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PHYLOGENY AND SPECIES RELATIONSHIPS IN *JASIONE* (*CAMPANULACEAE*) WITH EMPHASIS ON THE '*MONTANA*-COMPLEX'

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The evolutionary relationships of several, mainly Iberian, *Jasione* taxa (*Campanulaceae*) were investigated using molecular data. A parsimony analysis of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA was partially successful in elucidating some interspecific alliances. One of the main clades included three morphologically rather disparate species from S Spain and Morocco which might indicate that these areas were refugia during the last glaciations. The fact that most of the other taxa fell in a large polytomy suggests a recent origin of these species. Amplified fragment length polymorphisms (AFLPs) were analysed for a larger sample set of two taxa from within the ITS polytomy to assess the partition of genetic variation between and within populations. All the British accessions named *J. montana* var. *montana* were found to be close to those so-named from Spain. However, individual accessions of *J. montana* var. *montana* from Argyll (Scotland), Devon (SW England) and the Shetland Islands proved to be genetically different from each other. This might suggest a low level of genetic diversification from a common progenitor due to a rapid northward migration. It is proposed that *J. montana* var. *bracteosa* and *J. maritima* var. *sabularia* merit recognition at higher rank. The possibility that *J. montana* vars *gracilis* and *latifolia* are mere repetitive segregants of *J. montana* var. *montana* is considered.

Keywords. AFLP, *Campanulaceae*, glaciation, internal transcribed spacer, ITS, *J. montana*-complex, *Jasione*, phylogeny, refugia, taxonomy.

INTRODUCTION

Jasione L. is a small genus of c.16 species of annual, biennial and perennial herbs in the *Campanulaceae*. Within the family, it occupies an intermediate position between true 'wahlenbergioids' (genera with *Wahlenbergia* Schrad. ex Roth affinities) and 'campanuloids' (genera with *Campanula* L. affinities) and does not appear to have any close extant relatives (Eddie, 1997; Eddie *et al*., 2003). The species grow throughout Europe, NW Africa and in N and W Turkey. The centre of maximum morphological diversity is in the Iberian Peninsula, where several extremely localized endemic taxa exist (Sales & Hedge, 2001b).

Sales & Hedge (2001b) attempted to resolve the current and substantially conflicting taxonomic accounts of Rivas Martínez (1976), Tutin (1976), Parnell (1980)

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and Greuter *et al.* (1984). Resolution was achieved by studying morphological variation within populations in the field, anatomical studies (Bokhari & Sales, 2001) and extensive research into typification and nomenclature (Sales & Hedge, 2001a). The resulting *Flora iberica* account (Sales & Hedge, 2001b) differs substantially from the previous treatments.

Jasione species are morphologically uniform with similar habit, leaves, and often sessile flowers of more or less the same size, shape and colour held in dense, centripetal heads, enveloped by 1–3 series of bracts. The anthers are fused at the base and all species appear to have a similar pollination mechanism. However, the pattern of geographical distribution and morphological variation is rather disparate: some species (e.g. *J. montana*) are widely distributed and highly polymorphic with many ecological variants (Parnell, 1980; Sales & Hedge, 2001b), while others such as *J. cavanillesii* C.Vicioso and *J. mansanetiana* R.Roselló & Peris have restricted distributions with little morphological variation (Sales & Hedge, 2001b). The natural habitats of *Jasione* range from sea level to c.3500m, from coastal dunes to alpine rocky crevices, including a wide variety of substrates. This diversity of ecological niches may be the reason for the numerous small morphological differences of unknown evolutionary significance, a common phenomenon throughout the *Campanulaceae* (Eddie & Ingrouille, 1999).

A molecular analysis of *Jasione* was undertaken in the hope of discovering new evidence that would reveal insights into its evolutionary patterns and help produce a better taxonomic ranking of the morphological diversity observed. This approach included the reconstruction of a molecular phylogeny using internal transcribed spacer (ITS) sequences at a higher taxonomic level, and applying population genetic markers, amplified fragment length polymorphisms (AFLPs), at a lower level.

The nomenclature and circumscription of the taxa is that adopted in *Flora iberica* (Sales & Hedge, 2001b).

Materials and Methods

Plant material

ITS analysis. Silica-gel dried leaf material of six different *Jasione* species from Spain, Portugal, Scotland and Turkey, and herbarium material of two further species, *J. corymbosa* Poir. ex Schult. (Morocco) and *J. foliosa* Cav. (Spain), was used for ITS sequence analysis. Additionally, 11 ITS sequences previously obtained by Jill Preston and deposited in the Royal Botanic Garden Edinburgh (RBGE) DNA bank were included in the analysis. Details of all accessions are given in Table 1. For taxa with a wide distribution (e.g. *J. montana* L. var. *montana, J. montana* L. var. *gracilis* Lange and *J. montana* L. var. *latifolia* Cav.), several samples were included to cover the range and to test infraspecific ITS variation. A total of 15 taxa out of the 23 in Sales & Hedge (2001b) were studied. Unfortunately, no material of *J. cavanillesii* C.Vicioso (Spain), *J. crispa* subsp. *tomentosa* (A.DC.) (Spain), *J. idaea* Stok. (Turkey), *J. mansanetiana* R.Roselló & Peris (Spain),

