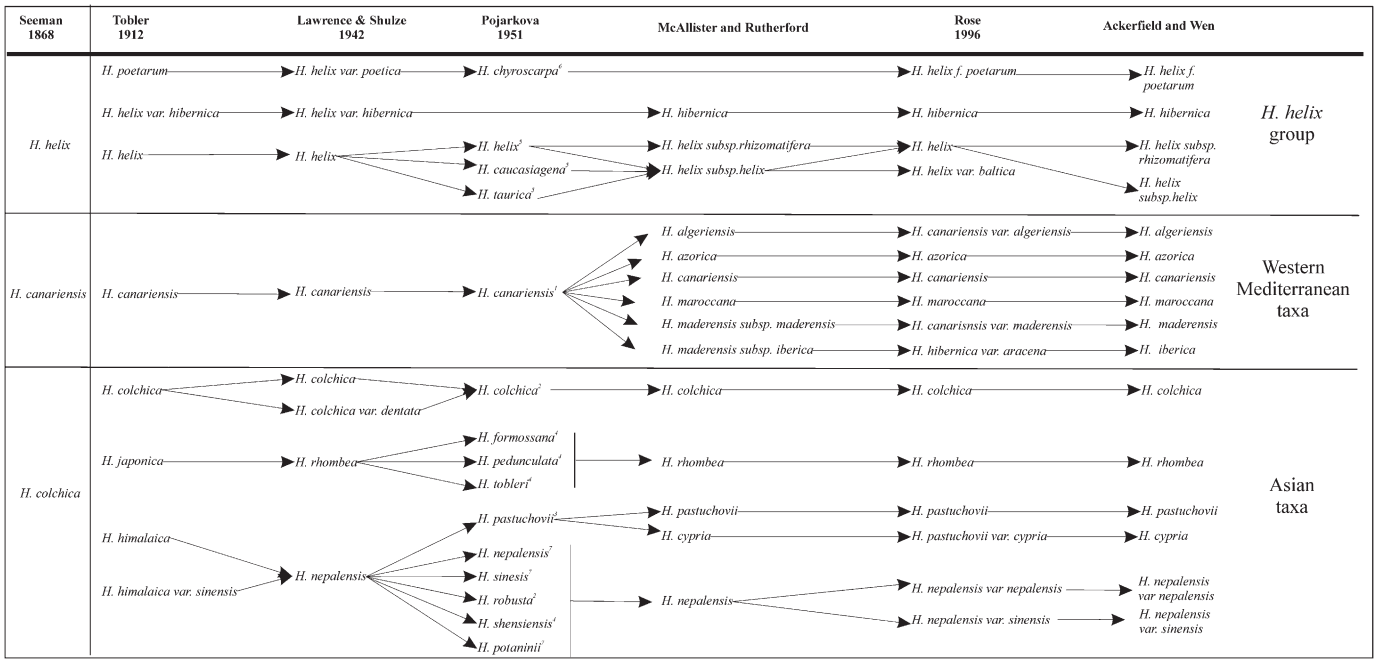


Fig. 1. Comparison of the seven major taxonomic treatments of *Hedera.* Last column indicates main groups affected by taxonomic controversy. Arrows indicate putative relatedness between taxa recognized by authors in consecutive taxonomic treatments. McAllister and Rutherford (McAllister, 1982; Rutherford, 1984, 1989; McAllister and Rutherford, 1990; Rutherford et al., 1993). Ackerfield and Wen (Ackerfield, 2001; Ackerfield and Wen 2002). [1] Ser. *Canariensis* Pojark. [2] Ser. *Robustae* Pojark. [3] Ser. *Pastuchovianae* Pojark. [4] Ser *Toblerianae* Pojark. [5] Ser. *Helix* Pojark. [6] Ser. *Chrysocarpae* Pojark. [7] Ser. *Sinenses* Pojark.

OSLO, P, PE, PPI, S, SANT, SNUA, SEV, SKK, TAIF, TAIPEI, TI, TNM, VAL, WAG, WU, Z; herbarium abbreviations are from http://sciweb.nybg.org/science2/IndexHerbariorum.asp). Specimens from the authors' collections from Austria, the Canary Islands, Cyprus, England, France, Germany, Italy, Morocco, Portugal, Spain, Tunisia, and Turkey, all of which have been deposited in the UPOS herbarium, were also examined. As a result, a total of 2473 specimens were studied under binocular magnification before selecting the samples included in the morphometric study. A special effort was made to represent the whole distribution range and morphological variability of each taxon and to include as many specimens per taxon as possible. However, the sampling of particular species, such as the Caucasian *H. pastuchovii* and the North African *H. algeriensis*, was limited due to their scarce representation in the herbarium collections. Also, incomplete or impossible-to-characterize herbarium specimens limited the sample size and necessitated the compilation of three independent data sets to reduce missing values (trichome, juvenile-phase, and adult-phase data sets; hereafter the TC, JP, and AP data sets, respectively). A total of 711 trichomes were measured; however, after excluding replicates (different observations of the same character in one specimen), the TC data set contained 351 individuals (Table 1; Appendix S1, see Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.1000115/DC1). The JP and AP data sets included 413 and 377 individuals, respectively (Table 1, Appendix S1). The within-species sample for each data set is specified in Table 1.

The prevalence of herbarium material over field collections of the studied material precluded us from ascribing specimens to specific populations with

TABLE 1. List of the 12 species recognized, specifying natural distribution, somatic chromosome number and ploidy level, and trichome type. Sample sizes included for the morphological study are specified in the last three columns, indicating the number of individuals examined for each of the three data sets. The number within parenthesis corresponds to the total number of replicates. For a detailed list of the studied material see Appendix S1 (see Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.1000115/DC1).

Taxa	Natural distribution	Ploidy level and somatic chromosome no.	Trichome type	Data set		
				Trichome	Juvenile-phase	Adult-phase
H. algeriensis	Algeria and Tunisia	$2n = 4x = 96$	Scale-like	5 (10)	7	5
H. azorica	Macaronesia, Azores Islands	$2n = 2x = 48$	Stellate (multiangulate)	24 (37)	21	14
H. canariensis	Macaronesia, Canary Islands	$2n = 2x = 48$	Scale-like	35 (50)	31	11
H. colchica	Caucasus, Turkey and Georgia	$2n = 8x = 192$	Scale-like	24 (53)	24	11
H. helix	Europe	$2n = 2X = 48$	Stellate (multiangulate)	64 (94)	111	74
H. hibernica	Atlantic Europe	$2n = 4x = 96$	Stellate (rotate)	27 (55)	66	66
H. maderensis	Macaronesia, Madeira	$2n = 6x = 144$	Scale-like	13 (29)	16	6
H. iberica	S Iberian Peninsula	$2n = 6x = 144$	Scale-like	29 (98)	44	31
H. maroccana	Morocco	$2n = 2x = 48$	Scale-like	27 (44)	20	19
H. nepalensis	E Hindu-Kush, Himalaya, and SW China	$2n = 2x = 48$	Scale-like	43 (106)	32	69
H. pastuchovii subsp. *pastuchovii*	Caucasus, Iran and Afghanistan	$2n = 6x = 144$	Scale-like	18 (41)	12	13
H. pastuchovii subsp. *cypria*	Cyprus	$2n = 6x = 144$	Scale-like	13 (32)	12	7
H. rhombea	Japan, Korea, and Taiwan	$2n = 2x = 48$	Scale-like	28 (62)	17	51

certainty. As a result, it was not possible to accomplish within-population sampling, and one single individual represented each population. Replicates within individuals were also limited due to specimen quality and herbarium preservation conditions, resulting in from one to nine observations within an individual. Using mean values calculated from replicates within a particular individual is desirable to better represent the within-individual variability. However, excluding specimens with only one observation would have resulted in important losses of valuable information and thereby reduced the power of the analysis (Quinn and Keough, 2002). Considering the high sensitivity of quantitative analysis to small sample size (Davis and Nixon, 1992; Luckow, 1995; Wiens, 1999) and the general aim of the present study, we considered it more appropriate to increase the species population sample size at the expense of the within-individual sample size. Therefore, for the analyses we used one randomly selected observation per specimen rather than mean values.

Character sampling—A survey was conducted to select the quantitative characters (continuous and meristic) to be analyzed. Character screening was primarily based on previous searches (Lum and Maze, 1989; McAllister and Rutherford, 1990; Rutherford et al., 1993; Ackerfield, 2001; Ackerfield and Wen, 2002) and our own observations. A pilot study including five samples per species and 66 characters was conducted to detect variable characters to increase the sample size. After this pilot study, 56 quantitative characters were analyzed: in trichomes, 15 characters were analyzed from which three combined variables were calculated; in the juvenile phase, nine quantitative characters and five combined variables were analyzed; and in the adult phase, 23 characters and one combined variable were analyzed (Table 2; Fig. 2).

Measurements—All characters were measured on dry, pressed specimens. Macromorphological leaf characters were measured in mature leaves from shoots older than 1 year. Macromorphological characters were measured using rulers and a Fowler electronic digital caliper (patent pending 54-100-000-1). The trichomes examined were taken from an interveinal space of the inner surface on the third or fourth apical leaf of 1-year shoots. The foliar trichomes were preferably taken from leaves of the juvenile phase. Samples (6 × 6 mm) of leaves were mounted with the inner surface up on an aluminum stub attached with silver paste. After 24 h of air-drying, each mounted sample was sputter-coated with gold (Balzers, SCD004). The samples were observed with a Hitachi S-300N scanning electron microscope (Hitachi, Ltd., Maidenhead, UK) and photographed with an IR chamberscope FC-65TI. Taking advantage of the single-plane orientation of the rays in the scale-like trichomes, we observed part of the sample of this group by mounting samples on microscope slides attached with cellophane tape. These samples were observed with a Nikon Eclipse E400 optical microscope and photographed with a Nikon DXM1200F digital camera.

Statistical analyses—Descriptive statistics (means, standard deviations, 75th percentiles, and ranges) and coefficients of variation were computed for the 56 variables. Multivariate analyses, such as principal component analysis (PCA), discriminant function analysis (DFA), and multivariate analysis of variance (MANOVA) were conducted on the data sets. The combined variables (ratios and percentages) were not considered in the PCA and DFA analyses to avoid redundancy and because they could mask whether the differences detected among groups were due to size or shape. For PCA and DFA, multivariate normality was assumed and not tested. Given that both analyses are relatively insensitive to such violations (Tabachnick and Fidell, 2007), our results should be considered as approximate.

The PCAs were conducted using a correlation matrix to scale the characters (Manly, 1994). Kaiser's measure of sampling adequacy and Bartlett's test of sphericity were performed to evaluate how suitable the data were for finding underlying structure. Rotation of the extracted component matrix was done with the Varimax orthogonal method. The component score coefficient matrix was compared to the rotated component matrix to detect possible nonlinearity problems (Hair et al., 2000). Principal components (PCs) with eigenvalues greater than or equal to one were retained.

The DFAs were performed using a within-groups covariance matrix and prior probabilities computed from size. For finding the variables that best discriminate the groups in each function, the structure matrix was interpreted. For evaluating the strength of the discriminant function for group identification, 30% of the cases were randomly excluded from the analyses (when a large enough within-group sample size was available) and used for validating the function. The implementation of the Cohen's kappa statistics in discriminant analysis (Titus et al., 1984) was used to evaluate the classification tables of the DFAs. This method computes the chance-corrected percentage of agreement between actual and predicted group memberships when group samples are equal or unequal, providing *P* values for each group's percentage of classification. For any given value of kappa, a *z* value is calculated from which the probability is

TABLE 2. List of quantitative characters (continuous and meristic) included in the morphological analyses.

Variable abbreviation	Character description
Juvenile phase data set	
IL(JP)	Internode length (cm)
PL(JP)	Petiole length (cm)
BL(JP)	Blade length (cm)
BW(JP)	Blade width (cm)
BL/BW(JP)	Blade length/width ratio
CLL(JP)	Central lobe length (cm)
FCLL(JP)	Free central lobe length (cm)
%BL(JP)	Percentage of blade lobulation: (FCLL/BL) × 100
CLW(JP)	Central lobe width (cm)
CLL/CLW(JP)	Central lobe length/width ratio
LLL(JP)	Lateral lobe length (cm)
CL/LLL(JP)	Central/lateral lobes length ratio
LLIL(JP)	Lateral lobe insertion length (cm)
%LL(JP)	Percentage of lateral lobulation: (FLLL/LLL) × 100
Adult phase data set	
IL(AP)	Internode length (cm)
PL(AP)	Petiole length (cm)
BL(AP)	Blade length (cm)
BW(AP)	Blade width (cm)
BL/BW(AP)	Blade length/width ratio
FLN	Flower number per umbel
PTL	Petal length (mm)
PTW	Petal width (mm)
SPL	Sepal length (mm)
SPW	Sepal width (mm)
SFL	Stamen filament length (mm)
AL	Anther length (mm)
SL	Stamen length (mm)
AW	Anther width (mm)
STL	Style length (mm)
PD2L	Peduncle length (cm)
PD1L	Pedicel length (cm)
FRN	Fruit number per umbel
DL	Disk length (mm)
FL	Fruit length (mm)
FW	Fruit width (mm)
SN	Seed number
SDL	Seed length (mm)
SDW	Seed width (mm)
Trichome data set	
RN	Ray number per trichome
TL	Trichome length (μm)
TW	Trichome width (μm)
TCP	Trichome central part (μm)
SL	Stalk length (μm)
LRL	Longest ray length (μm)
FLRL	Free longest ray length (μm)
BLRL	Basal longest ray length (μm)
LLRL	Lateral longest ray length (μm)
LRW	Longest ray width (μm)
SRL	Shortest ray length (μm)
FSRL	Free shortest ray length (μm)
BSRL	Basal shortest ray length (μm)
LSRL	Lateral shortest ray length (μm)
SRW	Shortest ray width (μm)
%CP	Percentage of central part: (TCP/TL) × 100
%FLR	Percentage of free longest ray: (FLRL/LRL) × 100
S/LRL	Shortest/longest rays length

