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A New Species of *Haplophyllum* A. Juss. (Rutaceae) from the Iberian Peninsula: Evidence from Morphological, Karyological and Molecular Analyses

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• *Background and Aims* The discovery of a new species, *Haplophyllum bastetanum* F.B. Navarro, V.N. Suárez-Santiago & Blanca **sp. nov.**, in the south-east of Spain has prompted the comparative study of species of the Iberian Peninsula, and others related, through morphological, cytogenetic, molecular, distributional and ecological characterization.
• *Methods* The morphological study involved a quantitative analysis of the species present in the Iberian Peninsula and a comparative analysis of the morphological characteristics between *H. bastetanum* and other related species. Mitotic analyses were made with root meristems taken from germinating seeds. Phylogenetic analyses of the internal transcribed spacer sequences of nuclear ribosomal DNA were performed using neighbour-joining (NJ) and maximum-parsimony methods.
• *Key Results Haplophyllum bastetanum* is a diploid species ($2n = 18$) distinguished primarily for its non-trifoliate glabrous leaves, lanceolate sepals, dark-green petals with a dorsal band of hairs, and a highly hairy ovary with round-apex locules. The other two Iberian species (*H. linifolium* and *H. rosmarinifolium*) are tetraploid ($2n = 36$) and have yellow petals. Both phylogenetic methods generated a well-supported clade grouping *H. linifolium* with *H. rosmarinifolium*. In the NJ tree, the *H. linifolium–H. rosmarinifolium* clade is a sister group to *H. bastetanum*, while in the parsimony analysis this occurred only when the gaps were coded as a fifth base and the characters were reweighted according to the rescaled consistency index. This latter group is supported by the sequence divergence among taxa.
• *Conclusions* The phylogenies established from DNA sequences together with morphological and cytogenetic analyses support the separation of *H. bastetanum* as a new species. The results suggest that the change in the number of chromosomes may be the key mechanism of speciation of the genus *Haplophyllum* in the Iberian Peninsula. An evolutionary scheme for them is propounded.

Key words: Rutaceae, *Haplophyllum*, taxonomy, morphology, cytogenetic analysis, ITS sequences, phylogenetic analysis, Iberian Peninsula.

INTRODUCTION

The family Rutaceae contains approx. 150 genera with some 900 species distributed throughout temperate and tropical regions, particularly in southern Africa and in Australia. Many of these species are of great economic interest, whether for food (*Citrus* sp.), medicine (oils from *Galipea*, *Toddalia*, *Ruta*, *Haplophyllum*, etc.), aesthetics (aromatic value of bergamot oil), wood, gardening, etc. Six subfamilies can be distinguished: Zanthoxyleae, Cusparieae, Toddaloideae, Rhabdodendroideae, Aurantioideae and Rutoideae. The last group, differentiated from the rest by having an ovary with two to five carpels separated in the apical part and connected only by styles (Heywood, 1978), includes the genera *Ruta* L., *Haplophyllum* A. Juss., *Dictamnus* L. and *Thamnosma* Torr. & Frém.

According to Townsend (1986), the genus *Haplophyllum* includes 66 species distributed throughout temperate and subtropical zones of Eurasia and the northern tropical zone of eastern Africa (Somalia). Many studies have examined the phytochemistry and medicinal attributes of some of these species, analysing their contents for alkaloids, lignanes, glycosides and flavonoids, etc. (Pascual-Villalobos and Robledo, 1999; Shaiq *et al.*, 2001; Nazrullaev *et al.*, 2002; Prieto *et al.*, 2002; Schinella *et al.*, 2002). However, few systematic and taxonomical studies have been completed and practically none from a molecular standpoint. Several authors have proposed different subdivisions of the genus into sections, as in the case of Spach (1849), Engler (1896), Vvdensky (1949) or Townsend (1966). In the latest review of the genus available, Townsend (1986) proposed three sections: sect. *Peganoides* (generally 3-locular ovary, dehiscent fruit); sect. *Indehiscentes* (5-locular ovary, indehiscent fruit); and sect. *Haplophyllum* (5-locular ovary and dehiscent fruit). This third section contains most of the species of the genus, including those on the Iberian Peninsula, which are the object of the present study.

Townsend (1968) recognized only seven species of the genus in *Flora Europaea*, of which only one species inhabited the Iberian Peninsula, *H. linifolium* (L.) G. Don. Later, Bolòs and Vigo (1974) estimated that sufficient differences existed to consider *H. rosmarinifolium* (Pers.) G. Don (=*Ruta rosmarinifolia* A. Juss. ex Pers.) as a subspecies [*H. linifolium* subsp. *rosmarinifolium* (Pers.) O. Bolòs & Vigo]. Townsend (1975) conferred only the status of variety to this latter taxon, although his most recent review (Townsend, 1986) accepted the proposal of Bolòs and Vigo (1974). In his later study Townsend (1986) proposed *H. linifolium* subsp. *africanum* C. Towns. as a new combination for North Africa. However, this subspecies was

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excluded from this study because enough evidence exists to consider it as a different species (V. N. Suárez-Santiago, F. B. Navarro and G. Blanca, unpubl. res.).

Following Don (1831–1834), we have considered *H. linifolium* and *H. rosmarinifolium* as different species based on our experience obtained during this study and following the criterion of the Rutaceae specialist (R. Morales, Real Jardín Botánico de Madrid, pers. comm.) from *Flora Iberica* (Vol. IX, unpubl. res.).

The discovery of a new species, *H. bastetanum*, in the Guadix-Baza Basin (south-east Spain) has prompted the present comparative study of species of the Iberian Peninsula, and others related, through morphological, cytogenetic, molecular, distributional and ecological characterization.

MATERIALS AND METHODS

Plant material

The plant material used in this study includes the three Spanish *Haplophyllum* species (*H. linifolium*, *H. rosmarinifolium* and *H. bastetanum*). Also, we selected a closely related species to our ingroup, *H. suaveolens* (DC.) G. Don, another one more distant, *H. coronatum* Griseb. and, finally, *H. blanchei* Boiss., which is closely related to the type species of the genus [*H. tuberculatum* (Forssk.) A. Juss.], according to the possible relationships amongst the species in the genus *Haplophyllum* proposed by Townsend (1986). *Ruta montana* (L.) L. was used as an outgroup in the molecular analyses.

Macromorphology

The morphological study involved a comparative analysis of the morphological characteristics of *H. bastetanum* and the other species, taking into account the monograph by Townsend (1986). In addition, a quantitative analysis was made of the species present in the Iberian Peninsula, using herbarium specimens (Appendix 1), except for measuring seed length and width, for which material was gathered from wild populations. The characters with the greatest variation among the different taxa were used, e.g. leaf width and length; leaf area; and number of glands on the upper leaf surface; length and width of the sepals, petals, and seeds; and the number of capsules per inflorescence. The number of foliar glands were calculated with a 4-mm^2 grid and leaf area was estimated by the WinFOLIA program, version 2002a. The terminology used basically follows Font Quer (1953), Stearn (1980) and Mabberley (1990).

For each of the variables studied, a one-way ANOVA was used for the statistical analysis. In some cases, a logarithmic transformation was made of the data (ln) to reduce heterocedasticity, which was confirmed by the Bartlett test. The Tukey multiple-comparisons test was used to test the significance of mean pairs. For the length and width of the leaves, the non-parametric Kruskal-Wallis test was used, as the data did not satisfy the principles of the variance analysis. In this case, the multiple-comparison test used was the T2-Tamhane, which does not assume equal variances. The statistical program used was STATGRAPHICS plus version 4.0. and SPSS version 11.0.

The seeds were photographed using a variable-pressure LEO 1430 VP scanning electronic microscope (SEM) in conventional mode, after gold–palladium coating (Thornill *et al.*, 1965).