TABLE 1. Accession details of *Jasione* samples used in ITS analysis

Taxon	RBGE DNA accession no.	Sample code	Voucher	Locality	GenBank no.
J. corymbosa Poir. ex Schult.	SP67	SP67	SL19271	Morocco: Kenitra	DQ222827
J. crispa (Pourr.) Samp. subsp. *crispa*	AM001501	SP1	*F. Sales* s.n.	Portugal: Beira Alta; Serra da Estrela; Manteigas Poço do Inferno	DQ222832
J. crispa (Pourr.) Samp. subsp. *mariana* (Wilk.) Rivas.-Mart.	AM001494	SP10	*F. Sales & I.C. Hedge* 01/79	Spain: Badajoz; Alange	DQ222833
J. crispa (Pourr.) Samp. subsp. *tristis* (Bory) G.Lopez	AM001492	SP12	*F. Sales & I.C. Hedge* 01/65	Spain: Granada; Sierra Nevada; Los Paredones del Velete	DQ222831
J. foliosa Cav.	AM001507	SP6	*M.F. Gardner & S.G. Gardner* 1460	Spain: Almería; Vélez-Blanco, Sierra de Maria	DQ222826
J. heldreichii Boiss. & Orph.	SP7	SP7	19694842	Turkey: Bolu	DQ222834
J. laevis Lam.	AM000926	DNA18R	*F. Sales & I.C. Hedge* 98/18	Spain: Cantabria; Picos de Europa, near the Aliva refuge	DQ222845
J. montana L. var. *bracteosa* Willk.	AM001495	SP70	*F. Sales & I.C. Hedge* 01/47	Spain: Andalucía; Málaga; Sierra de Tejeda; between Sedella and Canillas del Aceituno	DQ222828
J. montana L. var. *gracilis* Lange	AM001128	DNA2R	*F. Sales & I.C. Hedge* 98/02	Portugal: Trás-os-Montes e Alto Douro; Bragança	DQ222840
J. montana L. var. *gracilis* Lange	AM000948	DNA38	*F. Sales & I.C. Hedge* 98/38	Spain: La Coruña; from Muros to Cabo Finisterre, Lariño & Lira	DQ222841
J. montana L. var. *latifolia* Pugsley	AM000929	DNA21	*F. Sales & I.C. Hedge* 98/21	Spain: Cantabria; near Ungera	DQ222837
J. montana L. var. *latifolia* Pugsley	AM000935	DNA98	*F. Sales & I.C. Hedge* 98/98	Spain: Pontevedra; Tállara	DQ222838
J. montana L. var. *latifolia* Pugsley	n.a.	DNA28	*F. Sales & I.C. Hedge* 98/28	Spain: Asturias; Cabo de Peñas	DQ222839
J. montana L. var. *montana*	AM000933	DNA25	*F. Sales & I.C. Hedge* 98/25	Spain: Cantabria; Picos de Europa, before Pembes	DQ222836
J. montana L. var. *montana*	AM000938	SP25	*F. Sales & I.C. Hedge* 98/73	UK: Scotland; Argyll & Bute, Kintyre peninsula, Carradale	DQ222835
J. maritima (Duby) Merino var. *maritima*	AM001135	DNA49	*F. Sales & I.C. Hedge* 98/49	Spain: La Coruña; near Muros, Mar de Lira beach	DQ222842
J. maritima (Duby) Merino var. *sabularia* (Cout.) Sales & Hedge	AM001140	DNA89	*F. Sales & I.C. Hedge* 98/89	Portugal: Beira Litoral; Ovar, Furadouro beach	DQ222844
J. maritima (Duby) Merino var. *sabularia* (Cout.) Sales & Hedge	n.a.	DNA79	*F. Sales & I.C. Hedge* 98/79	Portugal: Douro Litoral, Vila do Conde	DQ222843
J. sessiliflora Boiss. & Reut.	AM000921	DNA13R	*F. Sales & I.C. Hedge* 98/13	Spain: León; Sierra del Teleno, near Morredero	DQ222830
J. supina Sieber ex Spreng.	AM001493	SP8	*A. Günner* s.n.	Turkey: Bolu	DQ222829

J. orbiculata Griseb. (Balkans), *J. penicillata* Boiss. (Spain), *J. sphaerocephala* Brullo, C.Marcenò & P.Pavone (Italy), or variants of *J. supina* Sieber ex Spreng. (Turkey) was available for this study. Voucher specimens for all analysed samples are deposited at E.

A pilot study to establish a suitable outgroup for the phylogenetic analyses was undertaken using sequences from nine different genera of *Campanulaceae*. Of these, eight were obtained from GenBank: *Adenophora remotiflora* Miq., GenBank AH008212; *Asyneuma japonicum* (Miq.) Briq., AH008214; *Campanula glomerata* L., AH006455; *Campanumoea javanica* Blume, AF134862; *Codonopsis pilosa* Chipp., AH008217; *Edraianthus graminifolius* (L.) A.DC., AH008215; *Hanabusaya asiatica* Nakai, AF177730/AF183432, and *Symphyandra hofmannii* Pant., AF183441/183442; the ninth sequence, for *Trachelium caeruleum* L. subsp. *caeruleum* (RBGE-AM001491) collected in Spain, was obtained during this study. Three other sequences (*Jasione montana* var. *montana* L., RBGE-AM000933; *J. laevis*, RBGE-AM000926, and *J. maritima* var. *maritima*, RBGE-AM001135), previously obtained by Jill Preston and deposited in the RBGE DNA Bank, were included in the pilot study. The phylogenetic trees were rooted on *Campanumoea* and *Codonopsis* (Eddie *et al*., 2003). As the pilot study showed *Trachelium caeruleum* subsp. *caeruleum* to be the closest taxon to *Jasione* (data not shown), it was used as the outgroup in the ITS sequence analysis.

AFLP analysis. For the AFLP analysis, silica-gel dried leaf material of 47 accessions was used: 24 of *Jasione montana* L. var. *montana*, six of *J. montana* L. var. *gracilis* Willk., six of *J. montana* var. *latifolia* Pugsley, six of *J. maritima* (Duby) Merino var. *maritima*, and five of *J. maritima* var. *sabularia* (Cout.) Sales & Hedge (Table 2). Samples of *J. montana* var. *montana* and *J. montana* var. *gracilis* were included from localities where they occur sympatrically (i.e. Spain: La Coruña; Cabo Finisterre, Lighthouse peninsula).