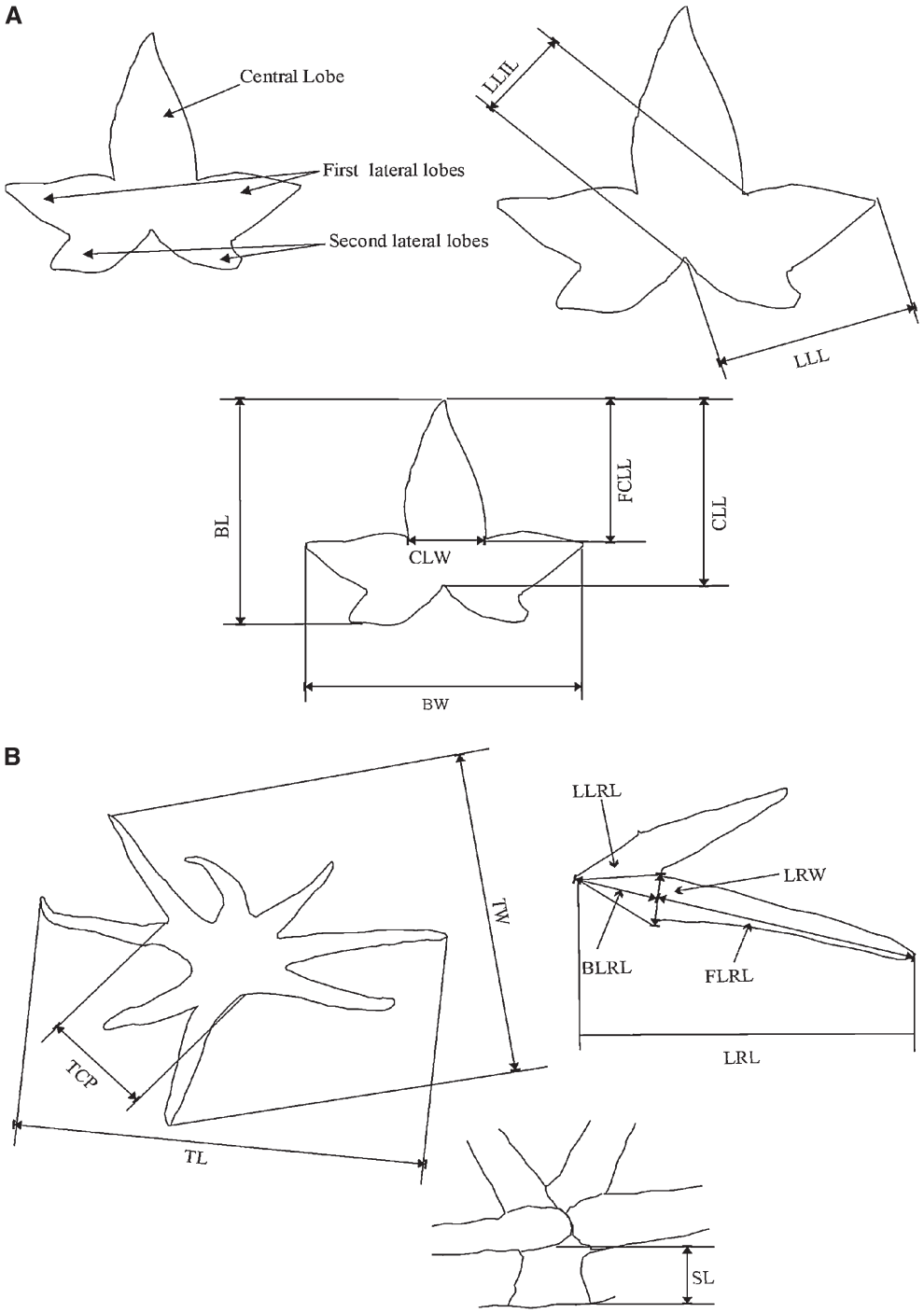


Fig. 2. Continuous quantitative characters examined for the multivariate analyses. (A) Macromorphological characters examined in leaves from the juvenile phase. (B) Micromorphological characters examined in trichomes. Abbreviations as in Table 2.

estimated. To evaluate the impact of sample size on the results obtained, we repeated the PCA and DFA analyses, replacing the missing data with mean values.

For MANOVA, the type III sum of squares was selected irrespective of the unplanned comparisons analyzed when data were unbalanced (Ayres and Thomas, 1990). Univariate analysis of variance (ANOVA) was conducted to assess the homogeneity of variances. For those variables that variance homogenization was not possible, Welch's robust ANOVA was used to evaluate statistical differences (Day and Quinn, 1989; Schürch et al., 2000). The Scheffé test for multiple comparisons of group means was used when more than two groups were evaluated, as it is one of the most conservative posthoc tests for unplanned comparisons (Day and Quinn, 1989).

The most discriminant characters were summarized for taxa in the form of box plot graphs. For species and group identification, quartiles were used.

All statistical analyses were performed using the software PASW statistics v.18 (SPSS, Chicago, Illinois, USA).

Delimiting and testing species*—Decision-making procedure*—A three-stage approach, consisting of three statistical analyses (PCA, DFA, MANOVA/ANOVA; Fig. 3), was performed to evaluate the hypothesis and to identify and

delimit species. The method used for decision-making was inspired by PAA (Davis and Nixon, 1992) and its extension (PAA-PSC) developed by Henderson (2005a).

Step 1. Structure of the given data set; if no prior information beside the hypothesis tested (that is, the species groups), then we proceeded to step 1.1; if the structure of the given data set is already known and groups are a priori defined, whether they coincide with the hypothesis tested or not, then we proceeded to step 1.2. Step 1.1: To explore possible unknown underlying structure within the given dataset that may obscure species hypotheses differences, we first conducted an exploratory multivariate analysis (PCA) (Fig. 3). If any internal grouping of the samples different from the hypothesis tested was detected, then we proceeded to step 1.2; if no internal grouping was detected, then we proceeded to step 1 (Fig. 3). Step 1.2 A confirmatory multivariate analysis (DFA) was performed using the original variables and preclassifying samples according to the groups a priori defined in step 1 or to the groups suggested by the PCA in step 1.1 (Fig. 3). If the discriminant function revealed statistical differences for group centroids and the classification of each group was significant with at least the 70% of cases correctly classified, then we proceeded to step 3.1 (see below); if the discriminant function revealed no statistical significance or if it was, but classifications were not significant and/or the percentage of cases correctly classified within each group was lower than the 70%, then we proceeded to step 2.

Step 2. A MANOVA was performed using the original variables and the DFA groups as the levels for the factor followed either by independent univariate analyses of the variance (ANOVAs or Welch's robust ANOVAs), if only two groups; or post hoc tests for multiple comparisons (i.e., Scheffé test), if more than two groups. If statistical support was retrieved, then we went on to step 3.2 (see below); if no statistical support was retrieved, then we proceeded to step 3.3 (see below).

Step 3. The groups detected through steps 1 and 2 were evaluated under the species criteria (see below). Step 3.1. The resulting groups were evaluated under species criterion 1 (see below); if the evaluation was positive, the groups were recognized and delimited as species; if the evaluation was negative, new subsets of data were generated with the groups that were subjected to reevaluation under the three stages approach. Step 3.2. The resulting groups were evaluated under species criterion 2 (see below); if the evaluation was positive, the groups were recognized and delimited as species; if the evaluation was negative, new subsets of data were generated with the groups that were subjected to reevaluation under the three stages approach. Step 3.3. The resulting groups (species already tested) were evaluated using the infraspecific operational criterion (see below); if the evaluation was positive, the groups were recognized and delimited as subspecies; if it was negative, the data set was discarded as being informative for the taxonomy of the particular group.

Diagnostic characters for delimiting the species identified through step 3.1 were obtained from the original variables that presented a factor loading (FL) equal to or greater than 0.50, providing enough sample sizes, in the significant discriminant function of the DFA (Hair et al., 2000). Diagnostic characters for species delimitation identified through step 3.2 were obtained from the significant original variables that presented no 75th percentile overlap. We applied an overlap upper-threshold of 25% for independent original variables because it seems more realistic than the application of a 5–10% threshold (Wiens and Servedio, 2000) for *Hedera*, a recently diverged group in which gene flow may occur at some frequency in wild populations, delaying fixation. Given the

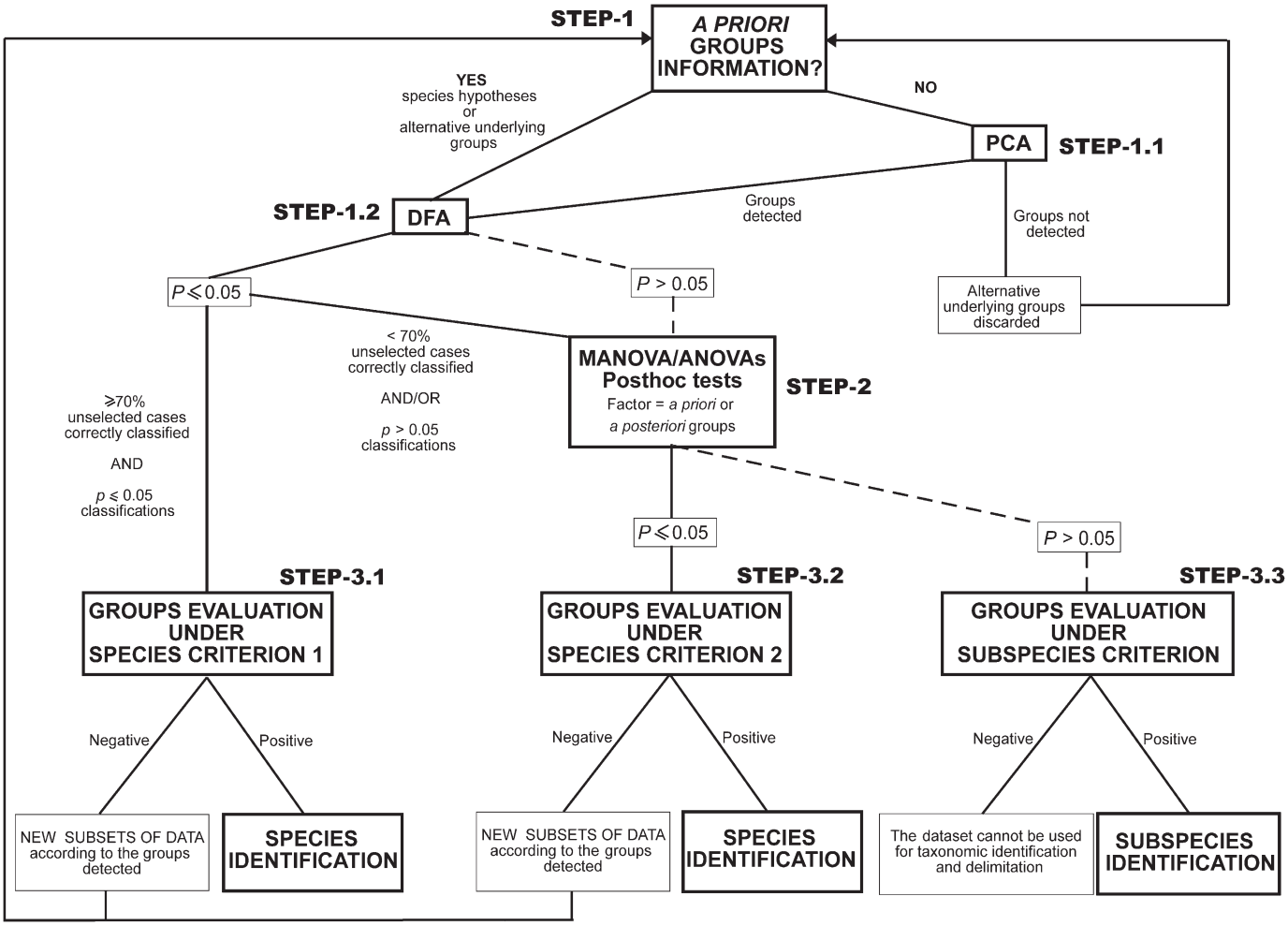


Fig. 3. Outline of the decision-making procedure performed for species identification and delimitation in *Hedera*. Species and subspecies criteria as specified in the text (Material and Methods).

multivariate significance, we adjusted the impact of assuming this relaxed threshold in type I error by considering more than one variables (at least four).

Species criteria—We delimited species under the GLC by using a relaxed implementation of the morphological diagnostic capability defined by Davis and Nixon (1992), with no differentiation between traits and characters (Nixon and Wheeler, 1990) and allowing a unique combination of quantitative characters. The explicit operational criteria were as follows. Species criterion 1: a group of samples was considered a species if it included the smallest aggregation of specimens with a unique combination of quantitative characters (i.e., significant differences among groups of samples were detected by DFA) with, at least, a 70% of statistically significant correct classification of unselected cases. If predicted memberships resulted in a nonsignificant and/or the correct classification was below the 70% for at least one group or there was no unique combination of quantitative characters but multivariate analysis supported the significance of the grouping, then species criterion 2 delimited a species if it constituted the smallest aggregation of specimens defined by at least four significant variables with no associated 75th percentile overlap or with overlap but with different inferred ploidy levels.