TABLE 1. *Origin of the material studied in cytogenetic analysis*

Species	Voucher	Origin
H. linifolium	GDA 47315	Jaén, Spain
H. linifolium	GDA 47314	Jaén, Spain
H. rosmarinifolium	GDA 47316	Almería, Spain
H. rosmarinifolium	GDA 47317	Granada, Spain
H. bastetanum	GDA 47502	Baza Basin, Spain
H. bastetanum	GDA 47497	Baza Basin, Spain

TABLE 2. *Origin of the material studied in molecular analysis and GenBank accession numbers*

Species	Voucher	Origin	GenBank accession no.
H. linifolium	GDA 47315	Jaén, Spain	AY484572
H. rosmarinifolium	GDA 47316	Almería, Spain	AY484574
H. bastetanum	GDA 47318	Baza Basin, Spain	AY484576
H. coronatum	MA 353234	Macedonia, Greece	AY484573
H. suaveolens	MA 181007	M. Stara, Bulgaria	AY484575
H. blanchei	MA 418591	Rutba, Iraq	AY484571
Ruta montana	GDA 43908	Granada, Spain	AY484577

Cytogenetic analysis

Mitotic analyses were made with root meristems taken from germinating seeds. These roots, pre-treated with 8-hydroxy-quinoleine and then fixed in ethyl alcohol–acetic acid (3 : 1), were later hydrolysed in 1 N HCl, stained in an acetic orcein solution, and then flattened for light microscopy (Darlington and La Cour, 1969). The seeds were taken from plants included in the Herbario de la Universidad de Granada (GDA) herbarium. The vouchers are shown in Table 1.

DNA extraction, polymerase chain reaction (PCR) and DNA sequencing

Total genomic DNA was extracted, following the CTAB method (Doyle and Doyle, 1987), from fresh leaves collected in the field for Spanish species (*Haploplyllum* and *Ruta*) or from herbarium material for *H. suaveolens*, *H. blanchei* and *H. coronatum* (Table 2).

The entire internal transcribed spacer (ITS) region (ITS1, 5.8S and ITS2) was amplified by PCR, using the primers N-nc18s10 and C26A (Wen and Zimmer, 1996). The PCR reactions were performed in a volume of 50 μL of mix containing 10 mM 10× PCR buffer, 2 mM $MgCl_2$, 200 μM of a mix of each dNTP, 0·5 μM of each primer, 2·5 μL of DMSO (dimethyl sulfoxide), 2·5 units of Taq DNA polymerase (Amersham Biosciences) and 50 ng of DNA. The

PCR conditions were five cycles of 94 °C for 1·5 min, 55 °C for 1 min and 72 °C for 1 min, each cycle decreasing 1 °C for the annealing temperature. Afterwards, 35 cycles were run: 1·5 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. After these 40 cycles, there was a 10-min extension period at 72 °C. For DNA from the herbarium material, the PCR conditions were changed, decreasing the annealing temperature from 55 °C to 50 °C in the first five cycles and from 50 °C to 48 °C in the following 35 cycles, while the DNA quantity was increased to 100–150 ng. Only single bands were detected from the PCR products on the agarose gels. These bands were excised from the gels and the DNA extracted using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The bands from the DNA of herbarium material (*H. coronatum*, *H. blanchei* and *H. suaveolens*) were very pale; therefore these PCR products were ligated into the pGEM-Teasy Vector System (Promega) and cloned in JM109 cells following the manufacturer's recommendations (Promega).

Automated sequencing of the purified double-stranded PCR products and plasmid clones was performed in both directions. The PCR products were sequenced using the primers N-nc18s10 and C26A (Wen and Zimmer, 1996), while the plasmid clones were sequenced with the generic primers T7 and SP6. Thermal-cycling reactions were performed using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing was then carried out on a 3700 Capillary DNA Sequencer. Nucleotide sequences were edited with the SEQMAN II v. 3.61 program of DNAstar software package (LASERGEN). BLAST searches confirmed that our products belonged to the Rutaceae family, based on their strong identity with other members of this family.

Sequence analysis

The multiple ITS sequences from all taxa were aligned using the CLUSTAL option of the MEGALIN program of DNAstar software package (LASERGEN), and after a visual inspection was done. Boundaries of the coding and spacer sequences were determined by comparison with published ITS sequences from Rutaceae and other Sapindales species (Becerra and Venable, 1999; Scott *et al.*, 2000). Positions with ambiguous alignments were treated following the method of Lutzoni *et al.* (2000), where first the homologous regions of the alignment containing ambiguously aligned sequences were delimited. Secondly, each of these regions was coded as a new character, replacing its respective ambiguous region. Thirdly, each of the coded characters was subjected to a specific step matrix to account for the differential number of changes needed to transform one sequence into another. The sequences reported in the present study have been deposited in GenBank (Table 2).

The G + C content and the sequence divergence among taxa were determined using MEGA version 2.1 (Kumar *et al.*, 2001); the latter was calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980) and the gaps were coded as missing data with the pairwise-deletion option.

Phylogenetic analysis

Phylogenetic analyses were performed using maximum-parsimony (MP) and neighbour-joining (NJ) methods; the latter method used MEGA version 2.1 (Kumar *et al.*, 2001) based on the K2P model (Kimura, 1980). Gaps were coded as missing data with the pairwise-deletion option, and, for testing the robustness of the clades, a bootstrap analysis (BS) (Felsenstein, 1985) was performed with 1000 replicates. The parsimony analysis involved branch-and-bound searches conducted with PAUP version 4.0b10 (Swofford, 2003) using the furthest-addition-sequence option. Character states were specified as unordered, except for the characters that replaced the ambiguous regions specified for their step matrices. Different rounds of weighting and searching were conducted. In the first round, all characters were specified as unweighted and, in the rest of the rounds, the characters were reweighted according to the rescaled consistency index (RC) (Farris, 1969, 1989) until reaching identical weights, lengths and topologies in two successive rounds. Indels were coded also as missing data as a fifth base. All optimal trees were saved. Bootstrap analysis was performed using 1000 replicates to estimate the support of the ITS data. Descriptive statistics reflecting the amount of phylogenetic signal in the parsimony analysis were given by the consistency index (CI) (Kluge and Farris, 1969), retention index (RI) (Swofford, 1993) and homoplasy index (HI). Additionally, the g1 statistic (Hillis and Huelsenbeck, 1992) was determined by calculating the tree-length distribution of 10 000 random trees, using RANDOM TREES under PAUP to assess the amount of phylogenetic signal in the dataset, in comparison to random noise.

Ecological characteristics

In this section, data are provided for distribution, bioclimatology, biogeography, and vegetation series in which *H. bastetanum* appears, following the terminology proposed by Rivas-Martínez *et al.* (1997), Rivas-Martínez and Loidi (1999) and Valle (2003). The threat of extinction was evaluated using the categories of the UICN (2001). The ecological distribution and behaviour of *H. linifolium* and *H. rosmarinifolium* were established by consulting the herbaria GDA, GDAC, MA, as well as the database of the project ANTHOS (Information system on plants in Spain; www.programanthos.com).

RESULTS

Macromorphology

The morphological characteristics used to differentiate *H. bastetanum* from the rest of the Iberian species and from the other related ones appear in Table 3. *Haploplyllum bastetanum* differs from the rest fundamentally in having dark-green petals and stamen filaments, fruit lobes without apical appendages, and seeds with predominantly longitudinal dorsal ridges. Furthermore, individually, this species differs from: *H. blanchei* in having soft hairs on the dorsal side of the petals, glabrous leaves, and densely

TABLE 3. *Principal features of the new Haplophyllum species and related species*

Traits	*H. linifolium*	*H. rosmarinifolium*	*H. bastetanum*	*H. coronatum*	*H. suaveolens*	*H. blanchei*
Branches and stems hair-covering	Hairy	Glabrous to glabrescent	Hairy	Hairy	Hairy	Glabrous to sublanate
Leaves						
Shape	Elliptical	Linear to narrowly spathulate	Oblanceolate to elliptical	Oblanceolate	Lanceolate to oblanceolate	Lanceolate-obovate to elliptical
Hair-covering	Hairy	Glabrous	Glabrous	Hairy	Hairy	Hairy
Trisect leaves	Yes	No	No	Yes	No	No
Sepals						
Shape	Ovate-lanceolate	Ovate-lanceolate	Lanceolate	Lanceolate	Lanceolate	Triangular
Hair-covering	Glabrescent to Hairy	Glabrous to Hairy	Hairy	Hairy	Hairy	Glabrous to ciliated
Arrangement	Overlapping	Overlapping	Not overlapping	Not overlapping	Not overlapping	Not Overlapping
Petals						
Colour	Yellow	Yellow	Dark green	Yellow	Yellow	Deep magenta
Hair-covering	Glabrous	Glabrous	Hairy	Hairy	Hairy	Glabrous
Filaments						
Colour	Yellow	Yellow	Dark green	Yellow	Yellow	Magenta
Monadelphy	No	No	No	No	No	Yes
Fruit						
Apex of lobes	Appendiculate	Appendiculate	Exappendiculate	Appendiculate	Appendiculate	Exappendiculate
Hair-covering	Hairy at the apex	Glabrous	Hairy	Glabrous or hairy	Glabrous	Glabrous
Ovules/loculi	4	4	4	4	4	2
Seed ridges	Transverse	Transverse	Longitudinal	Transverse	Tranverse	Transverse
Chromosome number ($2n$)	36	36	18	–	–	–