DNA extraction, PCR and sequencing

DNA was extracted from 0.3g of leaf material dried in silica-gel (Chase & Hills, 1991) using a modified CTAB method of Doyle & Doyle (1987). Eppendorf tubes containing the leaf material were held in liquid nitrogen prior to processing. The complete ITS region was amplified using the modified primers 'ITS5P' (forward: 5′-GGA AGG AGA AGT CGT AAC AAG G-3′) and 'ITS8P' (reverse: 5′-CAG CTT CTC CAG ACT ACA-3′) (Möller & Cronk, 1997a). Shorter PCR products were obtained using 'ITS4' (reverse: 5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al*., 1990) for samples of *Jasione sessiliflora* Boiss. & Reut. (DNA13R), *J. laevis* (DNA18R) and *J. montana* var. *gracilis* (DNA2R). PCR and cycle sequencing conditions were as described by Möller & Cronk (1997a). All sequences have been submitted to GenBank (see Table 1). Sequencing products were analysed on an ABI Prism 377 Automatic DNA Sequencer (Applied Biosystems). Output from the DNA sequences was edited using Sequence Navigator™ version 1.0.1 (Applied

TABLE 2. Sample codes and locality information for *Jasione* samples used in AFLP analysis

Taxon	Sample code	Voucher	Locality
J. montana L. var. *gracilis* Lange	gracilis Spain-1	*F. Sales & I.C. Hedge* 98/100	Spain: Orense; Serra do Xurés
J. montana L. var. *gracilis* Lange	gracilis Spain-2, 3, 4	*F. Sales & I.C. Hedge* 98/39, 98/45, 98/47	Spain: La Coruña; from Muros to Cabo Finisterre, Lariño & Lira
J. montana L. var. *gracilis* Lange	gracilis Spain-5, 6	*F. Sales & I.C. Hedge* 98/58, 98/61	Spain: La Coruña; Cabo Finisterre, Lighthouse peninsula
J. montana L. var. *latifolia* Pugsley	latifolia-19, 20, 21, 22, 23, 24	*F. Sales & I.C. Hedge* 98/27, 98/29, 98/30, 98/31, 98/32, 98/33	Spain: Asturias; Cabo de Peñas
J. montana L. var. *montana*	montana Spain-7, 8, 9	*F. Sales & I.C. Hedge* 98/37, 98/44, 98/46	Spain: La Coruña; from Muros to Cabo Finisterre, Lariño & Lira
J. montana L. var. *montana*	montana Spain-10, 11	*F. Sales & I.C. Hedge* 98/60, 98/62	Spain: La Coruña; Cabo Finisterre, Lighthouse peninsula
J. montana L. var. *montana*	montana Spain-12	*F. Sales & I.C. Hedge* 98/26	Spain: Cantabria; Picos de Europa, before Pembes
J. montana L. var. *montana*	montana Devon-36, 37, 38, 39, 40, 41	*Natasha de Vere* s.n.	UK: England; Devon, close to the Start Point Lighthouse
J. montana L. var. *montana*	montana Argyll-13, 14, 15, 16, 17, 18	*F. Sales & I.C. Hedge* 98/73, 98/74, 98/75, 98/76, 98/77, 98/77A	UK: Scotland; Argyll & Bute, Kintyre peninsula, Carradale
J. montana L. var. *montana*	montana Shetland-42, 43, 44, 45, 46, 47	*Walter Scott* s.n.	UK: Scotland; Shetland Islands, east side of Linga Island
J. maritima (Duby) Merino var. *maritima*	maritima-30, 31, 32, 33, 34, 35	*F. Sales & I.C. Hedge* 98/50, 98/51, 98/52, 98/53, 98/54, 98/55	Spain: La Coruña; near Muros, Mar de Lira beach
J. maritima (Duby) Merino var. *sabularia* (Cout.) Sales & Hedge	sabularia-25, 26, 27, 28, 29	*F. Sales & I.C. Hedge* 98/82, 98/83, 98/84, 98/85, 98/86	Portugal: Douro Litoral, Vila do Conde

Biosystems) followed by manual alignment adjustments in PAUP* version 4.0b10 (Swofford, 2002).

Data analysis

ITS sequences. Sequence boundaries of both ITS spacers in all taxa were determined by comparison with published rDNA sequence data for *Daucus carota* L. and *Vicia faba* L. (Yokota *et al*., 1989). Sequence characteristics were calculated using PAUP* version 4.0b10. For all phylogenetic analyses a two-step heuristic search was performed:

1 10,000 random additions with no swapping
2 TBR swapping, with MULTREES and STEEPEST DESCENT on the saved trees from the first round, with COLLAPSE min invoked.

Branch support bootstrap (BS) (Felsenstein, 1985) in PAUP* version 4.0b10 and decay indices (DI) using AutoDecay 4.02 (Eriksson, 1999) were calculated, the former on 1000 replicates of random addition, with TBR on and MULTREES off, the latter using factory settings. All characters were treated as unordered and unweighted. Alignment gaps were treated as missing data, but were coded as additional characters and in- or excluded from analyses.

AFLP analysis. AFLP fingerprinting was performed as described by Vos *et al*. (1995) with minor modifications (Huang & Sun, 1999). Table 3 lists the names and sequences for the adapters, pre-selective primers and the three selective primer combinations used in the AFLP analysis. Multiplexed fluorescent-labelled AFLP products were analysed using GeneScan® version 3.1.2 (Applied Biosystems) and fragments ranging from 35 to 450 base pairs (bp) were scored using Genotyper® version 2.0 (Applied Biosystems). Electropherograms generated by Genotyper® were carefully checked individually in order to avoid possible misinterpretations due to electropherogram misalignments. DNA fragments were scored as present (1) or absent (0).

An UPGMA dendrogram was generated based on Nei and Li (NL) genetic distances (Nei & Li, 1979). Internal support was assessed with 1000 bootstrap replicates in PAUP* version 4.0b10. To illustrate further the relationships between the taxa, a principal coordinates (PCO) analysis was performed on distances calculated from Jaccard's coefficient of the community using Le Progiciel R version 4.0d (Casgrain & Legendre, 1999).