When internal structure was detected within a given validated species but not satisfying the above-described criteria, we considered the recognition of infraspecific entities (herein referred as subspecies) appropriate for practical considerations. We applied partial reproductive isolation as the main operational criterion inferred from different ploidy levels or strong geographical discontinuities as indicative of ongoing speciation process in the absence of clear morphological divergence. As a result, we recognize a group of samples as a subspecies in the absence of multivariate significance if at least four variables revealed statistical difference, whether a 75th percentile overlap was revealed, if and only if geographical segregation and/or different inferred ploidy levels existed.

Molecular testing of morphological species: The genealogical sorting index—To evaluate the power of morphology to detect ancestor-descendant relationships, we tested the taxa identified and delimited using the above-described procedure in a molecular context. The exclusive genetic ancestry of the species was explored using the genealogical sorting index (gsi; Cummings et al., 2008). This statistical method estimates the amount of accumulated genetic ancestry among samples in gene-tree genealogies given a species tree. The method provides a *P* value for the probability that a random mating retrieved the same (or a stronger) violation of monophyly as the one obtained from the taxonomic structure mating of the given species tree. The null hypothesis is that the mating inferred from the species tree is that expected at random. The phylogenetic uncertainty among the gene trees is incorporated into the calculation of exclusivity by bootstrapping (gsi_T; Cummings et al., 2008).

For testing the genetic ancestry of the *Hedera* species, the ITS (internal transcribed spacer) region from rDNA of the nuclear genome was selected rather than plastid regions because the ITS spacer has retained the hybridization events detected in the evolution of the genus (Vargas et al., 1999; Valcárcel et al., 2003a). Thirty-four ITS sequences were taken from the GenBank database (Appendix S1); 30 belong to the genus *Hedera* and the remaining four to other Araliaceae genera (*Brassaiopsis*, *Dendropanax*, *Euaraliopsis*, and *Trevesia*; Wen et al., 2001) as the most likely closest relatives (Valcárcel et al., 2003a; Valcárcel, 2008). The 30 ITS accessions of *Hedera* represent all the species with one to five populations per species. Phylogenetic reconstructions were performed under two different methods (maximum parsimony, MP; Bayesian inference, BI). Posterior probabilities and nonparametric bootstrapping were calculated to assess branch supports. The selection of the evolutionary model that best fits the data were performed as implemented in the program jModelTest 1.1b (Posada, 2008). When different models were selected by different criteria, the model selected by the Akaike information criterion (AIC) (Akaike, 1974) was selected for the analyses. The BI analysis was performed as implemented in the program MrBayes 3.0b (Huelsenbeck and Ronquist, 2001). For the BI, two runs with four Markov chain Monte Carlo (MCMC) iterations, each initiated from random starting trees were performed over 5 million generations, sampling trees every 100 generations, and retaining branch lengths. Likelihood convergence was determined by direct observation over generations and trees retrieved before reaching the likelihood stationary were discarded. The MP was run under no constraints as implemented in the program TNT (Goloboff et al., 2003) using heuristic traditional searches with random starting trees and tree-bisection-reconnection branch swapping. An initial search with 10 000 random addition replicates was run retaining two trees per replicate. Once the initial search finished, swapping was completed on the trees retained in the previous analysis. The nonparametric bootstraps were calculated with traditional searches using 1000 bootstrap replicates and 10 000 random additions per replicate.

For the gsi calculations, classes were assigned according to the morphological species herein recognized. The four non-*Hedera* tips were removed from the trees, and the remaining 30 *Hedera* tips were assigned to their respective species class. The first 1000 postburn-in trees obtained in the BI analysis were used to calculate the gsi_T statistics of each class. The *P* value associated to the gsi_T calculated for each class was estimated over 1000 permutations. Because of the present-study sample size and given that the power of the gsi test is sensitive to the within-species sample size (Cummings et al., 2008), only species represented by three or more samples were evaluated. The analysis was conducted with the program R using APE (Paradis et al., 2004) and genealogical sorting (A. L. Bazinet, University of Maryland; M.C. Neel, University of Maryland; K. L. Shaw, Cornell University; and M. P. Cummings, University of Maryland; unpublished manuscript).

RESULTS

Main results are summarized below as well as in Table 3. Detailed descriptions of result are provided in the appendices in the Supplemental Data with the online version of this article; decision-making procedure results (online Appendix S2), coefficients of variation (Appendix S3), discriminant morphological characters (Appendix S4), and brief *Hedera* species descriptions (Appendix S5)

Delimiting and testing species hypotheses—The analysis of the three complete data sets including all samples of *Hedera* ($N_{TC} = 351$, $N_{JP} = 414$, $N_{AP} = 384$) under the three-stage multivariate analysis decision process revealed two groups (Fig. 4). One group included all the samples with stellate trichomes (hereafter called stellate group; A subset of data, Table 3; Appendix S5), and the second group included all the samples with scale-like trichomes (hereafter called scale-like group; B subset of data, Table 3; Appendix S5). These two groups were statistically detected and supported by the TC data set (PCA/DFA: Wilks' lambda = 0.204, $P < 0.001$), with a percentage of unselected cases correctly classified of the 71% ($k = 0.5$, $P = 0.01$) for the stellate group and the 100% ($k = 1$, $P < 0.001$) for the scale-like group. This samples grouping was also statistically supported by the JP data set (DFA: Wilks' lambda = 0.779, $P < 0.001$; MANOVA: $F_{14,344} = 8.6$, $P < 0.001$), with a percentage of unselected cases correctly classified of the 79% ($k = 0.5$, $P < 0.001$) for the stellate group and the 74% ($k = 0.5$, $P < 0.001$) for the scale-like group, as well as by the AP data set (DFA: Wilks' lambda = 0.803, $P < 0.001$; MANOVA: $F_{5,346} = 18.4$, $P < 0.001$), with a percentage of unselected cases correctly classified of the 71% ($k = 0.2$, $P = 0.002$) for the stellate group and the 75% ($k = 0.5$, $P < 0.001$) for the scale-like group. Discrimination between the stellate and the scale-like groups was based on eight original variables obtained from the TC data set: free longest ray length (−0.8 FL), free shortest ray length (−0.7 FL), longest ray length (−0.6 FL), shortest ray length (−0.6 FL), trichome width (−0.5 FL), trichome length (−0.5 FL), ray number (0.6 FL), and stalk length (−0.5 FL). Additional support was provided by two variables from the JP data set (blade length [0.8 FL] and blade width [0.5 FL]) and one from the AP data set (blade length [0.6 FL]).

A subset of data: The stellate group—The analysis of the three complete data sets including all samples of the stellate group of *Hedera* ($N_{TC} = 115$, $N_{JP} = 199$, $N_{AP} = 154$) under the three-stage multivariate analysis decision process revealed two groups (Fig. 5A). One group included all the samples with rotate stellate trichomes (hereafter called rotate stellate group; A_1 subset of data, Table 3; online Appendices S2 and S5), and the other group included all the samples with multiangulate stellate trichomes

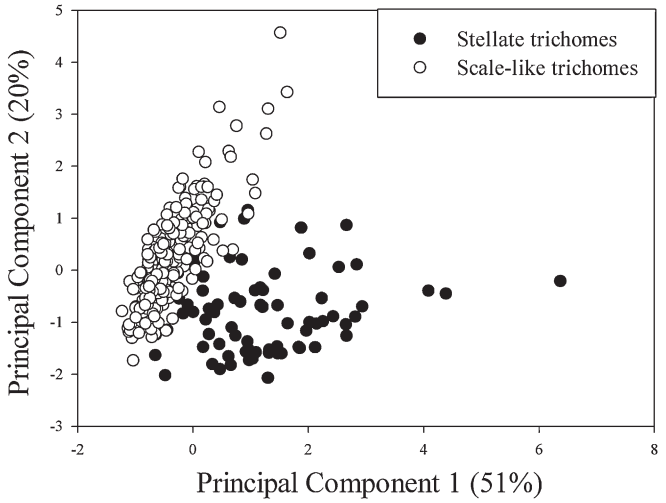
TABLE 3. Main results of the multivariate statistical approach applied for delimitation and evaluation of *Hedera* species. The first column indicates the source of data (TC, trichome data set; JP, juvenile phase data set; AP, adult phase data set). The next three columns summarize the PCA results specifying the number of original variables analyses (N_V), the number of specimens analyzed including missing data (N_{Sim}), the number of specimens analyzed replacing missing data by mean values (N_{Sem}), and the name of the groups detected in the PCA (Groups). The next four columns summarize the DFA results specifying the test value and level of significance of the discriminant function (Wilk's λ), the percentage of unselected cases correctly classified for each of the groups (% Corr. class.), the Cohen's kappa and levels of statistical significance for each of the groups, and the number of discriminant variables with a factor loading ≥ 0.5 (N_{DV}). The next three columns summarize the MANOVA results specifying the number of original variables analyzed (N_V), the number of specimens analyzed (N_S), the name of the groups considered in the factor (Groups), the statistics and level of significance (F); M_1 indicates the MANOVA conducted including specimens with lobate leaves and M_2 indicates the MANOVA performed including specimens both with lobate and entire leaves. The next two columns specify the number of significant original variables (N_{SV}) detected in the univariate tests (ANOVAs, Welch's robust ANOVAs and Scheffé test for multiple comparisons), and how many of them present no 75th percentile overlap (N_{SVno}). The last column includes the name of the original variables that best distinguish the groups; the superscripts indicate whether the variables were the most discriminant variables detected in the DFAs (D) or significant variables with no 75th percentile overlap detected in the univariate tests (M).

Data set	Principal component analysis\			Discriminant function analysis				MANOVA			Univariate tests		
	N_V, N_{Sim} (N_{Sem})	N_{PCs} (%Var.)	Groups	Wilks' λ	% Corr. class.	Cohen's k	N_{DV}	N_V, N_S	Groups	F	N_{SV}	N_{SVno}	Significant variables
Hedera													
TC	15, 351 (307)	3 (81)	A, B	0.2***	A: 71, B: 100	A: 0.5**, B: 1***	8	—	—	—	—	—	$FLRL^D$, $FSRL^D$, LRL^D, SRL^D, TW^D, TL^D, RN^D, SL^D
JP	9, 414 (359)	—	—	0.8***	A: 79, B: 74	A: 0.5***, B: 0.5***	2	14, 344	A, B	8.6***	9	7	BL^{DM}, BW^{DM}, BL/BW^M, CLL^M, $\%BL^M$, CLL/W^M, IL^M, PL^M, $\%LL^M$
AP	4, 384 (357)	—	—	0.8***	A: 71, B: 75	A: 0.2**, B: 0.5***	1	5, 346	A, B	18.4***	3	0	BL^D
A subset of data: Stellate group													
TC	15, 115 (71)	3 (80)	A_1, A_2	0.2***	A_1: 89, A_2: 77	A_1: 0.8***, A_2: 0.8***	5	—	—	—	—	—	RN^D, TCP^D, $LSRL^D$, SL^D, $BSRL^D$
JP	9, 199 (163)	—	—	0.8**	A_1: 88, A_2: 18	A_1: 0.8***, A_2: −1.1$^{n.s.}$	v	14, 148	A_1, A_2	3.2***	6	0	—
AP	4, 154 (149)	—	—	0.9*	A_1: 79, A_2: 30	A_1: 0.6***, A_2: −0.2$^{n.s.}$	—	5, 143	A_1, A_2	2.6*	1	0	—
A_2 subset of data: Multiangulate stellate group													
TC	15, 45(45)	4 (80)	No groups	0.4$^{n.s.}$	—	—	—	18, 45	Sp_2, Sp_3	2.1*	2	1	LRW^M
JP	9, 133 (120)	2 (79)	Sp_2, Sp_3	0.8**	Sp_2: 50%, Sp_3: 94	Sp_2: −0.5 $^{n.s.}$, Sp_3: 0.8***	—	14, 120	Sp_2, Sp_3	8.7***	10	3	IL^M, $\%BL^M$, $LLIL^M$
AP	4, 88 (84)	—	—	0.9*	Sp_2: 58, Sp_3: 96	Sp_2: −0.1 $^{n.s.}$, Sp_3: 0.9***	—	5, 84	Sp_2, Sp_3	14***	3	2	BW^M, BL/BW^M
B subset of data: Scale-like group													
TC	14, 236 (236)	3 (74)	B_1, B_2	0.7***	B_1: 62, B_2: 89	B_1: 0.4***, B_2: 0.9***	—	17, 236	B_1, B_2	8.3***	11	0	
JP	9, 215 (150)	—	—	0.6***	B_1: 81, B_2: 81	B_1: 0.7***, B_2: 0.5**	2	M_1:14, 150, M_2:4,215	B_1, B_2	9.7***, 21***	10, 2	3, 0	$LLIL^{DM1}$, CLW^D, CLL/W^{M1}, C/LLL^{M1}
AP	4, 230 (208)	—	—	0.8***	B_1: 34, B_1: 93	B_1: −0.6$^{n.s.}$, B_2: 0.7***	—	5, 203	B_1, B_2	9.4***	2	2	BL/BW^M, BW^M
B_1 subset of data: Western group													
TC	14, 109 (103)	4 (82)	B_{11}, B_{12}	0.5***	B_{11}: 54, B_{12}: 86	B_{11}: 0.3$^{n.s.}$, B_{12}: 0.7***	—	17, 109	B_{11}, B_{12}	3.3***	12	0	
JP	9, 118 (90)	—	—	0.6***	B_{11}: 55, B_{12}: 100	B_{11}: 0.4***, B_{12}: 0.8***	—	M_1:14,90, M_2:4, 103	B_{11}, B_{12}	M_1:4.1***, M_2:12***	M_1:10, M_2:3	M_1:3, M_2:0	BW^{M1}, LLL^{M1}, $LLIL^{M1}$
AP	4, 78 (78)	—	—	0.7*	B_{11}: 20, B_{12}: 90	B_{11}: −1.4$^{n.s.}$, B_{12}: 0.9***	—	4, 54	B_{11}, B_{12}	4.9**	2	2	BL^M, BW^M
B_{11} subset of data: *Hedera algeriensis* (Sp_4) plus *H. canariensis* (Sp_5)													
TC	14, 40 (40)	4 (82)	No groups	0.3**	Sp_4: 40, Sp_5: 97	Sp_4: e $^{n.s.}$, Sp_5: 1**	—	17, 40	Sp_4, Sp_5	2.8**	2	2	$LSRL^M$, RN^M
JP	9, 38 (24)	1 (77)	—	0.8$^{n.s.}$	—	—	—	M_1:14, 24, M_2: 4, 36	Sp_4, Sp_5	M_1:2$^{n.s.}$, M_2:0.5***	M_1: 1, M_2: 0	M_1: 1, M_2: 0	$\%BLL^{M1}$
AP	4, 16 (15)	—	—	0.4*	Sp_4: 60, Sp_5: 100	Sp_4: 0 $^{n.s.}$, Sp_5: 1**	—	5, 15	Sp_4, Sp_5	*3.2*	2	2	IL, BL
B_{12} subset of data: *Hedera maderensis subsp. iberica* (Sp_6) plus subsp. *maderensis* (B_{121}) plus *H. maroccana* (B_{121})													
TC	14, 69	4 (83)	Sp_6, B_{121}	0.1***	Sp_6: 92, B_{121}: 100	Sp_6: 0,9***, Sp_5: 1***	1	17, 69	Sp_6, B_{121}	48.2***	11	5	LRW^D, $LLRL^M$, LRL^M, SRL^M, $FSRL^M$, S/LRL^M
JP	9, 80 (67)	—	—	0.6**	Sp_6: 50, B_{121}: 67	Sp_6: 0,3**, Sp_5: 0.7***	—	14, 67	Sp_6, B_{121}	5.8***	4	3	$\%BLL^M$, CLL/CLW^M, $\%LL^M$

TABLE 3. Continued.