hairy ovary and capsule; *H. suaveolens* also in the hair-covering of the ovary and capsule, and in the glabrous leaves; *H. coronatum* in having glabrous and non-trifoliate leaves; *H. rosmarinifolium* in the hair-covering of the ovary and capsule, lanceolate sepals, a band of hairs on the petals and non-linear leaves; and *H. linifolium* fundamentally in the glabrous leaves, the absence of trifoliate leaves, free sepals, a band of hairs on the petals, and uniformly hairy ovary and capsule. Some of these differences can be appreciated in Figs 1 and 2. For the species present in the Iberian Peninsula, the results of the biometric analysis are listed in Table 4. Statistically significant differences were found (significance level of 99·9 %), except for leaf length; nevertheless, neither the leaf width nor area differed significantly in *H. linifolium* or *H. bastetanum*, and the same was true for sepal width and petal length in *H. rosmarinifolium* and *H. bastetanum*.

Chromosome count

Haploplyllum linifolium and *H. rosmarinifolium* are both tetraploid species ($2n = 36$), while *H. bastetanum* is diploid ($2n = 18$). These chromosome numbers have not been reported previously.

Sequence analysis

The characteristics of the ITS sequences are summarized in Table 5. Sequence alignments of the seven species analysed resulted in a 656-bp-long data matrix and required the insertion of 25 gaps (69 positions) of 1–9 bp in length, 19 of the gaps being due to the size difference between *Haplophyllum* species and *R. montana* (Table 5). For the parsimony analysis, 13 ambiguous regions were delimited and excluded from phylogenetic analysis (86 positions), but these were replaced by their respective coded characters, which were added to the end of the alignment. Therefore, 583 characters were used in the parsimony analysis. A step matrix was applied to each new coded character for reflecting the nature of the changes from one sequence to another within each ambiguous region.

For the entire ITS region, the pairwise sequence divergence varied from 0·3 % to 7·8 % (average 3·4 %) between *Haplophyllum* species, where the lowest divergences were between Spanish species. Including *Ruta montana*, the extreme divergence reached 18·8 % (*R. montana vs. H. suaveolens*) and the mean divergence was 7·4 % (Table 6A). The mean divergence among Spanish species and the other *Haplophyllum* species was 3·7 % (Table 6B).

Phylogenetic analysis

The original NJ tree using the K2P model (Fig. 3A) shows a strongly supported clade grouping Spanish *Haplophyllum* species together (BS = 94 %). Within this clade, *H. linifolium* and *H. rosmarinifolium* are placed together (BS = 95 %) and both are sister groups of *H. bastetanum*. In the original tree the rest of the groupings have low bootstrap values, so that the clade formed by *H. coronatum* and *H. blanchei* and the clade that groups these latter species with the Spanish species fails to appear in the consensus tree (Fig. 3B).

In the parsimony analysis of aligned ITS sequences, the number of variable and informative characters, and the g1 statistic value varied depending on the treatment of ambiguous region and gaps (Table 7A). When the characters were

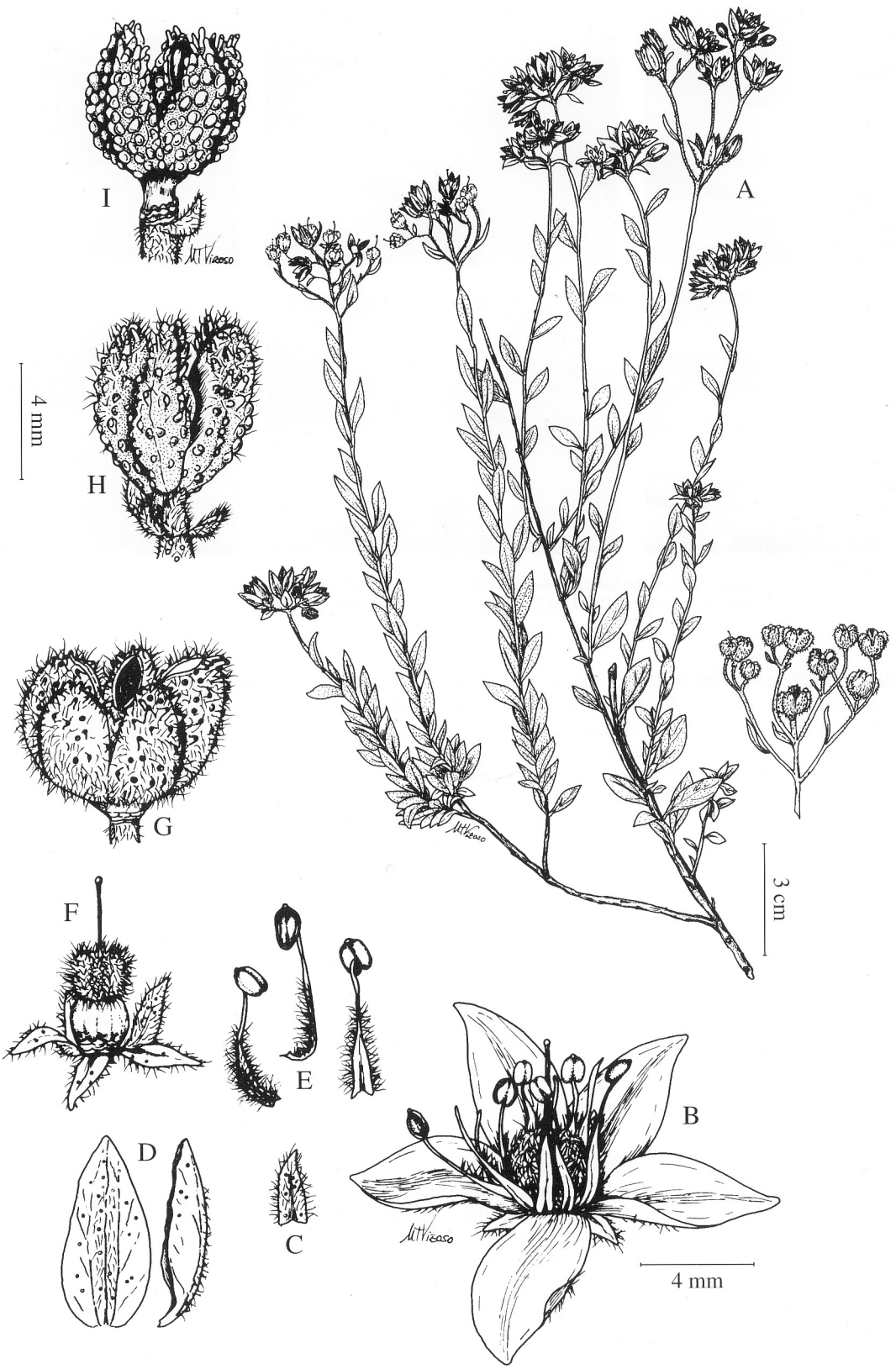

FIG. 1. *Haplophyllum bastetanum* (GDA 47318, GDA 47497, GDA 47502): (A) flowering and fruiting plant; (B) flower; (C) sepal; (D) petals; (E) stamens; (F) ovary; (G) capsule. *Haplophyllum linifolium* (GDA 47314): (H) capsule. *Haplophyllum rosmarinifolium* (GDA 47317): (I) capsule.