RESULTS

ITS sequence analysis

Alignment of the ITS of the 21 ingroup taxa and the outgroup taxon resulted in a 557bp long data matrix (with the 5.8S gene excluded). The length of ITS1 (excluding taxa with missing data) and ITS2 was, on average, 275 and 260.5bp, respectively.

TABLE 3. AFLP adapters and primer sequences

Adapter/primer	Sequence 5′–3′
Adapter	
*Eco*RI adapter	CTCGTAGACTGCGTACC
*Mse*I adapter	GACGATGAGTCCTGA
Pre-selective primers	
*Eco*RI + 1 primer	AGACTGCGTACCAATTCA
*Mse*I + 1 primer	GACGATGAGTCCTGAGTAAC
Selective primer combinations	
Pair 1 *Eco*RI + 3 primer	GACTGCGTACCAATTC**ACT**
*Mse*RI + 3 primer	GATGAGTCCTGAGTAA**CTA**
Pair 2 *Eco*RI + 3 primer	GACTGCGTACCAATTC**AAG**
*Mse*RI + 3 primer	GATGAGTCCTGAGTAA**CAG**
Pair 3 *Eco*RI + 3 primer	GACTGCGTACCAATTC**AAG**
*Mse*RI + 3 primer	GATGAGTCCTGAGTAA**CTG**

Alignment of all taxa required the insertion of 34 indels (insertion and deletion events) of 1–4bp length, 17 in both ITS1 and ITS2, of which 6 and 2, respectively, were potentially informative.

Due to ambiguous and missing data at the 5′ and 3′ end of ITS1 in some accessions, 18 and 32 characters, respectively, were excluded from parsimony analyses (Fig. 1). Of the remaining 507 unambiguously alignable sites, 377 (74.4%) were constant, 49 (9.6%) were potentially informative phylogenetically, and 81 (16%) were autapomorphies, unique to individual taxa.

Pair-wise comparisons of individual taxa across both spacer regions revealed 0–10% sequence divergence in the ingroup, and 19.6–21.5% divergence between ingroup and outgroup taxa. Maximum divergence among *Jasione* accessions was 48 character changes (10%) between *J. corymbosa* and *J. sessiliflora*. Several samples had identical sequences across species boundaries (a group of three taxa: *J. crispa* subsp. *mariana* SP10, *J. montana* var. *gracilis* DNA2R and *J. montana* var. *montana* SP25; and a group of seven taxa: *J. montana* var. *montana* DNA25, *J. montana* var. *latifolia* DNA28 and DNA98, *J. montana* var. *gracilis* DNA38, *J. maritima* var. *maritima* DNA49 and *J. maritima* var. *sabularia* DNA79 and DNA89) (Fig. 3).

Phylogenetic analysis

Parsimony analysis of unambiguously aligned ITS sequences yielded six most parsimonious trees. Removal of the gap matrix did not affect tree topology, but resulted in lower branch support values. The tree had a length of 170 steps with a consistency index (CI) of 0.9059. The retention index (RI) was 0.8367, and thus the rescaled consistency index (RC) was 0.758. Bootstrap values for individual clades ranged from 56 to 100% (Fig. 2). The average number of nucleotide substitutions per

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                                  10        20        30        40        50        60        70        80
                           ITS1   .         .         .         .         .         .         .         .
Trachelium caeruleum SP11  AAACGACCCGCGAACACGTT-GAA-CAACACATCCGGGGGGACGTGGATGCTGGACAAGGGCGAGAGCCCTCCA--CGCCC-G--
J.foliosa SP6              -AACGACCCSTGAACACGTTAAGGACAAA-CATCAAGGGGGCATTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.corymbosa SP67           -AACGACCCGTGAACACGTT-AAAACAAA-CATCAAGGGGTGGGTGCA-GGGGGAGAAGGGCGCGAGCCC-CCAAAC-CCCTGCA
J.montana var.bracteosa SP70 -AACGACCCGTGAACACGTT-AAAACAAA-CATCAAGGGGTGGGTGCG-GAGGGAGAAGGGCGCGAGCCC-CCAAAC-CCCTGCA
J.supina SP8               -AACGACCCGTGAACACGTTTAAA-CAAA-CATC-GGGGGGCACTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.sessiliflora 13R         -AACGACCCGTGAACACGTTTAAA-CAAA-CATC-GGGGGGCACTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.crispa ssp.tristis SP12  -AACGACCCNCGAACACGTTTAGG-CAAA--ATC-GGGGGGCGCTGCA-GTGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.crispa ssp.crispa SP1    -AACGACCCGCGAACATGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.crispa ssp.mariana SP10  -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.heldreichii SP7          -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.montana var.montana SP25 -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.montana var.montana DNA25 -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.montana var.latifolia DNA21 -AACGACCCGCGAACATGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.montana var.latifolia DNA28 -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.montana var.latifolia DNA98 -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.montana var.gracilis 2R  -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.montana var.gracilis DNA38 -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.maritima var.maritima DNA49 -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.maritima var.sabularia DNA79 -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.maritima var.sabularia DNA89 -AACGACCCGCGAACACGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA
J.laevis 18R               -AACGACCCGCGAACATGTTTAAA-CAAA--ATC-GGGGGGCGCTGCA-GCGGGAGAAGGGCGAGAGCCC-CCAAAC-CCCTGCA