Data set	Principal component analysis\			Discriminant function analysis				MANOVA			Univariate tests		
	N_V, N_{Sim} (N_{Sem})	N_{PCs} (%Var.)	Groups	Wilks' λ	% Corr. class.	Cohen's *k*	N_{DV}	N_V, N_S	Groups	*F*	N_{SV}	N_{SVno}	Significant variables
AP	4, 62 (44)	—	—	$0.9^{n.s.}$	—	-	—	5, 44	Sp_6, B_{121}	*n.s.*	1	0	-
B_{121} subset of data: *Hedera maderensis* (Sp_7) plus *H. maroccana* (Sp_8)													
TC	14, 40 (40)	4 (82)	Sp_7, Sp_8	0.1***	Sp_7: 100, Sp_8: 100	Sp_7: 0.8**, Sp_8: 1***	1	17, 40	Sp_7, Sp_8	10***	10	4	$SRW^{D,M}$, RN^M, TCP^M, $\%CP^M$
JP	9, 35 (35)	—	—	0.2**	Sp_7: 75, Sp_8: 50	Sp_7: 0.8***, Sp_8: 0.6**	—	14, 35	Sp_7, Sp_8	30.1***	5	2	CLL^M, CLL/W^M
AP	4, 25 (24)	—	—	0.6*	Sp_7: 40, Sp_8: 84	Sp_7: $0.4^{n.s.}$, Sp_8: 1**	—	5, 24	Sp_7, Sp_8	3*	1	0	—
B_2 subset of data: Eastern group													
TC	14, 127 (127)	3 (78)	Sp_9, B_{21}	0.7*	Sp_9: 25, B_{21}: 91	Sp_9: $-1.1^{n.s.}$, B_{21}: 1***	—	17, 127	Sp_9, B_{21}	4.3***	10	7	$\%CP^M$, TL^M, TW^M, LRL^M, SRL^M, $FSRL^M$
JP	9, 97 (93)	—	—	0.6**	Sp_9: 60, B_{21}: 93	Sp_9: $-0.1^{n.s.}$, B_{21}: 0.8***	—	M_1: 14, 59, M_2: 4, 93	Sp_9, B_{21}	M_1:6.5***, M_2:3.7**	M_1:9, M_2:2	M_1:4, M_2:1	$FCLL^{M1}$, $\%BLL^{M1}$, CLL/W^{M1}, C/LLL^{M1}, BL/BW^{M2}
AP	4, 152 (149)	—	—	0.6***	Sp_9: 59, B_{21}: 96	Sp_9: $-0.2^{n.s.}$, B_{21}: 0.8***	—	5, 149	Sp_9, B_{21}	21***	5	1	BL^M
B_{21} subset of data: *Hedera colchica* (Sp_{10}) plus *H. cypria* (B_{211}, Sp_{13}) plus *H. nepalensis* (B_{211}, Sp_{11}) plus *H. pastuchovii* (B_{211}, Sp_{12})													
TC	14, 99 (99)	3(78%)	No groups	0.3**	Sp_{10}: 55, Sp_{11}: 73, Sp_{12}: 0, Sp_{13}: 33	Sp_{10}: 0.5***, Sp_{11}: 0.8***, Sp_{12}: $0^{n.s.}$, Sp_{13}: $0.1^{n.s.}$	—	17, 99	Sp_{10}, Sp_{11}, Sp_{12}, Sp_{13}	2.2***	9	0	—
JP	9, 80 (78)	2(80%)	Sp_{10}, B_{211}	0.8**	Sp_{10}: 75, B_{211}: 86	Sp_{10}: $0.1^{n.s.}$, B_{211}: 0.6***	—	4, 77	Sp_{10}, B_{211}	7.8***	4	0	IL^M, PL^M, BL^M, BW^M
AP	4, 100 (100)	—	— [1]	0.9*	Sp_{10}: 18, B_{211}: 99	Sp_{10}: $-5^{n.s.}$, B_{211}: 0.9***	—	5, 100	Sp_{10}, B_{211}	2.8*	2	0	BW^M, BL/BW^M
B_{211} subset of data: *H. cypria* (B_{2111}) plus *H. nepalensis* (Sp_{11}) plus *H. pastuchovii* (B_{2111})													
TC	14, 74 (74)	4(81%)	Sp_{11}, B_{2111}	$0.4^{n.s.}$	—	—	—	17, 74	Sp_{11}, B_{2111}	$1.2^{n.s.}$	5	1	LRW^M
JP	9, 56 (54)	—	—	$0.6^{n.s.}$	—	—	—	M_1: 14, 54, M_2: 4, 45	Sp_{11}, B_{2111}	M_1: $1.6^{n.s.}$, M_2: $1.6^{n.}$	M_1: 7, M_2: 2	M_1: 0, M_2: 0	$BL/BW^{M1, M2}$, CLL^{M1}, $FCLL^{M1}$, $\%BL^{M1}$, $CLL/W^{M1, M2}$, LLL^{M1}, C/LLL^{M1}
AP	4, 89 (89)	—	—	$0.9^{n.s.}$	—	—	—	5, 89	Sp_{11}, B_{2111}	2.9*	2	0	BW^M, BL/BW^M
B_{2111} subset of data (Sp_{12}): *H. cypria* (Ssp_{121}) plus *H. pastuchovii* (Ssp_{122})													
TC	14, 31 (31)	4(85%)	Ssp_{121}, Ssp_{122}	$0.4^{n.s.}$	—	—	—	17, 31	Ssp_{121}, Ssp_{122}	$1.7^{n.s.}$	4	0	LRL^M, $FLRL^M$, $\%FLRL^M$, $\%CP^M$
JP	9, 24 (24)	—	—	$0.9^{n.s.}$	—	—	—	M_1: 14, 15, M_2: 4, 24	Ssp_{121}, Ssp_{122}	M_1: $0.4^{n.s.}$, M_2: $0.4^{n.s.}$	M_1: 0, M_2: 0	M_1: 0, M_2: 0	—
AP	4, 20 (20)	—	—	0.4**	Ssp_{121}: 57, Ssp_{122}: 77	Ssp_{121}: $0.7^{n.s.}$, Ssp_{122}: 0.8**	—	5, 20	Ssp_{121}, Ssp_{122}	$4.3^{n.s.}$	2	0	BW^M, BL/BW^M

Note: Variables' abbreviations are specified in Table 2. Levels of statistical significance * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$. Species abbreviations are as follows: A_1: *H. hibernica*, Sp_2: *H. azorica*, Sp_3: *H. helix*, Sp_4: *H. algeriensis*, Sp_5: *H. canariensis*, Sp_6: *H. iberica*, Sp_7: *H. maderensis*, Sp_8: *H. maroccana*, Sp_9: *H. rhombea*, Sp_{10}: *H. colchica*, Sp_{11}: *H. nepalensis*, Ssp_{12}: *H. pastuchovii* subsp. *pastuchovii*, Ssp_{13}: *H. pastuchovii* subsp. *cypria*. The results presented correspond to the analyses performed by discarding individuals with missing data. In all cases, the analyses conducted by replacing missing data with mean values gave similar results (online Appendix S2).



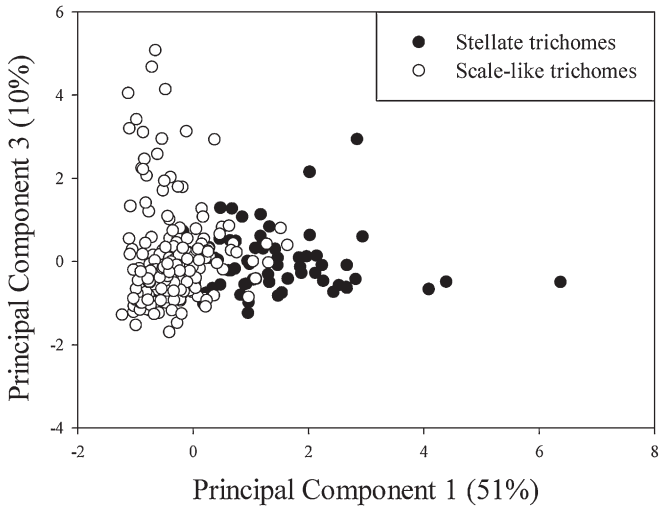
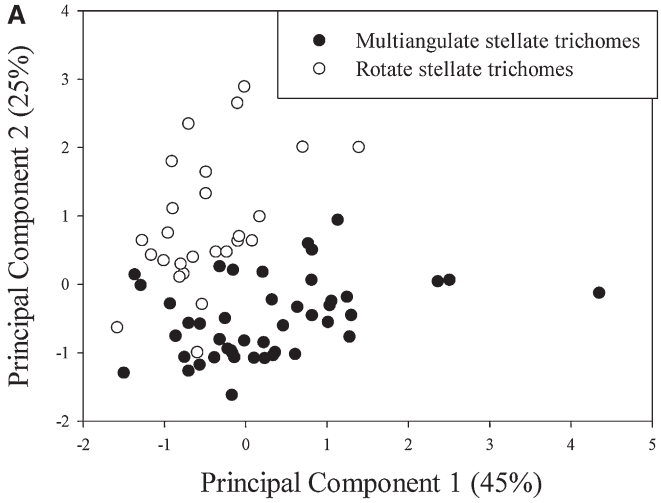


Fig. 4. Scatterplot of the first three principal components extracted in the principal component analysis of the complete trichome data set (15 variables, 307 samples excluding missing data) of *Hedera*. The samples were labeled according to the two main trichome types (stellate, and scale-like). Percentage of total variance explained by each component is specified in parenthesis.



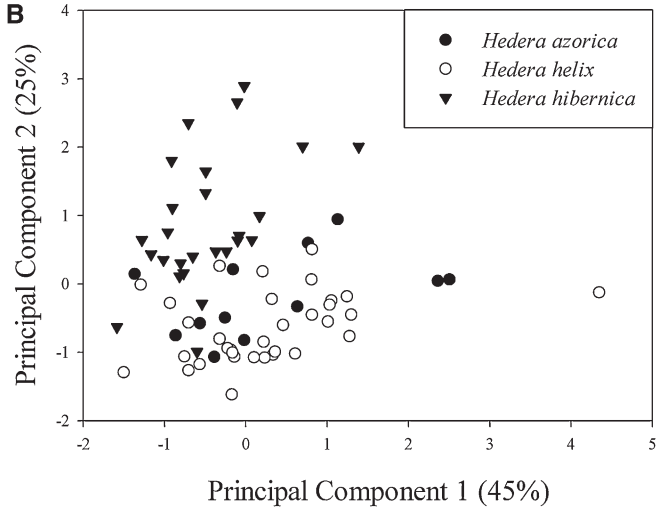


Fig. 5. Scatterplot of the first two principal components extracted in the principal component analysis of the trichome data set (15 variables, 71 samples excluding missing data) including all specimens of the stellate group of *Hedera*. (A) Distribution of samples when labeled according to two subgroups of trichomes (multiangulate, and rotate). (B) Distribution of samples when labeled according to the species hypotheses tested (*H. azorica*, *H. helix*, and *H. hibernica*). Percentage of total variance explained by each component is specified in parenthesis.

(hereafter called multiangulate stellate group; A_2 subset of data, Table 3; Appendices S2 and S5). The rotate stellate group (A_1 subset of data, Table 3) coincides with one of the species hypotheses tested (*H. hibernica* as recognized by McAllister and Rutherford; Fig. 5B), while the multiangulate stellate group (A_2 subset of data, Table 3) included two of the species hypotheses tested (*H. azorica* and *H. helix*; Fig. 5B). The multiangulate and rotate stellate groups were statistically detected and supported by the TC data set (PCA/DFA: Wilks' lambda = 0.228, $P < 0.001$), with a percentage of unselected cases correctly classified of the 89% ($k = 0.8$, $P < 0.001$) for the rotate stellate group and the 77% ($k = 0.8$, $P < 0.001$). These two groups were also statistically supported by the JP (DFA: Wilks' lambda = 0.785, $P = 0.003$; MANOVA: $F_{14,148} = 3.2$, $P < 0.001$), and the AP (DFA: Wilks' lambda = 0.902, $P = 0.03$; MANOVA: $F_{5,143} = 2.6$, $P = 0.03$) data sets.