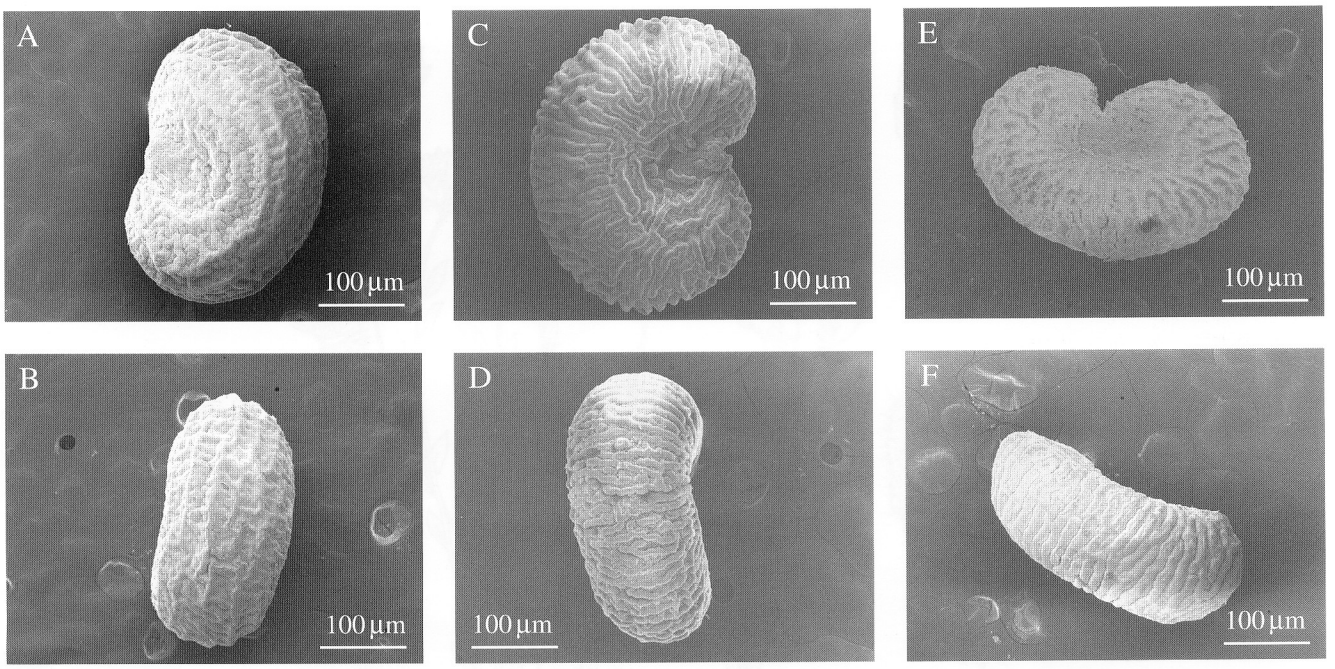

FIG. 2. Scanning electron microphotographs of the Spanish *Haplophyllum* seeds. (A and B) *H. bastetanum* (GDA 47502); (C and D) *H. linifolium* (GDA 47314); (E and F) *H. rosmarinifolium* (GDA 47317); (A, C and E) lateral view; (B, D and F) dorsal view. Scale bars = 100 µm.

TABLE 4. *Results of biometric analysis: interval of extreme values, mean (±1 s.e.) and* n = *number of samples of the differential parameters analysed for the species of the genus* Haplophyllum *present in the Iberian Peninsula*

	H. linifolium	*H. rosmarinifolium*	*H. bastetanum*
Stems diameter under inflorescence (mm)	1·7–3·8 [2·56 ± 0·09 (N = 39)^a]	0·8–2·2 [1·51 ± 0·04 (N = 45)^b]	0·6–2·1 [1·16 ± 0·04 (N = 56)^c]
Leaf width (mm)	2·1–8·2 [5·22 ± 0·25 (N = 38)^a]	1·0–3·0 [1·86 ± 0·06 (N = 55)^b]	2·5–7·5 [4·79 ± 0·16 (N = 42)^a]
Leaf length (mm)	6·0–30·0 [16·55 ± 1·08 (N = 31)^a]	8·0–33·0 [16·66 ± 0·99 (N = 31)^a]	11·0–18·0 [13·73 ± 0·37 (N = 31)^a]
Foliar surface (cm^2)	0·3–1·3 [0·74 ± 0·05 (N = 23)^a]	0·1–0·5 [0·31 ± 0·02 (N = 23)^b]	0·5–1·4 [0·85 ± 0·04 (N = 27)^a]
Foliar glands (number mm^{-2})	0·2–1·7 [0·76 ± 0·07 (N = 33)^a]	2·2–5·2 [3·32 ± 0·15 (N = 30)^b]	1·0–3·7 [1·98 ± 0·10 (N = 47)^c]
Sepal length (mm)	1·2–2·0 [1·6 ± 0·04 (N = 32)^a]	0·8–1·3 [1·05 ± 0·02 (N = 32)^b]	2·0–3·8 [2·75 ± 0·07 (N = 35)^c]
Sepal width (mm)	1·0–1·8 [1·2 ± 0·03 (N = 31)^a]	0·7–1·2 [0·92 ± 0·02 (N = 32)^b]	0·8–1·2 [0·97 ± 0·02 (N = 33)^b]
Petal length (mm)	8·0–11·5 [9·72 ± 0·21 (N = 27)^a]	5·0–8·5 [6·86 ± 0·21 (N = 28)^b]	5·8–8·5 [7·11 ± 0·16 (N = 29)^b]
Petal width (mm)	3·9–6·1 [4·86 ± 0·09 (N = 31)^a]	3·0–5·0 [3·95 ± 0·09 (N = 31)^b]	2·0–4·0 [2·97 ± 0·09 (N = 34)^c]
Seed length (mm)	1·6–1·9 [1·79 ± 0·01 (N = 37)^a]	1·5–1·9 [1·73 ± 0·01 (N = 32)^b]	1·8–2·4 [2·00 ± 0·02 (N = 38)^c]
Seed width (mm)	1·3–1·5 [1·42 ± 0·01 (N = 31)^a]	1·3–1·5 [1·37 ± 0·01 (N = 30)^b]	1·4–1·6 [1·50 ± 0·01 (N = 30)^c]
Capsule no./inflorescence	4–53 [20·24 ± 1·32 (N = 59)^a]	1–20 [7·09 ± 0·47 (N = 46)^b]	1–15 [4·84 ± 0·38 (N = 58)^c]

Letters after the samples show significant differences at a confidence level of 0·05 %.

treated as unweighted, all parsimony analyses yielded the same three most parsimonious trees. One of the strict consensus trees is shown in Fig. 4A; the CI, RI and RC, with the HI values of each analysis, are shown in Table 7B. The strict consensus tree supports, on one hand, the clade composed of *H. blanchei* and *H. coronatum*, and, on the other hand, the clade formed by *H. linifolium* and *H. rosmarinifolium*, although in this latter case the bootstrap value is not high. Both clades, together with *H. bastetanum*, form a trichotomy that it is separate from *H. suaveolens*. The bootstrap values in both analyses are summarized in Table 7B.

After the characters were reweighted according to the rescaled consistency index (RC), the trees differed whether or not the gaps were used as a fifth base. In both analysis, the bootstrap values were higher than in the unweighted analysis (Table 7B), but in the former analysis (Fig. 4B) the trichotomy between *H. bastetanum*, *H. coronatum–H. blanchei* clade and *H. linifolium–H. rosmarinifolium* clade was unresolved, whereas in the latter analysis this trichotomy was resolved with *H. bastetanum* joining the remainder of the Spanish species in a clade (BS = 54 %; Fig. 4C).

Distribution and ecological characterization

To date, only two *H. bastetanum* populations are known, with a total of roughly 100 individuals distributed

TABLE 5. *Sequence characteristics of ITS1, 5.8S and ITS2 regions of six studied taxa of* Haplophyllum *and* Ruta montana

Parameter	ITS1	5.8S	ITS2	ITS1 + 5.8S + ITS2
Length range (total) (bp)	216–248	164	219–232	612–637
Length mean (total) (bp)	240·6	164	221·9	626·4
Length range (*Haplophyllum*) (bp)	244–248	164	217–225	625–637
Length mean (*Haplophyllum*) (bp)	244·6	164	220·2	628·8
Length *Ruta montana* (bp)	216	164	232	612
Aligned length (bp)	249	164	243	656
G + C content range (total) (%)	64·4–71·7	54·8–56·1	65·1–69·8	62·2–67
G + C content mean (total) (%)	68·8	55·4	67·6	64·9
G + C content range (*Haplophyllum*) (%)	67·2–71·7	54·8–56·1	65·9–69·8	63·6–67
G + C content mean (*Haplophyllum*) (%)	69·5	55·4	68·1	65·3
G + C content *Ruta montana* (%)	64·4	55·4	65·1	62·2
Number of indels (*Haplophyllum*)	4	0	3	7
Number of indels (total)	13	0	12	25
Size of indels (*Haplophyllum*)	1	0	1–7	1–7
Size of indels (total)	1–7	0	1–9	1–9

TABLE 6. *Pairwise divergence between ITS region sequences from six* Haplophyllum *species and* Ruta montana

A	1	2	3	4	5	6
H. blanchei						
H. linifolium	0·044					
H. coronatum	0·044	0·019				
H. rosmarinifolium	0·045	0·003	0·019			
H. suaveolens	0·078	0·048	0·050	0·048		
H. bastetanum	0·043	0·005	0·018	0·005	0·046	
Ruta montana	0·185	0·164	0·175	0·164	0·188	0·160
B		S				R
S		0·004				
R		0·037				0·058

Sequences were compared specifying gaps as missing data with the pairwise deletion option: (A) combined ITS region distance matrix; (B) average distance matrix within and between groups of Spanish and non-Spanish *Haplophyllum* species.