                                  90        100       110       120       130       140       150       160       170
                                  .         .         .         .         .         .         .         .         .
Trachelium caeruleum SP11  CGGCCCCTCGCTTGCGGCGCCGGAGTTGGCAAGCGAGAGCGCGCGACCTCCGGCTCCCAAGAAACAAACCCCCGACGC--GATCC
J.foliosa SP6              CGCTCCCTTCCTTGCGGTGCCGGTGCGAGCTAGCGTAAGCGAGCGACTGTCGGCACCCAAGAAACGAACCCC-GGCGC--AATCC
J.corymbosa SP67           CGCTCCCTTCCTTGCGGTGCCGGTGCGAGCGAGCGTAAGCGAGCGACTGTCGGCACCCAAGAAACGAACCCCCGGCGC--AGGCC
J.montana var.bracteosa SP70 CGCTCCCTTCCTTGCGGTGCCGGTGCGAGCGAGCGTAAGCGAGCGACTGTCGGCACCCAAGAAACGAACCCCCGGCGC--AGCCC
J.supina SP8               CTCTCCCTTCCTTGTGGTTCCGGCGCGAGCGAGCGTTAGCGAGCGACTGCTGGGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.sessiliflora 13R         CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGCTAGCGAGCGACTGTCGGGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.crispa ssp.tristis SP12  CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGA-TGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.crispa ssp.crispa SP1    CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.crispa ssp.mariana SP10  CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.heldreichii SP7          CTCTCCCTTCCTTGCGGTTTCGGTGCGAGCGAGCGTAAGCGAGCAACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.montana var.montana SP25 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.montana var.montana DNA25 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.montana var.latifolia DNA21 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.montana var.latifolia DNA28 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGYCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.montana var.latifolia DNA98 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.montana var.gracilis 2R  CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.montana var.gracilis DNA38 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.maritima var.maritima DNA49 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.maritima var.sabularia DNA79 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.maritima var.sabularia DNA89 CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
J.laevis 18R                CTCTCCCTTCCTTGCGGTTCCGGTGCGAGCGAGCGTAAGCGAGCGACTGCCGTGACCCAAGAAACGAACCCC-GGCGCAAAATCC
```

FIG. 1. ITS alignment matrix of 21 *Jasione* accessions and the outgroup *Trachelium caeruleum* subsp. *caeruleum*. 18bp and 32bp at the 5′ and 3′ end of ITS1 are excluded. Unresolved bases were coded according to PAUP* 4.0b10 (Swofford, 2002); N = A/C/G/T, S = G or C, Y = C/T.

```
                                  180       190       200       210       220       230       240       250
                                   .         .         .         .         .         .    ITS2 .         .
Trachelium caeruleum SP11         GCGTCAA--AAAACATTTAACTCGAGGGCGTGCCGCCGTCCCGCCGCTCCCGTTCGCGGGCGCGATCGCGTCGCCCCCCCAAACC
J.foliosa SP6                     GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGTCACATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----ATC
J.corymbosa SP67                  GCGCCAAGGAAAACATTAAACTTGAGAGCGTGCTGCCGTCACATCGCCCCCGTTTGCGGGTGCGATCGCGTCGCCCCC----ACC
J.montana var.bracteosa SP70      GCGCCAAGGAAAACATTAAACTTGAGAGCGTGCTGCCGTCACATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----ACC
J.supina SP8                      GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGATGCGATCGCGTCGCCCCC----AAC
J.sessiliflora 13R                GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGATGCGATCGCGTCGCCCCC----AAC
J.crispa ssp.tristis SP12         GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.crispa ssp.crispa SP1           GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.crispa ssp.mariana SP10         GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.heldreichii SP7                 GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.montana var.montana SP25        GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.montana var.montana DNA25       GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.montana var.latifolia DNA21     GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.montana var.latifolia DNA28     GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.montana var.latifolia DNA98     GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.montana var.gracilis 2R         GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.montana var.gracilis DNA38      GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.maritima var.maritima DNA49     GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.maritima var.sabularia DNA79    GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.maritima var.sabularia DNA89    GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC
J.laevis 18R                      GCGCCAAGGAAAACTTTAAACTTGAGAGCGTGCTGCCGGCCCATCGCCCCCGTTCGCGGGTGCGATCGCGTCGCCCCC----AAC

                                      260       270       280       290       300       310       320       330       340
                                       .         .         .         .         .         .         .         .         .
Trachelium caeruleum SP11         ACGGGCACCTCAGGGCGCT-CGCTTGC--TTGGCGGGGAGCGGACATTGGCCTCCCGTGCCCTCGCGGCGCGGCTGGCTCAAAAT
J.foliosa SP6                     TAAAAACGTGCACCCACGTGCGC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCGGGCGCGGCTGGCTAAAATA
J.corymbosa SP67                  TTAAAACGCGCACCCACGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCTGGGCGCGGCTGGCTAAAATA
J.montana var.bracteosa SP70      TTAAAACGCGCACCCACGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCTGGGCGCGGCTGGCTAAAATA
J.supina SP8                      -TAAATCGTGCACCCAGGTGCAC-AGCTTTTGGTGGGGAGCGGATAATGGCCTCCCGTGCC-TCTTGGTGCGGCTGGCTGAAAAT
J.sessiliflora 13R                TGAAATTGTGCACCCAGGCGCAC-TGCTTTTGGTGGGGAGCGGATAATGGCCTCCCGTGC--TCCTGGTGCGGCTGGCTGAAAAT
J.crispa ssp.tristis SP12         TGAAATCGTGCGCCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.crispa ssp.crispa SP1           TTAAATCGTGCACCCAGGTGCGC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.crispa ssp.mariana SP10         TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.heldreichii SP7                 TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.montana var.montana SP25        TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.montana var.montana DNA25       TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.montana var.latifolia DNA21     TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.montana var.latifolia DNA28     TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.montana var.latifolia DNA98     TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.montana var.gracilis 2R         TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.montana var.gracilis DNA38      TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.maritima var.maritima DNA49     TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.maritima var.sabularia DNA79    TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.maritima var.sabularia DNA89    TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
J.laevis 18R                      TTAAATCGTGCACCCAGGTGCAC-TGCTTTTGGTGGGGAGCGGATATTGGCCTCCCGTGCC-TCCTGGTGCGGCTGGCTGAAAAT
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FIG. 1. (*Cont'd*).