Evaluation of the rotate stellate group (A_1 subset of data, Table 3) under species criterion 1 resulted in the identification and delimitation of one species with no subspecific subdivision (*H. hibernica*; Fig. 6, Appendices S4 and S5) based on five significant original variables obtained from the TC data set: ray number (0.6 FL; Fig. 6A), trichome central part (0.5 FL), lateral shortest ray length (0.5 FL), stalk length (−0.5 FL) and basal shortest ray length (0.5 FL). Additional support was obtained from six significant original variables with 75th overlap of the juvenile phase (blade length, blade length/width ratio, free central lobe length, percentage of blade lobulation, central lobe length/width ratio and central/lateral lobe length ratio; Fig. 6B–F) and one from the adult phase (internode length).

Evaluation of the *H. azorica* and *H. helix* groups (A_2 subset of data, Table 3) under species criterion 2 resulted in the identification and delimitation of two species with no subspecific subdivision (*H. azorica* and *H. helix*, Appendices S4 and S5) based on three significant original variables with no 75th percentile overlap

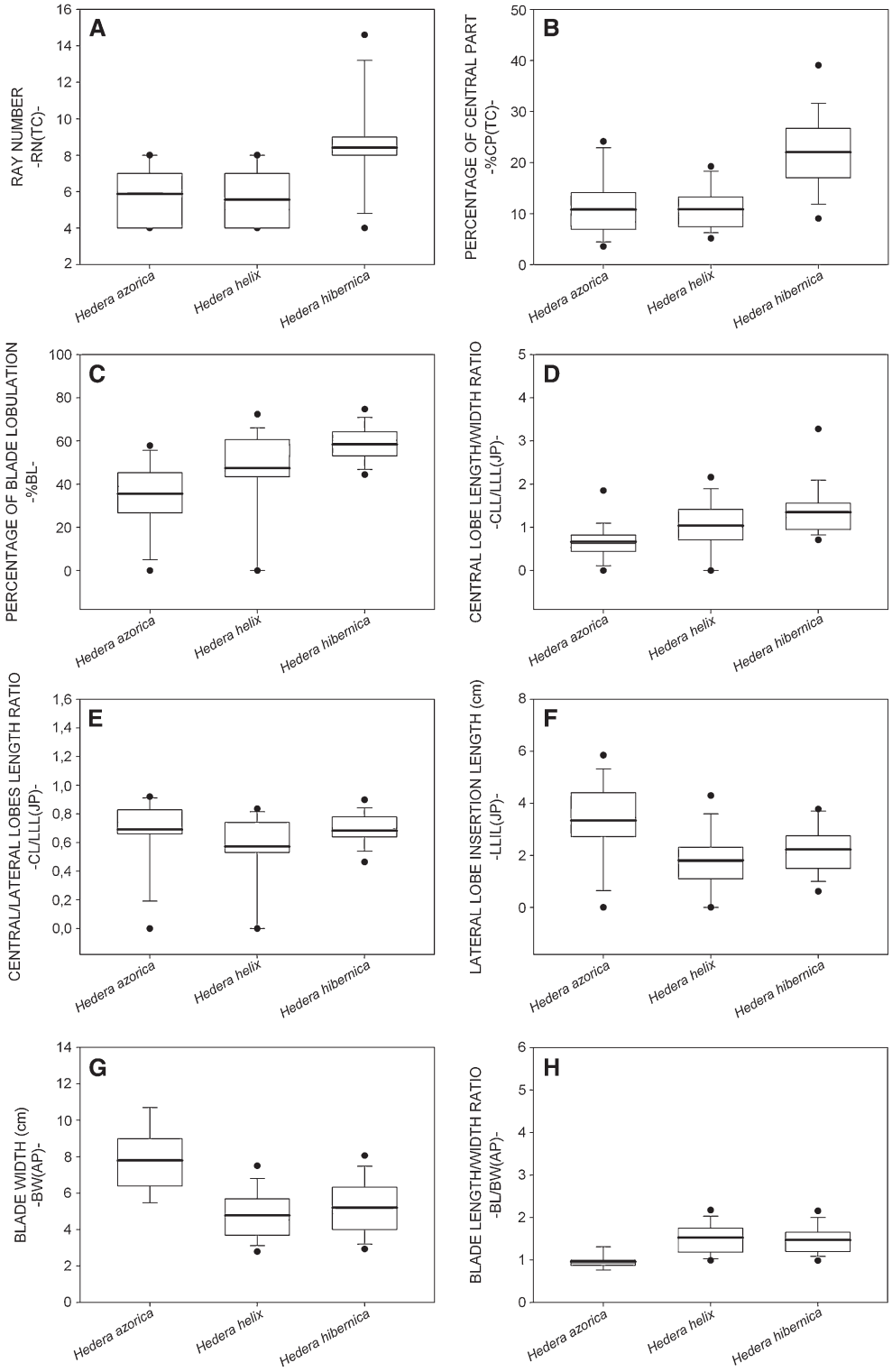


Fig. 6. Box plots of the eight most discriminant characters (two from trichomes, four from leaves of the juvenile phase, and two from the leaves of the adult phase) obtained for the diagnosis of the three species of *Hedera* recognized within the stellate group (*H. azorica*, *H. helix*, and *H. hibernica*). (A) Character "ray number" measured on trichomes ($N = 115$). (B) Character "percentage of central part" measured in trichomes ($N = 112$). (C) Character "percentage of blade lobulation" measured in lobate leaves from the juvenile phase ($N = 171$). (D) Character "central lobe length/width ratio" measured in lobate leaves from the juvenile phase ($N = 169$). (E) Character "central/lateral lobes length ratio" measured in lobate leaves from the juvenile phase ($N = 171$). (F) Character "lateral lobe insertion length" measured in lobate leaves from the juvenile phase ($N = 169$). (G) Character "blade width" measured in leaves from the adult phase ($N = 154$). (H) Character "blade length/width ratio" measured in leaves from the adult phase ($N = 154$). Boxes represent 75th percentiles, bars indicate 5th and 95th percentiles, and horizontal lines indicate mean values.

obtained from the JP (internode length; percentage of blade lobulation, Fig. 6C; lateral lobe insertion length, Fig. 6F), and two from the AP (blade width, Fig. 6G, and blade length/width ratio, Fig. 6H) data sets. Additional support was obtained from one significant original variable with no 75th percentile overlap of the TC data set (longest ray width).

B subset of data: The scale-like group—The analysis of the three complete subsets of data including all samples of the scale-like group of *Hedera* (N_{TC} = 236, N_{JP} = 215, N_{AP} = 230) under the three-stage multivariate analysis decision process revealed two groups (Table 3; Appendix S2). One group included all samples from the western Mediterranean basin s.l. (hereafter called the western group; B_1 subset of data, Table 3; Appendices S2 and S5), and the other group included all samples from the eastern extreme of the Mediterranean basin together with the Asian samples (hereafter called the eastern group; B_2 subset of data, Table 3; Appendices S2 and S5). These two groups were statistically supported by the JP data set (DFA: Wilks' lambda = 0.585, $P < 0.001$; MANOVA: $F_{14,150} = 9.7$ and $F_{4,215} = 13$, $P < 0.001$), with a percentage of unselected cases correctly classified of the 81% for both groups (B_1: $k = 0.7$, $P < 0.001$; B_2: $k = 0.5$, $P = 0.001$). This samples grouping was also statistically supported by the TC (PCA/DFA: Wilks' lambda = 0.658, $P < 0.001$; MANOVA: $F_{17,236} = 8.3$, $P < 0.001$), and the AP (DFA: Wilks' lambda = 0.8, $P < 0.001$; MANOVA: $F_{5,203} = 9.4$, $P < 0.001$) data sets. Discrimination between the western and eastern groups was based on four significant original variables obtained from the JP data set—lateral lobe insertion length (−0.6 FL) and central lobe width (−0.6 FL), central lobe length/width ratio and central/lateral lobe length ratio—three of them with no 75th percentile overlap (Table 3). Additional support was obtained from two significant original variables with no 75th percentile overlap of the AP data set (blade width and blade length/width ratio).

B_1 subset of data: The western group—The analysis of the three complete subsets of data including all samples of the western group (N_{TC} = 109, N_{JP} = 118, N_{AP} = 78) under the three-stage multivariate analysis decision process revealed two groups (*H. algeriensis* plus *H. canariensis* vs. *H. maderensis* plus *H. maroccana*; B_{11} and B_{12}, respectively, Table 3; Appendix S2). These two groups were statistically detected and supported by the TC (PCA/DFA: Wilks' lambda = 0.54, $P < 0.001$; MANOVA: $F_{17,109} = 3.3$, $P < 0.001$), and the JP (PCA/DFA: Wilks' lambda = 0.588, $P < 0.001$; MANOVA: $F_{14,90} = 4.1$ and $F_{4,103} = 12$, $P < 0.001$) data sets. Additional support was retrieved by the AP data set (DFA: Wilks' lambda = 0.74, $P = 0.01$). Discrimination between both groups was based on five significant original variables no 75th percentile overlap (Fig. 7), three obtained from the JP data set (blade width, Fig. 7C; lateral lobe length, Fig. 7F and lateral lobe insertion length), and two from the AP data set (blade length, Fig. 7G and blade width, Fig. 7H). Successive runs of the three stages multivariate statistical decision process with these two subsets of data (B_{11} and B_{12}, Table 3) allowed the identification and delimitation of five species (Table 3, Appendix S2)

Evaluation of the *H. algeriensis* and *H. canariensis* groups (B_{11} subset of data, Table 3) under species criterion 2 resulted in the identification and delimitation of two species with no subspecific subdivision (*H. algeriensis* and *H. canariensis*; Table 3, Appendices S4 and S5) based on two significant original variables with no 75th percentile overlap obtained from the TC (lateral shortest ray length and ray number; Table 3), one from the JP (percentage of blade lobulation, Fig. 7D), and two from the AP (internode length and blade width, Fig. 7H) data sets.

Evaluation of the Iberian hexaploid group (B_{12} subset of data, Table 3, Appendix S2) under species criterion 1 resulted in the identification and delimitation of one species with no subspecific subdivision, *H. iberica* (McAllister) Ackerfield and Wen (Appendices S4 and S5) based on six significant original variables obtained from the TC data set (lateral longest ray length, longest ray width, shortest ray length, free shortest ray length and shortest/longest rays length, Fig. 7B); five of them with no 75th percentile overlap (Table 3). Additional support was obtained from three significant original variables with no 75th percentile overlap of the JP data set (percentage of blade lobulation, Fig. 7D, central lobe length/width ratio, Fig. 7E and percentage of lateral lobulation).

Evaluation of the Madeiran hexaploid group (*H. maderensis* subsp. *maderensis*) together with the *H. maroccana* group (B_{121} subset of data, Table 3, Appendix S2) under species criterion 1 resulted in the identification and delimitation of two species with no subspecific subdivisions (*H. maderensis* K. Koch ex Rutherford and *H. maroccana* McAllister; Table 3, Appendices S4 and S5). These species were supported by four significant original variables with no 75th percentile overlap obtained from the TC data set (ray number, trichome central part, Fig. 7A; shortest ray width and percentage of central part), two from the juvenile phase (central lobe length; central lobe length/width ratio, Fig. 7E), and one with 75th percentile overlap obtained from the AP data set (blade length, Fig. 7G).

B_2 subset of data: Eastern group—Successive runs of the three complete subsets of data including all samples of the western group (N_{TC} = 127, N_{JP} = 97, N_{AP} = 152) under the three-stage multivariate statistical decision process allowed the identification and delimitation of four species (*H. rhombea*, Sp_9; *H. colchica*, Sp_{10}; *H. nepalensis,* Sp_{11}; *H. pastuchovii*, Sp_{12}; Fig. 8, Table 3; Appendix S2).

Evaluation of the *H. rhombea* group (B_2 subset of data, Table 3) under species criterion 2 resulted in the identification and delimitation of one species with no subspecific subdivision (*H. rhombea* (Miq.) Bean; Table 3, Appendices S4 and S5) based on seven significant original variables with no 75th percentile overlap obtained from the TC data set (percentage of central part; trichome length; trichome width; longest ray length; free longest ray length; shortest ray length; and free shortest ray length), and five from the JP data set (free central lobe length, Fig. 8C; percentage of blade lobulation, Fig. 8D; central lobe length/width ratio, Fig. 8E; central/lateral lobe length, Fig. 8F; and blade length/width ratio), and one from the adult phase (blade length).

Evaluation of the *H. colchica* group under species criterion 2 resulted in the identification and delimitation of one species with no subspecific subdivision (*H. colchica* K.Koch; Table 3, Appendices S4 and S5) based on four significant variables with no 75th percentile overlap obtained from the JP data set. Additional support was obtained from two significant variables with 75th percentile overlap of the AP data set (blade width, Fig. 8G; and blade length/width ratio, Fig. 8H).