Numbers and letters along the top and left margins of each matrix correspond to samples. 1, *H. blanchei*; 2, *H. linifolium*; 3, *H. coronatum*; 4, *H. rosmarinifolium*; 5, *H. suaveolens*; 6, *H. bastetanum*; S, Spanish *Haplophyllum* species; R, non-Spanish *Haplophyllum* species.

throughout the Guadix-Baza Basin (Granada Province, Spain). From a biogeographical standpoint, this territory belongs to the Guadiciano-Bastetano district (Guadiciano-Bacense sector, Betica province) where *H. bastetanum* grows on carbonate soils in dry and semi-arid mesomediterranean bioclimate levels. This plant forms part of the low woody scrublands and esparto (*Stipa tenacissima* L.) grasslands belonging to the vegetation series *Paeonio coriaceae–Querceto rotundifoliae S.* and *Rhamno lycioidis–Querceto cocciferae S.*, where this species is often found refuged in the interior of esparto plants, presumably because of the influence of livestock. The degree of threat, according to UICN (2001) categories, is CR A2acd; B2ab(i,ii,iii,iv,v); C2a(i).

The other two Iberian species have a larger number of populations and distribution areas. *Haploplyllum linifolium* extends throughout the interior zones of the Iberian Peninsula, fundamentally in the centre and south, on roadsides and in crops, growing on sedimentary clayey-carbonate substrates. *Haploplyllum rosmarinifolium* is a more thermophilous species, which extends throughout Mediterranean coastal zones, forming part of the low, woody scrublands, on limestones and dolomites, in dry and semi-arid places.

DISCUSSION

With the use of morphometric, cytogenetic, molecular and ecological data, *H. bastetanum* can be distinguished from related *Haplophyllum* species as follows.

Morphology

Several key traits combined differentiate *H. bastetanum* from the rest of the species: glabrous leaves; lanceolate sepals; dark-green petals and stamen filaments; dorsal band of hairs on the petals; densely hairy ovary and capsule; capsule lobes without apical appendages; and seeds with fundamentally longitudinal dorsal ridges (Table 3 and Figs 1 and 2).

With respect to the species present in the Iberian Peninsula, the results of the quantitative analysis (Table 4) support the existence of three taxa with different characteristics (*H. bastetanum, H. linifolium* and *H. rosmarinifolium*). However, *H. bastetanum* shares some characters with *H. linifolium* (leaf length, width and area) and with *H. rosmarinifolium* (sepal width and petal length).

Cytology

Haploplyllum bastetanum is a diploid species ($2n = 18$) like other species of the genus, which have been only scantily studied: *H. patavinum* (L.) G. Don (Cappelletti, 1929; Negodi, 1939), *H. dauricum* (L.) G. Don (Mesicek and Sojak, 1972; Hanelt, 1973), *H. obtusifolium* Ledib. (Guerra, 1984, 1985), *H. perforatum* (M. Bieb) Kar. & Kir. (Ghaffari, 1986, 1987), *H. latifolium* Kar. & Kir. (Zakironova and Nafanailova, 1992). However, *H. linifolium* and *H. rosmarinifolium* are two tetraploid species ($2n = 36$); this ploidy

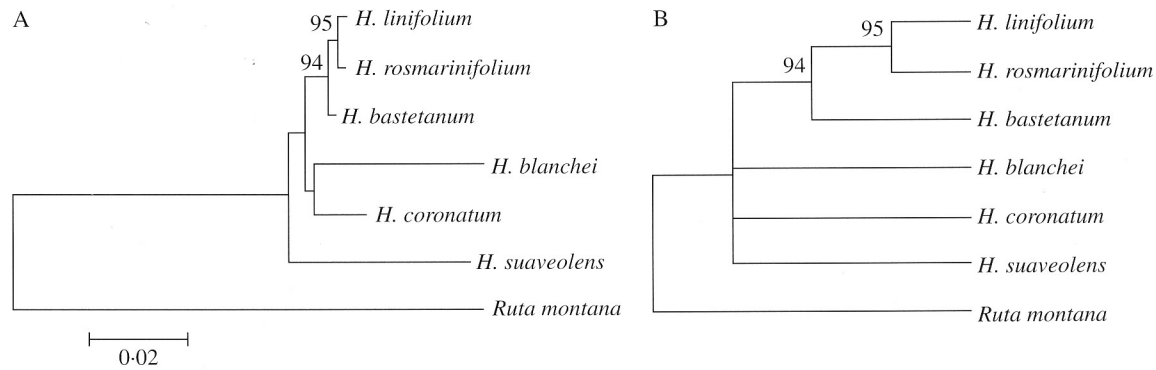

FIG. 3. Original NJ tree (A) and bootstrap consensus NJ tree (B) generated by the ITS matrix using K2P model. Numbers are bootstrap values.

TABLE 7. *Comparison of results from the different phylogenetic analyses*

		Nc/Gm	C/Gm	C/Gb			
(A) Comparison of variable and informative characters and g1 statistic value among data matrices with different treatments of ambiguous regions and gaps							
Variable characters		126	121	131			
Informative characters		15	17	17			
g1		−0·4514	−0·49669	−0·4484			
					MP		
Clade	NJ % bootstrap	Nc/Gm/Uw % bootstrap	C/Gm/Uw % bootstrap	C/Gb/Uw % bootstrap	Nc/Gm/W % bootstrap	C/Gm/W % bootstrap	C/Gb/W % bootstrap
(B) Tree scores and bootstrap values (%) of clades obtained from different phylogenetic reconstruction methods							
1	95	62	59	67	64	69	73
2	94	**25**	**35**	**38**	**34**	**41**	54
3	**44**	77	74	78	87	88	81
4	**46**	59	62	60	98	100	100
MPTs	–	3	3	3	3	3	2
Tree length	–	145	188	199	130·5	170·5	181·5
CI	–	0·945	0·9521	0·955	0·994	0·996	0·996
RI	–	0·556	0·55	0·55	0·912	0·921	0·921
RC	–	0·525	0·524	0·525	0·907	0·917	0·917
HI	–	0·055	0·0479	0·045	0·006	0·004	0·004

NJ, neighbour joining; MP, maximum parsimony; Nc, ambiguous region not coded; C, ambiguous region coded; Gm, gaps treated as missing data; Gb, gaps treated as fifth base; Uw, characters specified as unweighted; W, characters specified as weighted.
In the clade column the numbers are the different clades of the trees: 1, *H. rosmarinifolium–H. linifolium* clade; 2, Spanish *Haplophyllum* clade; 3, *H. blanchei–H. coronatum* clade; 4, *H. rosmarinifolium–H. linifolium–H. bastetanum–H. blanchei–H. coronatum* clade.
MPTs, number of most parsimonious trees; CI, consistency index; RI, retention index; RC, rescaled consistency index; HI, homoplasy index.
Bootstrap values <50 %, which appear only in the original NJ tree and in the bootstrap-consensus tree including groups compatible with 50 % majority-rule consensus, are printed in bold.

level has previously gone unreported for any *Haplophyllum* species. Thus, the changes in ploidy level may be the basic mechanism for evolutionary diversification in Spanish *Haplophyllum* species.