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                                  350        360        370        380        390        400        410        420
                                   .          .          .          .          .          .          .          .
Trachelium caeruleum SP11         TGAGTCCCCCT-CGGAGGACGCACGACAAGTGGTGGTTGATAACAAGGCCCTCGCGTCCCGTCGTGCGGCAACCCTTCGCGGGAT
J.foliosa SP6                     GGAGACCCC-TGCGGAGGATGCACGACAAGTGGTGGTTGATAACAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTCTGCGGGAA
J.corymbosa SP67                  AGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAACAAGGCCCT---GTTCCGTCGTGTGCCACTCCTCTGCAGGAA
J.montana var.bracteosa SP70      AGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAACAAGGCCCT---GTTCCGTCGTGTGCCACTCCTCTGCAGGAA
J.supina SP8                      GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.sessiliflora 13R                GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.crispa ssp.tristis SP12         GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.crispa ssp.crispa SP1           GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.crispa ssp.mariana SP10         GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.heldreichii SP7                 GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.montana var.montana SP25        GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.montana var.montana DNA25       GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.montana var.latifolia DNA21     GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.montana var.latifolia DNA28     GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.montana var.latifolia DNA98     GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.montana var.gracilis 2R         GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.montana var.gracilis DNA38      GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.maritima var.maritima DNA49     GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.maritima var.sabularia DNA79    GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.maritima var.sabularia DNA89    GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA
J.laevis 18R                      GGAGACCCC-TGCGAAGGATGCACGACAAGTGGTGGTTGATAATAAGGCCCTCGCGTCCCGTCGTGTGCCAATCCTTTGCAGGAA