Evaluation of the Chinese diploid *H. nepalensis* group and the hexaploid *H. cypria-H. pastuchovii* group (B_{2111} subset of data, Table 3) under species criterion 2 resulted in the identification and delimitation of two species (*H. nepalensis* K.Koch and

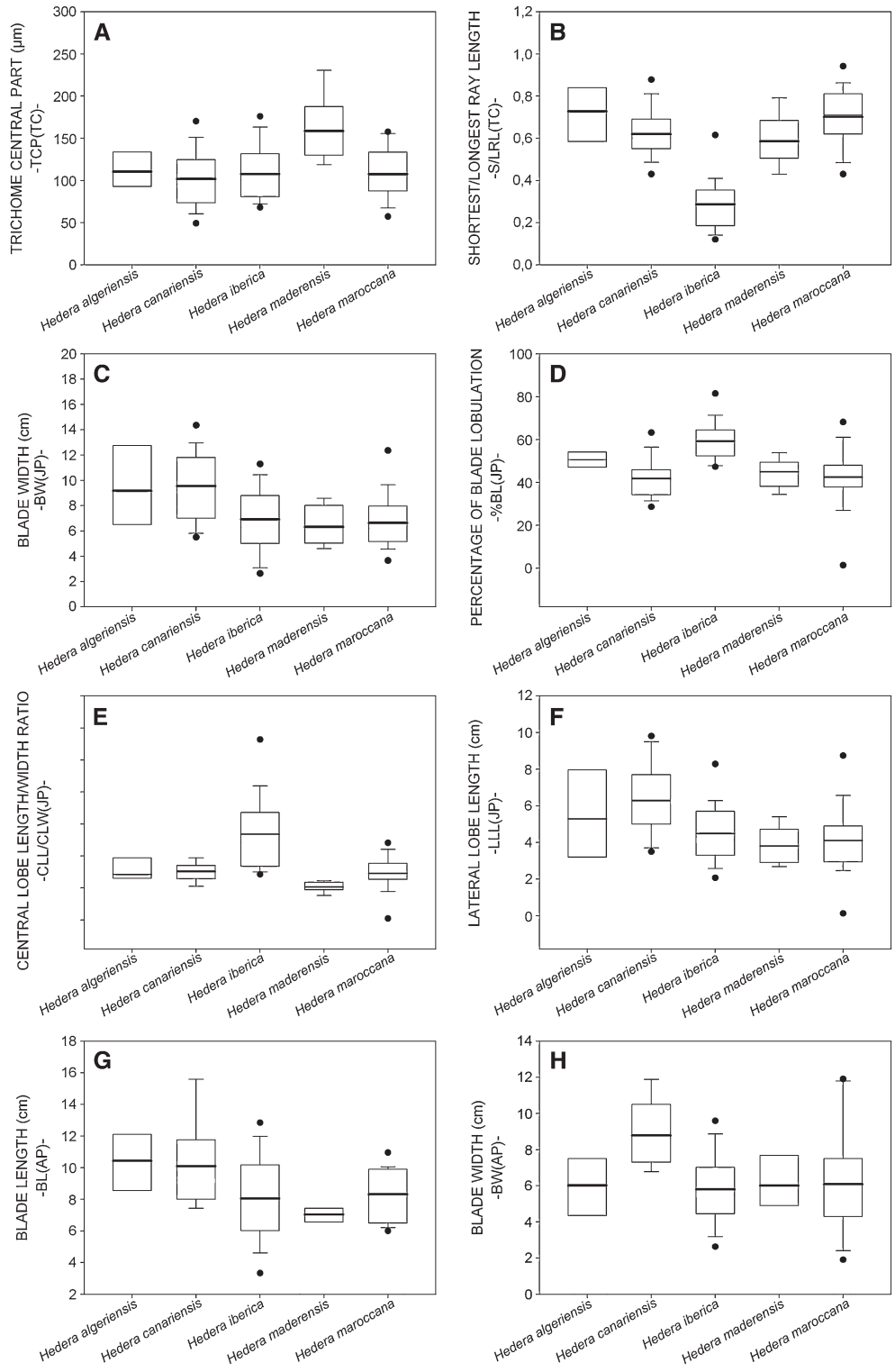


Fig. 7. Box plots of the eight most discriminant characters (two from trichomes, four from leaves of the juvenile phase, and two from the leaves of the adult phase) obtained for the diagnosis of the five species of *Hedera* recognized within the western group of the scale-like trichomes (*H. algeriensis*, *H. canariensis*, *H. iberica*, *H. maderensis*, and *H. maroccana*). (A) Character "trichome central part" measured in trichomes ($N = 109$). (B) Character "shortest/longest ray length" measured in trichomes ($N = 109$). (C) Character "blade width" measured in entire and lobate leaves from the juvenile phase ($N = 118$). (D) Character "percentage of blade lobulation" measured in lobate leaves from the juvenile phase ($N = 91$). (E) Character "central/lateral lobes length ratio" measured in lobate leaves from the juvenile phase ($N = 91$). (F) Character "lateral lobe length" measured in lobate leaves from the juvenile phase ($N = 91$). (G) Character "blade length" measured in leaves from the adult phase ($N = 66$). (H) Character "blade width" measured in leaves from the adult phase ($N = 66$). Boxes represent 75th percentiles, bars indicate 5th and 95th percentiles, and horizontal lines indicate mean values.

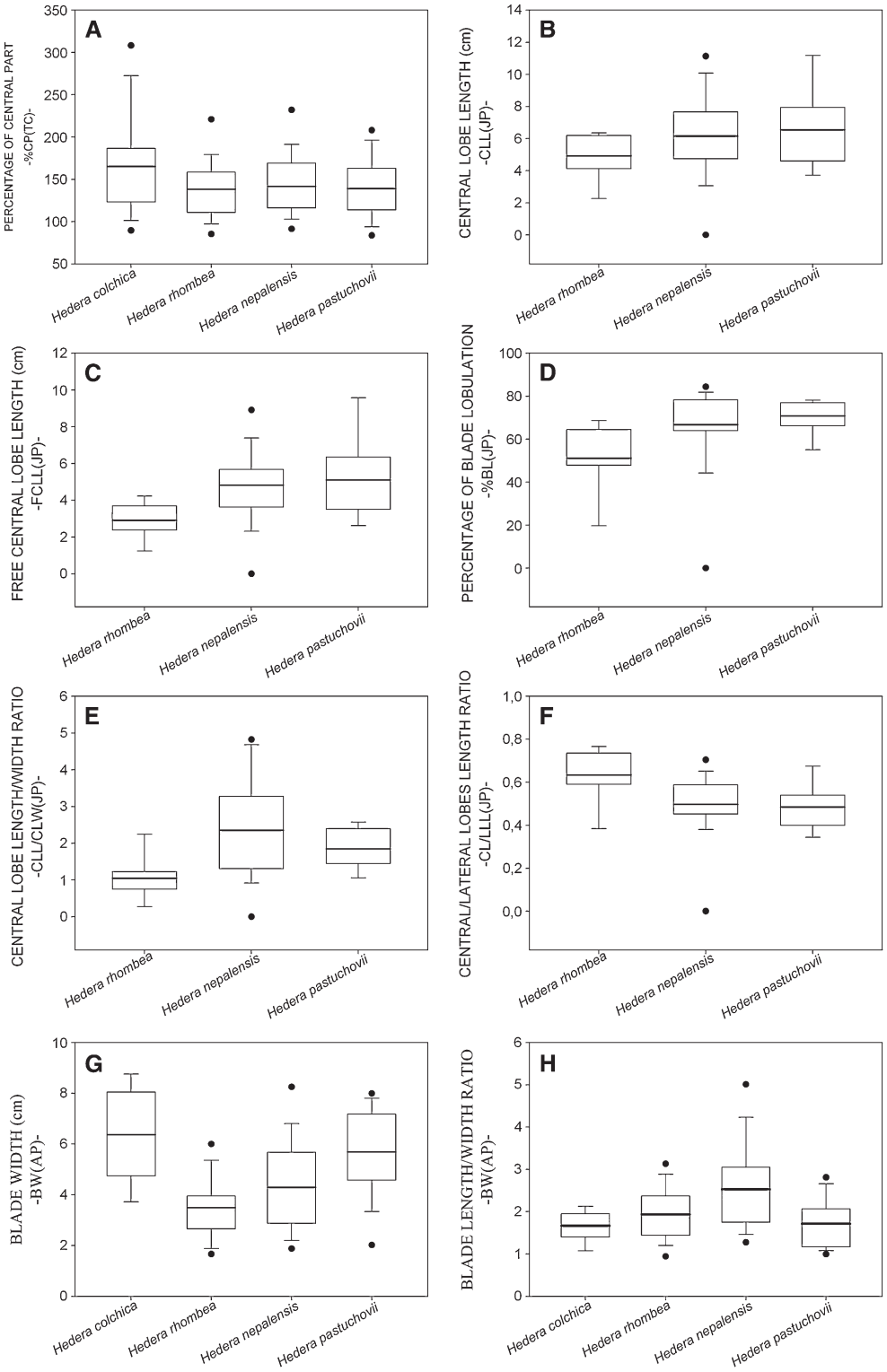


Fig. 8. Box plots of the eight most discriminant characters (one from trichomes, five from leaves of the juvenile phase, and two from the leaves of the adult phase) obtained for the diagnosis of the four species of *Hedera* recognized within the eastern group of the scale-like trichomes (*H. colchica*, *H. nepalensis*, *H. pastuchovii*, and *H. rhombea*). (A) Character "percentage of central part" measured in trichomes ($N = 127$). (B) Character "central lobe length" measured in lobate leaves from the juvenile phase ($N = 61$). (C) Character "free central lobe length" measured in lobate leaves from the juvenile phase ($N = 61$). (D) Character "percentage of blade lobulation" measured in lobate leaves from the juvenile phase ($N = 61$). (E) Character "central lobe length/width ratio" measured in lobate leaves from the juvenile phase ($N = 60$). (F) Character "central/lateral lobes length ratio" measured in lobate leaves from the juvenile phase ($N = 61$). (G) Character "blade width" measured in leaves from the adult phase ($N = 151$). (H) Character "blade length/width ratio" measured in leaves from the adult phase ($N = 151$). Boxes represent 75th percentiles, bars indicate 5th and 95th percentiles, and horizontal lines indicate mean values.

H. pastuchovii Woronow; Table 3, Appendices S4 and S5) based on seven significant variables with 75th percentile overlap obtained from the JP data set (blade length/width ratio; central lobe length, Fig. 8B; free central lobe length, Fig. 8C; percentage of blade lobulation, Fig. 8D; central lobe length/width ratio, Fig. 8E; lateral lobe length and central/lateral lobe length ratio, Fig. 8B), two from the AP data set (blade width, Fig. 8G and blade length/width ratio, Fig. 8H), and one with no 75th percentile overlap obtained from the TC data set (longest ray width).

Evaluation of the tested species *H. pastuchovii* under the subspecies criterion resulted in the identification and delimitation of two subspecies (*H. pastuchovii* Woronow subsp. *pastuchovii*, from the Caucasus and subsp. *cypria* (McAllister) Hank from Cyprus; Table 3, Appendices S4 and S5) based on four significant variables with 75th percentile overlap obtained from the TC data set (longest ray length, free longest ray length, percentage of free longest ray and percentage of central part), and two from the AP data set (blade width, Fig. 8G and blade length/width ratio, Fig. 8H).

Quantification of lineage divergence—The null hypothesis was rejected for three of the four evaluated species (Fig. 9). The amount of genetic ancestry for these three species ranged between 0.4 and 0.7 (*H. helix*, $gsi_T = 0.416$; *H. hibernica*, $gsi_T = 0.518$; *H. pastuchovii*, $gsi_T = 0.704$). The probability that the genealogical ancestry of the samples as inferred from the gene trees (given a species tree) was due to random mating is less than 1% (*H. helix*, $P < 0.001$; *H. hibernica*, $P = 0.003$; *H. pastuchovii*, $P < 0.001$). *Hedera maroccana* was the only tested species for which the null hypothesis was not rejected ($gsi_T = 0.180$, $P = 0.112$), and therefore, from the samples studied, it cannot be accepted as a natural species in terms of ancestor–descendant relationship.

DISCUSSION

Hedera taxonomic implications—The present study provides statistical support for the recognition of 12 species as well as the two major groups traditionally recognized in *Hedera* based on trichomes: the stellate group (three species) and the scale-like group (nine species) (Table 1; online Appendices S3, S4). Additional subdivision of the stellate group into two subgroups is also supported by multivariate quantitative variation of trichomes (multiangulate subgroup, two species; rotate subgroup, one species), in agreement with McAllister and Rutherford (1983). Two subgroups were also detected within the scale-like group on the basis of trichome and leaf characters from the juvenile phase that present geographical congruence; a western group (five species from the western Mediterranean basin) and an eastern group (four species from the eastern Mediterranean basin and Asia). Interestingly, this geographical clustering correlates with the two diversity centers proposed for *Hedera* based on cytogenetic and molecular data (Vargas et al., 1999; Valcárcel et al., 2003a).