Molecular analyses

No sequence information for the ITS region has been reported previously either for *Haplophyllum* or for *Ruta* species. The size of ITS1, ITS2 and the 5·8S coding sequences in *Haplophyllum* species and *Ruta montana* lie within the range of those reported previously for other angiosperms (ITS1, 187–298; ITS2, 187–252; 5·8S, 163–164; see Baldwin *et al.*, 1995) and other taxa within the order Sapindales (Ackerly and Donoghue, 1998; Becerra and Venable, 1999; Scott *et al.*, 2000; Suh *et al.*, 2000; Edwards and Gadek, 2001; Becerra, 2003). Among *Haplophyllum* species, the ITS has evolved mainly by base substitutions, where only seven indel events (one to seven gaps) appeared. High G+C content among ITS sequences is found in *Haplophyllum* (65·3 %) as has been noted in other Sapindales (Suh *et al.*, 2000) and in angiosperms in general (from 50 % to 75 %; see Baldwin *et al.*, 1995).

The divergence of ITS sequences is reportedly sufficient to provide phylogenetic signals in various families, as for instance in Apiaceae (Downie and Katz-Downie, 1996), Asteraceae (Baldwin, 1992, 1993), Rosaceae (Campbell *et al.*, 1995) and Aceraceae (Suh *et al.*, 2000). In these

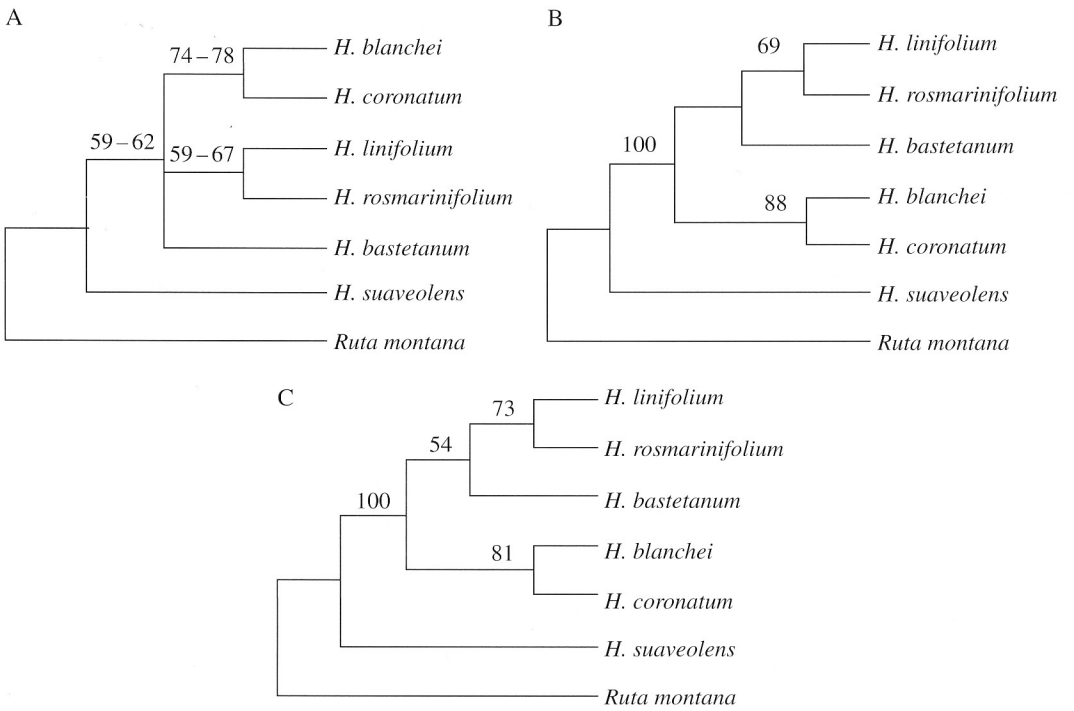

FIG. 4. Phylogenetic trees generated by ITS matrix using maximum-parsimony method: (A) strict consensus tree of the three maximally parsimonious trees obtained in all analyses with characters specified as unweighted; (B) bootstrap consensus tree including groups compatible with 50 % majority rule consensus reached when the ambiguous regions were coded, gaps specified as missing data and characters were reweighted according to rescaled consistency index; (C) bootstrap consensus tree obtained when the ambiguous regions were coded, gaps specified as fifth base and characters were reweighted according to rescaled consistency index. Numbers above branches are the bootstrap values (%) (in A the range of bootstrap values found are shown for each clade in the different analyses with unweighted characters; see Table 7B).

groups, ITS sequences have proven most valuable for examining relationships within genera and among the more closely related genera. Within species, ITS sequences can be useful for investigating relationships among allopatric or disjunct populations (Baldwin, 1993). In this study, the divergence of ITS sequences and the phylogenetic analysis support the idea that *H. bastetanum* is a different species from *H. rosmarinifolium* and *H. linifolium*. In all the phylogenetic trees (both the NJ and the parsimony trees) the only two Spanish species described (*H. linifolium* and *H. rosmarinifolium*) appear together and differ from *H. bastetanum* with high bootstrap values. This result is supported by the pairwise sequence-divergence values, so that this value between *H. rosmarinifolium* and *H. linifolium* is 0·3 %, whereas each of them diverge from *H. bastetanum* by 0·5 %.

The NJ tree represented the *H. rosmarinifolium*-*H. linifolium* clade as a sister clade of *H. bastetanum* (BS = 94 %), while this grouping appeared with a bootstrap value only slightly higher than 50 % (54 %) in the parsimony analysis when the gaps were specified as fifth base and the characters were reweighted according to their rescaled consistency index (RC). The existence of this clade is supported by the sequence-divergence values, and thus, on one hand, the lowest divergence values were between the three Spanish taxa and, on the other hand, the sequence-divergence values between the Spanish species and the other *Haplophyllum* species studied were higher than the average pairwise divergence of all analysed *Haplophyllum* species. The existence of a clade formed by the Spanish species is supported also by biogeographical data, so that these species are geographically isolated from other species.

The high level of the ITS-sequence conservation among closely related diploid and polyploid species may indicate an origin by autopolyploidy of the polyploid species from diploids (Baldwin, 1992; Wendel, 2000). In the case of Spanish *Haplophyllum* species, the three species show high levels of sequence conservation (low sequence-divergence values, high structural-sequence conservation), which, together with the biogeographical data, suggest the probable origin of *H. rosmarinifolium* and *H. linifolium* from *H. bastetanum* or from an ancestor to this species.

Evolution of the genus Haplophyllum *in the Iberian Peninsula*

The high level of sequence conservation, in addition to the grouping of *H. rosmarinifolium* with *H. linifolium* as well as these two with *H. bastetanum* in the phylogenetic analysis, suggest that the change in the number of chromosomes may be the key mechanism for speciation on the Iberian Peninsula. From *H. bastetanum* (or from an ancestor $2n = 18$), a tetraploid species could have

TABLE 8. *Key of the genus* Haplophyllum *on the Iberian Peninsula*

1.	Sepals lanceolate; petals dark green, with a band of hairs on the dorsal side; stamen filaments green; capsule lobes with tubercular glands at the apex, without prominent appendages; seeds with predominantly longitudinal ridges, narrower than the spaces separating them	***H. bastetanum* sp. nov.**
1′.	Sepals ovate-lanceolate; petals yellow, sometimes with a brownish or greenish dorsal band, glabrous; stamen filaments yellow; capsule lobes with prominent appendages at the apex; seeds with transversal ridges, wider than the spaces between them	2
2.	Herbaceous perennial, woody at the base; leaves hairy, elliptical; capsule with hairy apex	*H. linifolium*
2′.	Woody; leaves glabrous, linear or narrowly spathulate; capsule glabrous	*H. rosmarinifolium*

originated by autopolyploidy and, perhaps, diversified by subsequent adaptation to different environmental conditions, giving rise to *H. rosmarinifolium* and *H. linifolium*.

The three aforementioned species, endemic to the Iberian Peninsula, are isolated from the rest of the species by the Mediterranean Sea. Apparently, *H. bastetanum* or its diploid ancestor reached the Iberian Peninsula from the north of Africa during the Messinian (some 5 million years ago), following the migration model proposed for other groups (Bocquet *et al.*, 1978; Blanca, 1981; Jeanmonod and Bocquet, 1981; Rosúa and Blanca, 1988).

According to the results of the present study, the following species is proposed.