                                      430        440        450        460        470        480        490        500
                                       .          .          .          .          .          .          .          .
Trachelium caeruleum SP11         TCGGCTCGTCGCAAGTGACCCTGGCGACGCA-CCT-TCACGATCCATCGCGCAAGCGGTGGTCGATGACGGATGCGCTCCGA
J.foliosa SP6                     T-GGCTC-T-AAAAGTGACCCTGTTGAGGCATCCTCCCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.corymbosa SP67                  T-GGCTC-T-ATAAGTGACCCTTTTGAGGCATCCTCCCAGGCACAAACGC--AAGTTGTGGCTGGCGATGG-TGCGCTCCGA
J.montana var.bracteosa SP70      T-GGCTC-T-AAAAGTGACCCTTTTGAGGCATCCTCCCAAGCACAAACGC--AAGTTGTGGCTGGCGATGG-TGCGCTCCGA
J.supina SP8                      T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCCCAAGCGCACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.sessiliflora 13R                T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCTCAAGCGCACACGC--AAGTTGTGGTTGGCGATGGGTGCCCTCCGA
J.crispa ssp.tristis SP12         T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.crispa ssp.crispa SP1           T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGCTGCGCTCCGA
J.crispa ssp.mariana SP10         T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.heldreichii SP7                 T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.montana var.montana SP25        T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.montana var.montana DNA25       T-GGCTC-T-GAAAGTGACCCTGTTGAAGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.montana var.latifolia DNA21     T-GGCTC-T-GAAAGTGACCCTGTTGAAGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.montana var.latifolia DNA28     T-GGCTC-T-GAAAGTGACCCTGTTGAAGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.montana var.latifolia DNA98     T-GGCTC-T-GAAAGTGACCCTGTTGAAGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.montana var.gracilis 2R         T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.montana var.gracilis DNA38      T-GGCTC-T-GAAAGTGACCCTGTTGAAGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.maritima var.maritima DNA49     T-GGCTC-T-GAAAGTGACCCTGTTGAAGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.maritima var.sabularia DNA79    T-GGCTC-T-GAAAGTGACCCTGTTGAAGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.maritima var.sabularia DNA89    T-GGCTC-T-GAAAGTGACCCTGTTGAAGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGGGTGCGCTCCGA
J.laevis 18R                      T-GGCTC-T-GAAAGTGACCCTGTTGAGGCACCCTCTCAAGCACACACGC--AAGTTGTGGTTGGCGATGG-TGC???????
```

FIG. 1. (*Cont'd*).

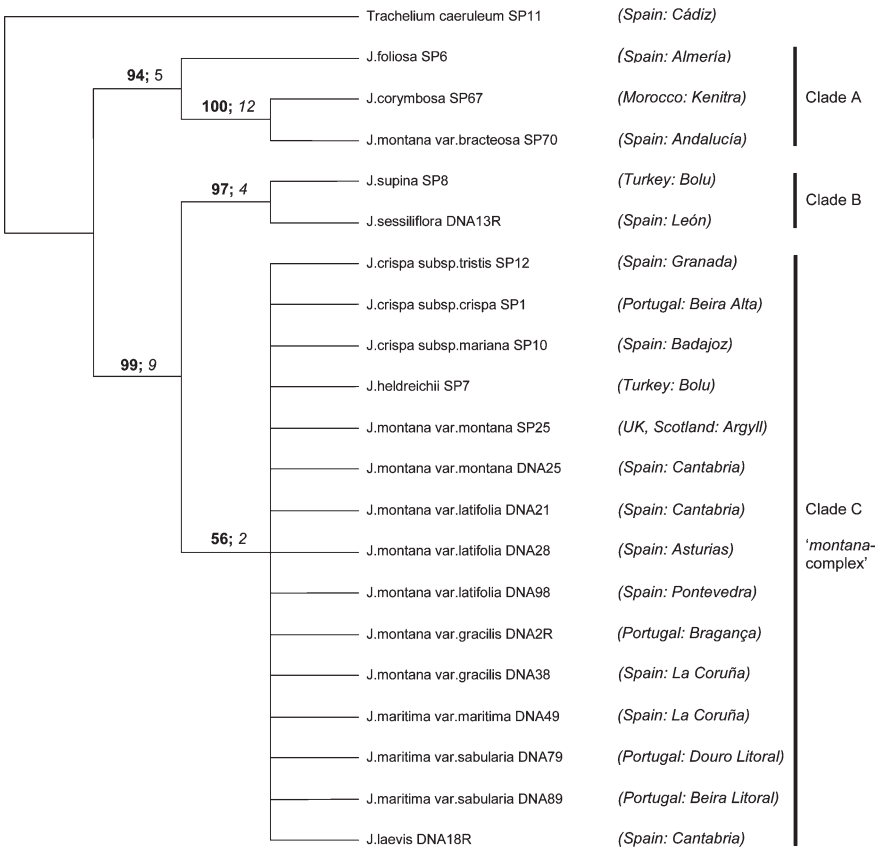

FIG. 2. Strict consensus tree based on six most parsimonious trees for 21 *Jasione* taxa and the outgroup *Trachelium caeruleum* subsp. *caeruleum* of 181 steps based on combined ITS1 and ITS2 sequence data plus alignment gap matrix. Numbers in bold are bootstrap values of 10,000 replicates; numbers in italics are decay indices.

character was, at 0.33, very low, indicating a very low saturation of base substitutions across the matrix. It is therefore unlikely that distortion of the phylogenetic signal due to reversal and double hits occurred.

The taxa formed two main clades: clade A and a second clade including clades B and C. Clade A consisted of *Jasione foliosa*, *J. corymbosa* and *J. montana* var. *bracteosa* (BS = 94%, DI = 5). Within this clade *J. corymbosa* and *J. montana* var. *bracteosa* formed a sister pair to *J. foliosa* (BS = 100%, DI = 12). Clade B consisted of *J. supina* and *J. sessiliflora* (BS = 97%, DI = 4) which was sister to the 'crown group' we term the '*montana*-complex' (clade C), including the remainder of the samples on a polytomy (BS = 56%, DI = 2).

However, within the '*montana*-complex' polytomy, the result of identical sequences (hard polytomy) and conflicting phylogenetic signals among the most parsimonious trees (soft polytomy), there was some variation in branch length, with

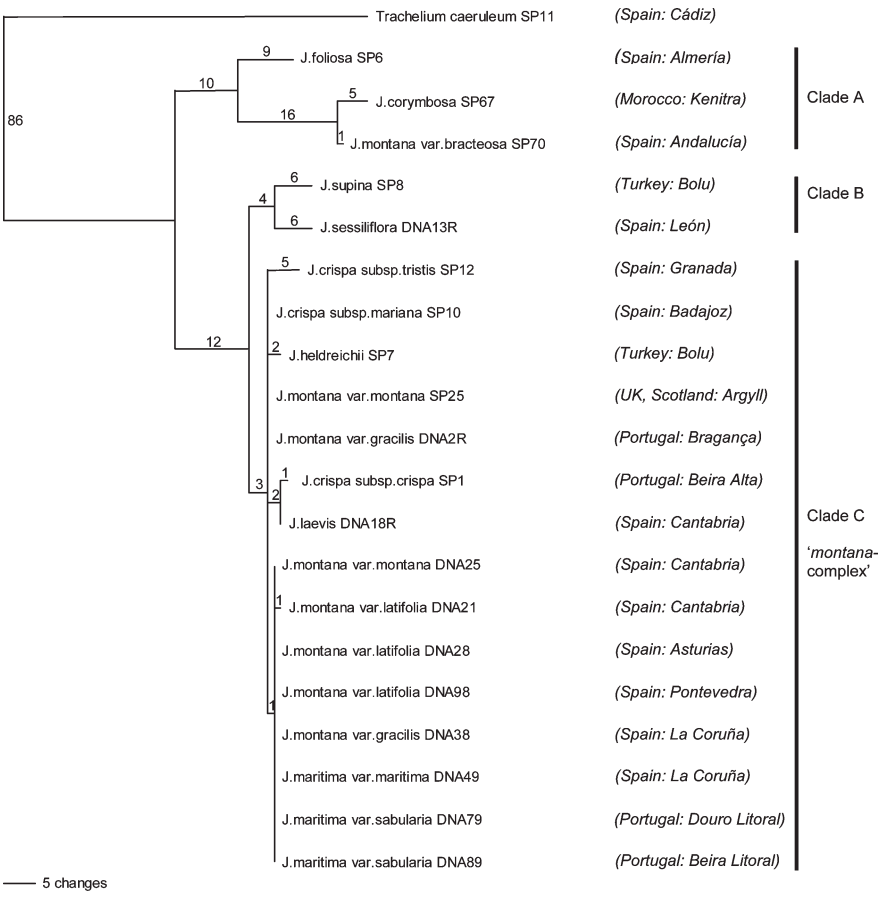

FIG. 3. Phylogram of one of six most parsimonious trees for 21 *Jasione* taxa and the outgroup *Trachelium caeruleum* subsp. *caeruleum* of 181 steps based on combined ITS1 and ITS2 sequence data plus alignment gap matrix. Numbers indicate number of changes shared among taxa.