The 12 species herein recognized are mainly in agreement with McAllister and Rutherford's proposal, with three exceptions. The first difference is that our results do not provide statistical support for the recognition of *H. helix* subsp. *rhizomatifera*; however, only a small taxon sample was analyzed due to the difficulties in identifying individuals with the features described by the authors (positive geotropism, small leaves and white nerves; McAllister, 1982; McAllister and Rutherford, 1983; Valcárcel et al., 2003b). The second main disagreement with McAllister and Rutherford's proposal concerns *H. maderensis*. McAllister (1988) observed certain morphological differences in leaves from the juvenile phase between the Madeiran and Iberian populations. However, considering the lack of character fixation and the presence of the same ploidy level, these authors recognized both groups of populations as subspecies (*H. maderensis* subsp. *maderensis* and subsp. *iberica*). In contrast, Ackerfield and Wen (2002), based on micro- and macromorphological differences detected by multivariate analyses, considered these two taxa as independent species. Our results confirm the species level as the most appropriate taxonomic rank, because multivariate differences were significant for features not only from the juvenile phase but also from the adult phase and trichomes. The fact that both subspecies share the same ploidy level is interpreted as additional evidence for the ancestor–descendant relationship already indicated by molecular data (Fig. 9; Vargas et al., 1999; Ackerfield and Wen, 2003; Valcárcel et al., 2003a). The third main disagreement with McAllister and Rutherford's proposal concerns the taxonomic status of the Cypriot ivies. McAllister and Rutherford recognized the populations from Cyprus as an independent species (*H. cypria*) morphologically close to the Caucasian *H. pastuchovii*. The results obtained do not provide statistical support for considering them as independent species, but they do support a distinction at the subspecies level (*H. pastuchovii* subsp. *pastuchovii* and subsp. *cypria*), in agreement with Hand (2004). However, disjunction, together with the significant univariate differences in the variances of some characters and the morphological homogeneity displayed by the subspecies *cypria*, might be indicative of an incipient process of island speciation. The subspecies status was supported by the same ploidy level and close phylogenetic relationship (Vargas et al., 1999; Ackerfield and Wen, 2003; Valcárcel et al., 2003a), which resulted in the detection of significant common ancestry even in the absence of monophyly (Fig. 9).

The close relationship between *H. nepalensis* and *H. pastuchovii* has seldom been discussed in the literature. Indeed, most of the controversy about these two taxa has affected the *H. pastuchovii-H. cypria* dilemma and the recognition of two geographical taxa of *H. nepalensis* (see below). However, the present study reveals faint morphological distinction between *H. pastuchovii* and *H. nepalensis*, as once stated by Woronow (1933). The typical spearhead-shaped leaves with long oblong, central lobes and two small rounded, lateral lobes that characterize the juvenile phase in *H. pastuchovii* are completely different from the typical leaves of *H. nepalensis*, which have 3–5 lobes, with triangular central lobes. However, departures from these typical morphotypes are frequent, and a continuum of variation between both taxa is detected near contact zones. The presence of distinctive combinations of morphological characters supports the existence of two different species, which confirms previous cytogenetic (two ploidy levels; Vargas et al., 1999) and molecular results (two distant lineages, Fig. 9; Vargas et al., 1999; Ackerfield and Wen, 2003; Valcárcel et al., 2003a). However, we stress the existence of intermediate forms in the contact area of Pakistan and the need for a larger sample size from both species for the establishment of robust boundary limits. Regarding the morphological variation within *H. nepalensis*, we could not evaluate the species subdivision into two different taxa ('*nepalensis*' and '*sinensis*' varieties or subspecies) because the admixture of the states of diagnostic characters makes unfeasible the circumscription of individuals into one of

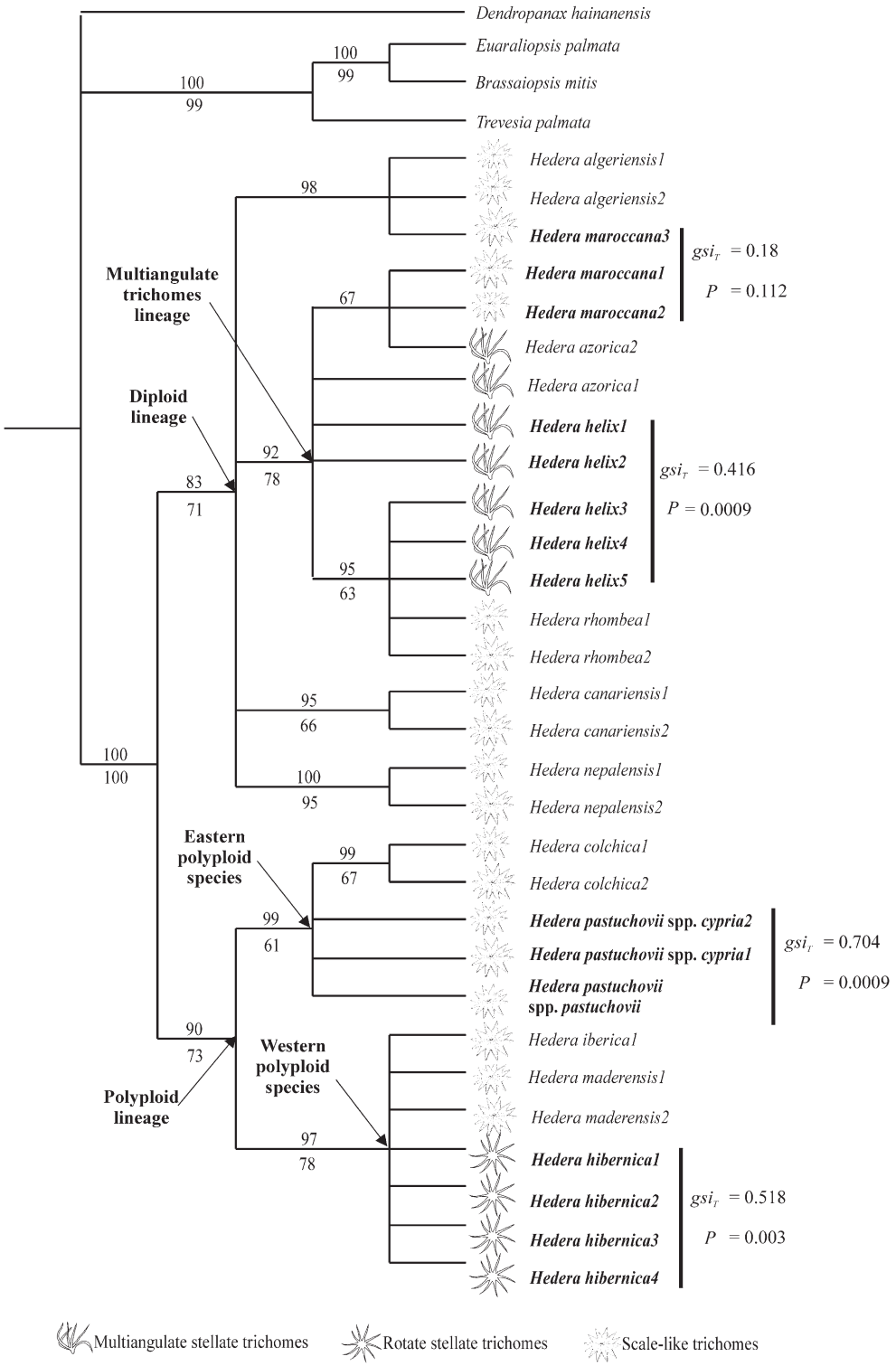


Fig. 9. Mapping of genealogical sorting index (gsi_T) on the majority rule consensus tree computed from the 37 500 postburn-in trees obtained in the Bayesian inference analysis of the 30 ITS samples of *Hedera* plus four other Araliaceae genera, using *Dendropanax* as the outgroup. The gsi_T and the *P* values associated are only indicated for species with three or more samples. The numbers given above and below branches indicate posterior probabilities and bootstrap supports, respectively.

these two taxa. It was not even possible to detect any alternative internal structure of morphological variation or further local differentiation within *H. nepalensis*. However, the large range of the species, together with the great morphological variability observed across it, suggests the need for further studies with a greater sample size.

Providing sample improvement, the distribution of quantitative morphological diversity seems to hold for the hypothesis that places the diversity center of *Hedera* in the Mediterranean basin (Vargas et al., 1999) rather than in Asia (Valcárcel et al., 2003a), which was traditionally thought to be the most likely center of differentiation based on a great number of Araliaceae genera.

Quantitative variation and some clues for Hedera species identification—A continuum of morphological variation underlying the discrete variation of characters traditionally used for the classification of ivies is inferred from the large congruence detected between the species herein recognized and the species considered in McAllister and Rutherford's proposal. Given this congruence, we suggest the use of discrete characters as a first approximation for ivy specimen identification. There are species-dependent discrete and/or qualitative characters that allow species to be distinguished when the specimen is typical. This is the case for the typical forms of *H. nepalensis* that present reddish-orange fruits and for the characteristic thick heart-shaped leaves of *H. colchica* that have a strong celery scent (McAllister, 1982). However, because these diagnostic characters very often fail for nonregular specimens of the above-cited species as well as for the remaining *Hedera* species, we propose the use of quantitative data for accurate identification (Appendices S2 and S4). The coefficients of variation (CVs) herein detected across the three data sets (trichomes, juvenile phase and adult phase; Appendix S3) confirmed the potential taxonomic significance of trichomes, followed by features of leaves from the juvenile phase, then by features of leaves from the adult phase. Therefore, we suggest starting specimen identification by examining trichomes (percentage of the central part, percentage of free longest ray, stalk length, ray number, shortest/longest ray length ratio and ray width). Once the trichome type is characterized, leaf features primarily from the juvenile phase (blade length/width ratio, central lobe length, central lobe width, central lobe length/width ratio, percentage of blade lobulation and central/lateral lobe length ratio) and from the adult phase (blade length, blade width, and blade length/width ratio) should be examined to obtain a precise identification (Figs. 6 to 8).

Quantitative morphology for species delimitation under the general lineage concept: Hedera case—A proper species definition (and consequent delimitations) should account for practical considerations (O'Hara, 1993) without neglecting the underlying evolutionary history. Therefore, and given that any morphological change has a molecular basis, we hold the use of the GLC using morphological divergence as the most functional contingent property for species delimitation. The specific operational criterion for the application of morphological divergence as the main contingent property may be case-dependent to improve the effectiveness of morphology to detect independent evolving lineages. We propose the morphological diagnostic capability as described by Davis and Nixon (1992, p. 427) in their implementation of the PSC of Nixon and Wheeler (1990):, that is, a "…unique combination of character states in comparable individuals…" for cases where unequivocal morphological divergence has been reached. We suggest the relaxed interpretation herein applied in cases of recently diverged groups or when loss of hierarchy due to persistence of gene flow and/or hybridization occurs. Taking advantage of the most important challenge of the GLC, the effectiveness of morphology to identify independently evolving lineages can be later evaluated by testing the species so far identified and delimited with the alternative contingent properties (i.e., reproductive isolation, reciprocal monophyly, etc.; de Queiroz, 2007a).

The complex diversity patterns indicated by morphological variation in *Hedera* appear to reflect its intricate evolutionary and biogeographic history (Valcárcel et al., 2003a). While molecular phylogenies reveal two or three main lineages with great cytogenetic congruence—diploids and polyploids (Vargas et al., 1999; Ackerfield and Wen, 2003; Valcárcel et al., 2003a) or diploids, western polyploids, and eastern polyploids (V. Valcárcel and J. Wen, National Museum of Natural History, Smithsonian Institution, unpublished manuscript)—morphological characters divide the genus into two main groups according to the type of trichomes (stellate vs. scale-like). However, ancestral reconstruction of trichome types in molecular phylogenies has revealed phylogenetic structure in trichome evolution (Valcárcel, 2008; V. Valcárcel and P. Vargas, unpublished manuscript). The effort made in this study to include all the representatives of *Hedera* and to increase sample size has revealed extensive variation and overlap for even the most traditionally accepted diagnostic characters (Appendices S2 and S4). All this morphological complexity is easily understood by means of the evolutionary history that the genus has undergone. Molecular phylogenies retrieved limited species monophyly and complex lineage relationships (Vargas et al., 1999; Valcárcel et al., 2003a; Fig. 9). In fact, considering nuclear and plastid phylogenies together, none of the *Hedera* species are monophyletic groups (Valcárcel, 2008). However, disagreement between gene trees and species trees is very often retrieved because of biological causes (reticulation, lineage sorting, mistaken orthology; Doyle, 1992). In particular, the lack of monophyly within *Hedera* has been attributed to the effects of hybridization and polyploidization (Vargas et al., 1999; Valcárcel et al., 2003a) as well as to the short time inferred for speciation processes (Ackerfield and Wen, 2003). When a short time is estimated for species divergence, the use of monophyly is not a suitable criterion for species identification (Knowles and Carstens, 2007) because lack of monophyly is expected (Rosenberg, 2003). In fact, in our study case, the genealogical sorting index retrieved statistical support for the common ancestry of most of the species analyzed, even in a nonmonophyletic context (e.g., *H. helix*, *H. hibernica*; Fig. 9). The common ancestry revealed by the gsi_T for the species described together with the different ploidy levels provides significant evidence for the naturalness of the species recognized based on quantitative morphology. However, the gsi results must be taken with caution as they are approximate, given that low sample numbers of species have been analyzed for just one DNA region. This is indicative that, despite the apparent disagreement between the phylogenetic lineages detected within ivies (Fig. 9; Valcárcel et al., 2003a; Valcárcel, 2008) and the taxonomic entities herein recognized, morphology seems to be a good indicator of lineages divergence and speciation events in *Hedera* evolution. This fact was also corroborated by a 90% correlation between identification based on morphological characters and the molecular haplotypes of 210 *Hedera* specimens (V. Valcárcel and P. Vargas in Grivet and Petit, 2002).

Methodological open issues—The application of the decision-making procedure herein performed for the delimitation of species using quantitative morphology does not retrieve major taxonomic novelties for the study case (compare our taxonomical

proposal, see Appendix S5, to McAllister and Rutherford's proposal or Ackerfield, 2001; Ackerfield and Wen, 2002; Fig. 1). However, it provides statistical support for the species previously recognized by other authors and provides a rigorous procedure to solve taxonomic status conflicts of particular controversial taxa (see taxonomic incongruence between both treatments). This quantitative decision-making procedure also provides useful practical tools for nontaxonomy-specialized users of species-based systems.