Haplophyllum bastetanum *F.B. Navarro, V.N. Suárez-Santiago & Blanca,* **sp. nov.** *(Fig. 1)*

Diagnosis. Ovarium atque fructus dense pilosi. Ovarii loculi 5, tetraovulati. Fructus maturus dehiscens (capsula). Differt ab speciebus *H. linifolium, H. rosmarinifolium, H. coronatum, H. suaveolens* et *H. blanchei* petalis atque staminalibus filamentis saturate viridibus. Ab *H. linifolium, H. rosmarinifolium, H. coronatum*, et *H. suaveolens* ovarii loculis apice rotundatis atque glandulis tuberculatis praeditis-cornibus atque prominentibus appendicibus carentibus-et seminibus longitudinaliter cristatis. Ab *H. linifolium* et *H. rosmarinifolium* insuper differt sepalis lanceolatis petalisque linea dorsali pilorum praeditis et numero chromosomatum ($2n = 18$); ab *H. suaveolens* et *H. blanchei*, ovario piloso foliisque glabris; ab *H. coronatum*, foliis trifoliolatis nullis. Adiectivum specificum ('bastetanum') geographicam originem, ut patet, innuere contendit.

Flowering. May–June.

Holotype. SPAIN, GRANADA: Guadix-Baza Basin, pr. Hernán Valle, 30SVG9638, 1040 m, 25 May 2002, esparto patches, low woody scrublands, on carbonate substrate in the dry mesomediterranean bioclimatic level, Leg.: *F. B. Navarro & M. N. Jiménez.*, Det.: *F. B. Navarro*, GDA 47318.

Description. Perennial herbaceous plant, woody at the base. Stems 10–50 cm, weak, bluish-green, reddish at the base, densely covered with short white, curved hairs, frequently with axillary and sterile basal shoots. Leaves (11) 13–14 (18) × (2·5) 3·5–7·0 (7·5) mm, simple, elliptical or oblanceolate, sessile or barely petiolate, acute, green-glaucous, glabrous, with dark glands. Inflorescences loose, often corymbose, pubescent; bracts lanceolate. Flowers actinomorphic, hermaphroditic, pentamerous. Sepals 2–3·5 (3·8) × (0·8) 1·0 (1·2) mm, lanceolate, dark green, with numerous hairs 1–1·5 mm. Petals (5·8) 6–8·5 × (2·0) 3·0 (4·0) mm, lanceolate or ovate-lanceolate, somewhat concave, dark green, somewhat keeled with a band of dorsal hairs, and dotted by yellowish glands. Stamen filaments 5–6 mm, free, gradually widening in the lower middle, green, hairy on the inner side, glandular. Ovary densely hairy, divided into 5 tetraovulate locules; style 3–3·5 mm, green, glabrous, narrow, twisted at maturity. Infructescence corymbose, with (1) 3–10 (15) fruits. Capsule 5-lobed, dehiscent, uniformly hairy, with only rounded and tuberculate glands at the apex of each lobe, without horns or prominent appendages. Seeds (1·8) 2·0 (2·4) × (1·4) 1·5 (1·6) mm, kidney-shaped, dark grey or black, reticulated, with ridges predominantly longitudinal especially on the dorsal side (Fig. 2).

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LITERATURE CITED

Ackerly DD, Donoghue MJ. 1998. Leaf size, sapling allometry, and Corner's rules: phylogeny and correlated evolution in maples (*Acer*). *The American Naturalist* **152**: 767–791.

Baldwin BG. 1992. Phylogenetic utility of the internal transcribed spacers of the nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* **1**: 3–16.

Baldwin BG. 1993. Molecular phylogenetics of *Calycadenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: chromosomal and morphological evolution re-examined. *American Journal of Botany* **80**: 222–238.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell ChS, Donoghue MJ. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on Angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82**: 247–277.

Becerra JX. 2003. Evolution of Mexican *Bursera* (Burseraceae) inferred from ITS, ETS, and 5S nuclear ribosomal DNA sequences. *Molecular Phylogenetics and Evolution* **26**: 300–309.

Becerra JX, Venable L. 1999. Nuclear ribosomal DNA phylogeny and its implications for evolutionary trends in Mexican *Bursera* (Burseraceae). *American Journal of Botany* **86**: 1047–1057.

Blanca G. 1981. Origen, evolución y endemismo en la sección *Willkommia* G. Blanca (Gén. *Centaurea* L.). *Anales del Jardín Botánico de Madrid* **37**: 607–618.

Bolòs O, Vigo J. 1974. Notes sobre taxonomia i nomenclatura de plantes. I. *Butlletí de la Institució Catalana d'Història Natural* **38** (Sec. Bot. 1): 61–89.

Bocquet G, Widler B, Kiefer H. 1978. The Messinian model-A new outlook for the floristics and systematics of the Mediterranean area. *Candollea* **33**: 269–287.

Campbell CS, Donoghue MJ, Baldwin BG, Wojciechowski MF. 1995. Phylogenetic relationships in Maloideae (Rosaceae): evidence from sequences of the internal transcribed spacers of nuclear ribosomal DNA and its congruence with morphology. *American Journal of Botany* **82**: 903–918.

Cappelletti C. 1929. Embriogenesi di Ruta patavina L. *Nuovo Giornale Botanico Italiano* **36**(1): 138.

Darlington CD, La Cour LF. 1969. *The handling of chromosomes.* London: Allen & Unwin.

Don G. 1831–1834. *A general history of the dichlamydeous plants.* 4 vols. London: JG & F Rivington *et al.*

Doyle JJ, Doyle JD. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Downie SR, Katz-Downie DS. 1996. A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal spacer sequences. *American Journal of Botany* **83**: 234–251.

Edwards KJ, Gadek PA. 2001. Evolution and biogeography of *Alectryon* (Sapindaceae). *Molecular Phylogenetics and Evolution* **20**: 14–26.

Engler A. 1896. Rutaceae. In: Engler A, Prantl K, eds. *Die natürlichen Pflanzenfamilien*, **3**(4): 95–201. Leipzig: Wilhelm Engelmann.

Farris JS. 1969. A successive approximations approach to character weighting. *Systematic Zoology* **18**: 374–385.

Farris JS. 1989. The retention index and homoplasy excess. *Systematic Zoology* **38**: 406–407.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Font Quer P. 1953. *Diccionario de botánica.* Barcelona: Ed. Labor.

Ghaffari SM. 1986. In: IOPB chromosome number reports 91. *Taxon* **35**: 407.

Ghaffari SM. 1987. Chromosome counts of some angiosperms from Iran. II. *Iran Journal of Botany Research* **3**: 183–188.

Guerra MdosS. 1984. New chromosome numbers in Rutaceae. *Plant Systematics and Evolution* **146**: 13–30.

Guerra MdosS. 1985. Cytogenetics of Rutaceae. III. Heterochromatin patterns. *Caryologia* **38**: 335–346.

Hanelt P. 1973. In: IOPB chromosome number reports 42. *Taxon* **22**: 647–654.

Heywood VH. 1978. *Flowering plants of the world.* London: Oxford University Press.

Hillis DM, Huelsenbeck JP. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* **83**: 189–195.

Jeanmonod D, Bocquet G. 1981. Remarques sur la distribution de *Silene mollissima* (L.) Pers. et des espèces affines en Méditerranée occidentale. *Candollea* **36**: 279–287.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111–120.

Kluge AG, Farris JS. 1969. Quantitative phyletics and the evolution of anurans. *Systematic Zoology* **40**: 315–328.

Kumar S, Tamura K, Jokobsen IB, Nei M. 2001. MEGA2: molecular evolutionary genetics analysis software. Temple: Arizona State University.

Lutzoni F, Wagner P, Reeb V, Zoller S. 2000. Integrating ambiguously aligned regions of DNA sequences in phylogenetic analyses without violating positional homology. *Systematic Biology* **49**: 628–651.

Mabberley DJ. 1990. *The plant-book.* Cambridge: Cambridge University Press.

Mesicek J, Sojak J. 1972. Chromosome studies in Mongolian plants. *Preslia* **44**: 334–358.

Nazrullaev SS, Bessonova IA, Akhmedkhodzhaeva KS. 2002. Estrogenic activity as a function of the chemical structure in *Haplophyllum* quinoline alkaloids. *Chemistry of Natural Compounds* **37**: 551–555.

Negodi G. 1939. Cariologia delle Rutaceae e delle Zygophyllaceae. *Scientia Genetica* **1**: 168–185.

Pascual-Villalobos MJ, Robledo A. 1999. Anti-insect activity of plant extracts from the wild flora in southeastern Spain. *Biochemical Systematics and Ecology* **27**: 1–10.

Prieto JM, Giner RM, Recio MC, Schinella G, Manez S, Ríos JL. 2002. Diphyllin acetylapioside, a 5-lipoxygenase inhibitor from *Haplophyllum hispanicum*. *Planta Medica* **68**: 359–360.