However, some of the steps of the decision-making process raised certain methodological issues that have to be addressed before applying this procedure to other groups. We argue that, providing statistical significance, the 70% of multivariate correct identification is a tolerable and more reliable threshold than assuming 100% for complex groups, where morphological discontinuities are unexpected. In our particular case of study, we detected statistical significance even for groups of specimens with 60% of correct classifications (B_1, B_{11}, SP_6; Table 3). However, the consideration of the Cohen's kappa statistics and the *P*-values gave us support to apply the threshold of 70% (Titus et al., 1984). In any case, we do not know exactly the preferred misidentification threshold that should be assumed. Therefore, we suggest the evaluation of the values of Cohen's kappa statistics and levels of significance (Titus et al., 1984), to determine the minimum cutoff of correct identification for a given genus. The minimum number of significant variables and the overlap threshold required is also a case-dependent issue. As the number of the considered significant variables (diagnostic characters) increases, the type I error may decrease contributing to more robust taxonomic proposals. However, any decrement of the type I error implies a diminishment of the power of the analysis (Hair et al., 2000), which is undesirable for taxonomic purposes. We suggest the combination of both parameters for providing the most accurate threshold, given the genus. For example, for groups displaying evidence for limited gene flow or long time of lineages divergence, we would suggest increasing the thresholds of nonoverlap to the limits of confidence intervals, to avoid possible overestimation of species number, which may allow reducing the number of significant variables required. However, further studies are needed to provide a methodology that allows researchers to evaluate whether a given threshold is statistically supported, given the data set analyzed.

This procedure assumes quantitative variation; however, the PCA conducted in step 1.1 (Fig. 3) could be replaced by an alternative multivariate exploratory analysis that allows qualitative data (i.e., cluster analysis, principal coordinates analysis) to detect possible hidden structure (Henderson and Ferreira, 2002; Henderson, 2004, 2006), when possible.

LITERATURE CITED

ACKERFIELD, J. 2001. Trichome morphology in *Hedera* (Araliaceae). *Edinburgh Journal of Botany* 58: 259–267.

ACKERFIELD, J., AND J. WEN. 2002. A morphometric analysis of *Hedera* L. (the ivy genus, Araliaceae). *Adansonia, series 3* 24: 197–212.

ACKERFIELD, J., AND J. WEN. 2003. Evolution of *Hedera* (the ivy genus, Araliaceae): Insights from chloroplast DNA data. *International Journal of Plant Sciences* 164: 593–602.

AKAIKE, H. 1974. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* 19: 716–723.

AYRES, P. M., AND D. L. THOMAS. 1990. Alternative formulations of the mixed-model ANOVA applied to quantitative genetics. *Evolution* 44: 221–226.

BERNARDOS, S., A. CRESPÍ, F. DEL REY, AND F. AMICH. 2005. The section *Pseudophrys* (*Ophrys*, Orchidaceae) in the Iberian Peninsula: A morphometric and molecular analysis. *Botanical Journal of the Linnean Society* 148: 359–375.

BROWER, A. V. Z. 1999. Delimitation of phylogenetic species with DNA sequences: A critique of Davis and Nixon's population aggregation analysis. *Systematic Biology* 48: 199–213.

CUMMINGS, M. P., C. N. MAILE, AND K. L. SHAW. 2008. A genealogical approach to quantifying lineage divergence. *Evolution* 62: 2411–2422.

DAVIS, J. I., AND K. C. NIXON. 1992. Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology* 41: 421–435.

DAY, R. W., AND G. P. QUINN. 1989. Comparisons of treatments after an analysis of variance in ecology. *Ecological Monographs* 59: 133–163.

DE QUEIROZ, K. 1998. The general lineage concept of species, species criteria, and the process of speciation: A conceptual unification and terminological recommendations. *In* D. J. Howard and S. H. Berlocher [eds.], Endless forms: Species and speciation, 57–75. Oxford University Press, Oxford, UK.

DE QUEIROZ, K. 2007a. Species concepts and species delimitation. *Systematic Biology* 56: 879–886.

DE QUEIROZ, K. 2007b. Towards an integrated system of clade nomenclature. *Systematic Biology* 56: 956–974.

DE SALLE, R., M. G. EGAN, AND M. SIDDALL. 2005. The unholy trinity: Taxonomy, species delimitation and DNA barcoding. *Philosophical Transactions of the Royal Society, B, Biological Sciences* 360: 1905–1916.

DOYLE, J. J. 1992. Gene trees and species trees: Molecular systematics as one-character taxonomy. *Systematic Botany* 17: 144–163.

DOYLE, J. J. 1995. The irrelevance of allele tree topologies for species delimitation, and a non-topological alternative. *Systematic Botany* 20: 574–588.

EYDE, R. H., AND C. C. TSENG. 1971. What is the primitive floral structure of Araliaceae? *Journal of the Arnold Arboretum* 52: 205–239.

GAVRILETS, S. 2003. Perspective: Models of speciation—What have we learned in 40 years? *Evolution* 57: 2197–2215.

GOLOBOFF, G., J. FARRIS, AND K. NIXON. 2003. TNT: Tree analysis using new technology. Program and documentation available from the authors at http://www.zmuc.dk/public/phylogeny.

GRIVET, D., AND R. J. PETIT. 2002. Phylogeography of the common ivy (*Hedera* sp.) in Europe: Genetic differentiation through space and time. *Molecular Ecology* 11: 1351–1362.

HAND, R. 2004. Supplementary notes to the flora of Cyprus IV. *Willdenowia* 34: 427–437.

HAIR, J. F., R. E. ANDERSON, R. L. TATHAM, AND W. C. BLACK. 2000. Multivariate analysis. Prentice Hall International, Upper Saddle River, New Jersey, USA.

HENDERSON, A. 2004. A multivariate analysis of *Hysopathe* (Palmae). *American Journal of Botany* 91: 953–965.

HENDERSON, A. 2005a. The methods of herbarium taxonomy. *Systematic Botany* 30: 456–459.

HENDERSON, A. 2005b. A multivariate study of *Calyptrogyne* (Palmae). *Systematic Botany* 30: 60–83.

HENDERSON, A. 2006. Traditional morphometrics in plant systematics and its role in palm systematics. *Botanical Journal of the Linnean Society* 151: 103–111.

HENDERSON, A., AND E. FERREIRA. 2002. A morphometric study of *Synechanthus* (Palmae). *Systematic Botany* 27: 693–702.

HIBBERD, S. 1872. The ivy, a monograph. Groombridge, London, UK.

HUELSENBECK, J. P., AND F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.

LAWRENCE, G. H., AND A. E. SCHULZE. 1942. The cultivated hederas. *Gentes Herbarum* 6: 107–173.

KNOWLES, L. L., AND B. C. CARSTENS. 2007. Delimiting species without monophyletic gene trees. *Systematic Biology* 56: 887–895.

LUCKOW, M. 1995. Species concepts: Assumptions, methods, and applications. *Systematic Botany* 20: 589–605.

LUM, C., AND J. MAZE. 1989. A multivariate analysis of the trichomes of *Hedera* L. *Watsonia* 17: 409–418.

MANLY, B. F. J. 1994. Multivariate statistical methods: A primer. Chapman Hall, London, UK.

MCALLISTER, H. A. 1982. New work on ivies. *International Dendrological Society* 1981: 106–109.

MCALLISTER, H. A. 1988. Canary and Algerian ivies. *Plantsman* 10: 27–29.

MCALLISTER, H. A., AND A. RUTHERFORD. 1983. The species of ivies. *Ivy Journal* 9: 45–54.

MCALLISTER, H. A., AND A. RUTHERFORD. 1990. *Hedera helix* L. and *H. hibernica* (Kirchner) Bean (Araliaceae) in the British Isles. *Watsonia* 18: 7–15.

MCDADE, L. A. 1995. Species concepts and problems in practice: Insight from botanical monographs. *Systematic Botany* 20: 606–622.

NIXON, K. C., AND Q. D. WHEELER. 1990. An amplification of the phylogenetic species concept. *Cladistics* 6: 211–223.

O'HARA, R. J. 1993. Systematic generalization, historical fate, and the species problem. *Systematic Biology* 42: 231–246.

PARADIS, E., J. CLAUDE, AND K. STRIMMER. 2004. APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* 20: 289–290.

POJARKOVA, A. I. 1951. The Chinese species of ivy and their taxonomic and geographic connections. *Natulae Systematicae ex Herbario Botanici Nomine V.L. Komarovii Academiae Scientiarum URSS* XIV: 224–264.

POSADA, D. 2008. jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* 25: 1253–1256.

QUINN, G. P., AND M. J. KEOUGH. 2002. Experimental design and data análisis for biologists. Cambridge University Press, Cambridge, UK.

RIM, K. H. 1994. Fossils of North Korea. Science and Technology Press, Pyongyang, Korea.

ROSE, P. Q. 1996. The gardener's guide to growing ivies. David and Charles, London, UK.

ROSENBERG, N. A. 2003. The shapes of neutral gene genealogies in two species: Probabilities of monophyly, paraphyly, and polyphyly in a coalescent model. *Evolution* 57: 1465–1477.

RUTHERFORD, A. 1984. The history of the Canary Islands ivy and its relatives. *Ivy Journal* 10: 13–18.

RUTHERFORD, A. 1989. The ivies of Andalusia (southern Spain). *Ivy Journal* 15: 7–17.

RUTHERFORD, A., H. A. MCALLISTER, AND R. R. MILL. 1993. New ivies from the Mediterranean area and Macaronesia. *Plantsman* 15: 115–128.

SCHÜRCH, S., M. PFUNDER, AND B. A. ROY. 2000. Effects of ants on the reproductive success of *Euphorbia cyparissias* and associated pathogenic rust fungi. *Oikos* 88: 6–12.

SEEMAN, B. 1868. Revision of the natural order *Hederaceae*. L. Reeve and Co., London, UK.

SITES, J. W. JR., AND K. A. CRANDALL. 1997. Testing species boundaries in biodiversity studies. *Conservation Biology* 11: 1289–1297.

SITES, J. W. JR., AND J. C. MARSHALL. 2003. Delimiting species: A Renaissance issue in systematic biology. *Trends in Ecology & Evolution* 18: 462–470.

SITES, J. W. JR., AND J. C. MARSHALL. 2004. Operational criteria for delimiting species. *Annual Review of Ecology Evolution and Systematics* 35: 199–227.

STEVENS, P. F. 1991. Character states, morphological variation, and phylogenetic analysis: A review. *Systematic Botany* 16: 553–583.

STEVENS, P. F. 2000. Botanical systematics 1950–2000: Change, progress, or both? *Taxon* 49: 635–659.

SUN, F. J., G. A. LEVIN, AND S. R. DOWNIE. 2006. A multivariate analysis of *Pseudocymopterus* (Apiaceae). *Journal of the Torrey Botanical Society* 133: 499–512.

TABACHNICK, B. G., AND L. S. FIDELL. 2007. Using multivariate statistics, 5th ed. Allyn and Bacon, Boston, Massachusetts, USA.

TAUTZ, D., P. ARCTANDER, A. MINELLI, R. H. THOMAS, AND A. P. VOGLER. 2003. A plea for DNA taxonomy. *Trends in Ecology & Evolution* 18: 70–74.

TEMPLETON, A. R. 2001. Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology* 10: 779–791.

THIELE, K. 1993. The holy grail of the perfect character: The cladistic treatment of morphometric data. *Cladistics* 9: 275–304.

TITUS, K., J. A. MOSHER, AND B. K. WILLIAMS. 1984. Chance-corrected classification for use in discriminant analysis: Ecological applications. *American Midland Naturalist* 111: 1–7.

VALCÁRCEL, V. 2008. Taxonomy, sistematics and evolution of *Hedera* L. (Araliaceae). Ph.D. dissertation, Universidad Pablo de Olavide, Sevilla, Spain [in English but two sections, introduction and conclusions, in Spanish].

VALCÁRCEL, V., O. FIZ, AND P. VARGAS. 2003a. Chloroplast and nuclear evidence for multiple origins of polyploids and diploids of *Hedera* (Araliaceae) in the Mediterranean basin. *Molecular Phylogenetics and Evolution* 27: 1–20.

VALCÁRCEL, V., A. RUTHERFORD, R. MILLER, AND H. A. MCALLISTER. 2003b. *Hedera* L. *In* G. Nieto Feliner [ed.], vol. X, 1–7. Departamento de publicaciones del CSIC, Madrid, Spain.

VARGAS, P., H. A. MCALLISTER, C. MORTON, S. L. JURY, AND M. J. WILKINSON. 1999. Polyploid speciation in *Hedera* (Araliaceae): Phylogenetic and biogeographic insights based on chromosome counts and ITS sequences. *Plant Systematics and Evolution* 219: 165–179.

WEN, J., G. M. PLUNKETT, A. D. MITCHELL, AND S. J. WAGSTAFF. 2001. The evolution of Araliaceae: A phylogenetic analysis based on ITS sequences of nuclear ribosomal DNA. *Systematic Botany* 26: 144–167.

WIENS, J. J. 1999. Polymorphism in systematics and comparative biology. *Annual Review of Ecology and Systematics* 30: 327–362.

WIENS, J. J. 2007. Species delimitation: New approaches for discovering diversity. *Systematic Biology* 56: 875–878.

WIENS, J. J., AND T. A. PENKROT. 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). *Systematic Biology* 51: 69–91.

WIENS, J. J., AND M. R. SERVEDIO. 2000. Species delimitation in systematics: Inferring diagnostic differences between species. *Proceedings of Royal Society of London, B, Biological Sciences* 267: 631–636.

WORONOW, G. 1933. Flora et systematica plantae vasculares. *Acta Instituti Botanici Academiae Scientiarum Unionis Rerum Publicarum Soveticarum Socialisticarum* 1: 216–217.