Rivas-Martínez S, Asensi A, Díez-Garretas B, Molero J, Valle F. 1997. Biogeographical synthesis of Andalusia (southern Spain). *Journal of Biogeography* **24**: 915–928.

Rivas-Martínez S, Loidi J. 1999. Bioclimatology of the Iberian Peninsula. *Itinera Geobotanica* **13**: 41–47.

Rosúa JL, Blanca G. 1988. Revisión del género *Salvia* L. sect. *Aethiopis* Benth. (Lamiaceae) en el Mediterráneo occidental. *Collectanea Botanica* (Barcelona) **17**(2): 205–236.

Schinella GR, Tournier HA, Prieto JM, Ríos JL, Buschiazzo H, Zaidenberg A. 2002. Inhibition of *Trypanosoma cruzi* growth by medical plant extracts. *Fitoterapia* **73**: 569–575.

Scott KD, McIntyre CL, Playford J. 2000. Molecular analyses suggest a need for a significant rearrangement of Rutaceae sub-families and a minor reassessment of species relationships within *Flindersia*. *Plant Systematics and Evolution* **223**: 15–27.

Shaiq M, Kashif M, Saleem M, Bakhsh R. 2001. Haplophytin-A and B: the alkaloidal constituents of *Haplophyllum acutifolium*. *Phytochemistry* **57**: 1277–1280.

Spach E. 1949. Conspectus generis *Haplophyllum*. *Annales des Sciencies Naturelles, Botanique III* **11**: 174–192.

Stearn WT. 1980. *Botanical Latin.* London: David & Charles.

Suh Y, Heo K, Park ChW. 2000. Phylogenetic relationships of maples (*Acer* L.; Aceraceae) implied by nuclear ribosomal ITS sequences. *Journal of Plant Research* **113**: 193–202.

Swofford DL. 1993. *PAUP: phylogenetic analysis using parsimony, version 3.1.1.* Champaign, IL: Illinois Natural History Survey.

Swofford DL. 2003. *PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4.* Sunderland, MA: Sinauer Associates.

Thornill JW, Matta RK, Wood WH. 1965. Examining three dimensional microstructures with the scanning electron microscope. *Grana Palynologica* **6**: 3–6.

Townsend CC. 1966. Towards a revision of *Haplophyllum* A. Juss. (Rutaceae): 1. *Kew Bulletin* **20**: 89–102.

Townsend CC. 1968. *Haplophyllum.* In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. *Flora Europaea, Vol.* 2. Cambridge: Cambridge University Press, 227–229.

Townsend CC. 1975. Towards a revision of *Haplophyllum* A. Juss. (Rutaceae): 3. *Kew Bulletin* **30**: 527–530.

Townsend CC. 1986. *Taxonomic revision of the genus* Haplophyllum (Rutaceae). *Hooker's Icones Plantarum, Vol. XL, parts I, II and III.* Kent: Bentham-Moxon Trustees.

UICN. 2001. *Categorías y criterios de la Lista Roja de la UICN: Versión 3.1.* Comisión de Supervivencia de Especies de la UICN: Gland.

Valle F. 2003. *Mapa de series de vegetación de Andalucía.* Madrid: Ed. Rueda.

Vvdensky A. 1949. *Haplophyllum.* In: Komarov VL, ed. *Flora of the U.R.S.S., Vol. 14.* Leningrad: Ed. Academiae Scientiarum URSS, 200–227.

Wen J, Zimmer EA. 1996. Phylogeny and biogeography of *Panax* L. (the ginseng genus, Araliaceae): inferences from ITS sequences of nuclear ribosomal DNA. *Molecular Phylogenetics and Evolution* **6**: 167–177.

Wendel JF. 2000. Genome evolution in polyploids. *Plant Molecular Biology* **42**: 225–249.

Zakironova RO, Nafanailova II. 1992. Chromosome numbers in members of some families of the Kazakhstan flora. *Botaniceskii Zcurnal* **77**: 71–72.

APPENDIX 1: HERBARIUM MATERIAL USED IN MORPHOMETRIC ANALYSIS

H. rosmarinifolium *(Pers.) G. Don*

SPAIN. ALICANTE: Benidorm, sierra Helada, 30SYH5469, 17 May 1980, *J. Fernández-Casas*, GDA 14005; Elche, 26 March 1989, *L. Gracia Vicente*, GDAC 30282; el Altet, 27 May 1972, *J. Borja & E. Valdés-Bermejo*, MA 410124. ALMERA: Lucainena, 11 June 1980, *G. Blanca & J. L. Rosúa*, GDAC 9284; Lucainena, 11 June 1980, *G. Blanca & J. L. Rosúa*, GDAC 6097; between Uleila del Campo and Benizalón, 30SWG6812, 700 m, 16 April 1988, *A. B. Robles, J. Peñas & C. Morales*, GDAC 28107; Níjar, cortijo de Bornos, 30SWF8683, 250 m, 30 April 1991, *M. J. M. Lirola & L. Gutiérrez*, GDA 25936; Tahal, between Los Yesos and Benizalón, 30SWG6710, 650 m, 21 May 2003, *Navarro, F.B.*, GDA 47316. GRANADA: Dúrcal river, 28 May 1983, *J. Guirado*, GDAC 15650; around Dúrcal, May 1974, *G. Blanca*, GDAC 260; Dúrcal, 30SVF5096, 850 m, 21 June 2003, *Navarro, F.B.*, GDA 47317. MURCIA: S^{a} Espuña, 21 March 1970, *S. Rivas-Goday & M. Ladero*, GDA 6188. VALENCIA: 26 Dec. 1944, GDA 38737; Segorbe, June 1886, *C. Pau*, MA 73713; Segorbe, 17 May 1912, *C. Pau*, MA 73712; Tous, Fontblanquilla, 30SYJ04, 520 m, 29 May 1995, *J. Riera, J. Güemes & E. Estrellas*, MA 589220.

H. linifolium *(L.) G. Don*

SPAIN. GRANADA: Near Moreda, 1100 m, 8 June 1989, *G. Blanca, C. Morales & C. Díaz de la Guardia*, GDAC 30945; sierra Elvira, 9 June 1979, *C. Morales*, GDAC 5665; cortijo de las Taulas, cañada del Carar, 9 June 1983, *J. Hurtado*, GDA 24731. **JAÉN**: Martos, 30SVG17, 600 m, 7 May 1988, *G. Blanca*, GDAC 28048; Torredelcampo, 30SVG28, 500 m, 17 April 1988, *G. Blanca*, GDAC 28047; Otíñar, 30SVG3471, 600 m, 2 June 1985, *E. García-Martínez*, GDA 25589; cerro Tallán, 30SVG3686, 400 m, 19 May 1987, *Carlos Fernández*, GDA 26896; between Torrequebradilla and Arroyo Hondo, 30SVG3686, 400 m, 15 May 2003, *Navarro, F.B.*, GDA 47315; between Torrequebradilla and Arroyo Hondo, 30SVG3686, 400 m, 3 June 2003, *Navarro, F.B.*, GDA 47314. MADRID: Between Aranjuez and Valdeagua, cerro Cabina, 30TVK4627, 600 m, 20 June 1989, *D. Sánchez-Mata & R. Gavilán*, GDAC 39641; Arganda, GDA 38738. VALLADOLID: Almaraz de la Mota, 30TUM1719, 800 m, 16 July 1980, *F. Amich, E. Rico & J. Sánchez*, GDA 13205.

H. bastetanum *F.B. Navarro, V.N. Suárez-Santiago & Blanca*

SPAIN. GRANADA: Hoya de Baza, Guadix, near Hernán Valle, 30SVG9638, 1040 m., 25 May 2002, *Navarro, F.B. & Jiménez, M.N.*, GDA 47318; Gorafe, near Baños de Alicún, 30SVG9151, 800 m, 11 June 2003, *F. B. Navarro*, GDA 47496; Gorafe, 30SVG9151, 800 m, 15 June 2003, *F. B. Navarro*, GDA 47497; Guadix, Hernán Valle, 30SVG9638, 1040 m, 1 May 2002, *F. B. Navarro & M. N. Jiménez*, GDA 47498; Guadix, Hernán Valle, 30SVG9638, 1040 m, 4 July 1998, *F. B. Navarro & M. N. Jiménez*, GDA 47502.