Jasione crispa subsp. *tristis* possessing, with five steps, the longest terminal branch (Fig. 3).

AFLP analysis

A total of 156 clearly distinguishable AFLP fragments were generated with the three primer combinations; 99 fragments (63%) were present in all samples and 57 (37%) generated unambiguously variable bands. All 47 *Jasione* accessions revealed unique multilocus genotypes, except for samples 42 and 45 of *J. montana* var. *montana* from the Shetland Islands.

Jaccard indices showed that the average genetic distance was lowest in the *Jasione montana* var. *montana* populations from the UK, with 0.0728, 0.0785 and 0.0908 for

the samples from Argyll, the Shetland Islands and Devon, respectively (Table 4). The populations from Spain showed, at 0.1342, a much higher value. For sympatric samples across taxa from the localities in Spain (Lariño & Lira and Cabo Finisterre) a slightly lower genetic distance was observed (0.1242 and 0.1303, respectively). The greatest genetic distance was among *J. montana* var. *gracilis* samples from Spain (0.1504). Populations of *J. montana* var. *latifolia, J. maritima* var. *maritima* and *J. maritima* var. *sabularia* from Spain and Portugal showed intermediate genetic distances of 0.1198, 0.1106 and 0.1215, respectively.

Figure 4 shows the UPGMA tree. In the phenogram, accessions of *Jasione maritima* var. *sabularia* clustered together (BS = 85%), appearing most distant from the rest of the samples. *Jasione maritima* var. *maritima* individuals also clustered together and were clearly separated from the *J. montana* accessions. Samples of *J. montana* var. *gracilis* repeatedly clustered with samples of *J. montana* var. *montana* from Spain, the latter appearing scattered across the tree. The six individuals of *J. montana* var. *montana* from Argyll formed a cluster very close to that containing *J. montana* var. *montana* from Spain and *J. montana* var. *gracilis*, but distant from samples from the other British localities (Devon and the Shetland Islands). Samples of *J. montana* var. *montana* from the Shetland Islands clustered with some accessions of *J. montana* var. *montana* from Spain. Individuals of *J. montana* var. *latifolia* formed a separate cluster close to a mixed cluster containing *J. montana* var. *montana* from Devon and Spain and some *J. montana* var. *gracilis* samples.

TABLE 4. Average genetic distances within *Jasione* groups and localities, based on AFLP data expressed as Jaccard index

Taxon	AFLP samples	Jaccard index	SD
J. montana var. *gracilis*	Different populations, gracilis Spain-1 to 6	0.1504	0.036
J. montana var. *montana*	Different populations, montana Spain-7 to 12	0.1342	0.035
J. montana var. *montana*	Single population, montana Argyll-13 to 18	0.0728	0.031
J. montana var. *latifolia*	Single population, latifolia-19 to 24	0.1198	0.022
J. maritima var. *sabularia*	Single population, maritima-25 to 29	0.1215	0.034
J. maritima var. *maritima*	Single population, maritima-30 to 35	0.1106	0.030
J. montana var. *montana*	Single population, montana Devon-36 to 41	0.0908	0.028
J. montana var. *montana*	Single population, montana Shetland-42 to 47	0.0785	0.043
J. montana var. *gracilis*	Lariño & Lira, Spain-2 to 4	0.1242	0.030
J. montana var. *montana*	Lariño & Lira, Spain-7 to 9		
J. montana var. *gracilis*	Cabo Finisterre, Spain-5, 6	0.1303	0.036
J. montana var. *montana*	Cabo Finisterre, Spain-10, 11		

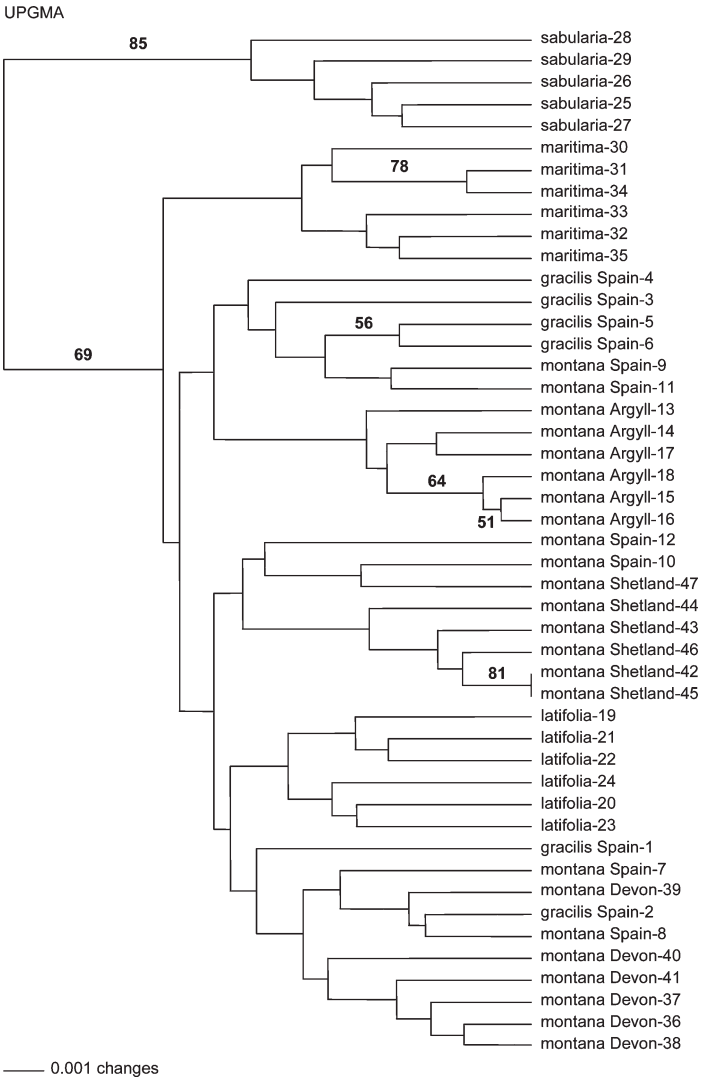

FIG. 4. UPGMA phenogram of 47 samples of populations of *Jasione montana* and *J. maritima* based on Nei & Li (1979) genetic distances of the AFLP data set. Sample codes are given in Table 2. Numbers above branches are bootstrap values of 1000 replicates.

PCO analysis

In the PCO analysis, the first three principal coordinates accounted for 35% of the total variance (PCO1 for 15.6%, PCO2 for 10.7% and PCO3 for 8.7%). Figure 5 shows a three-dimensional plot of the first three principal coordinates. *Jasione maritima* var. *sabularia* and *J. maritima* var. *maritima* formed clearly distinguishable clusters separate from the varieties of *J. montana*. Accessions of *J. maritima* var. *sabularia* clustered very distantly from the rest of the samples under study, while

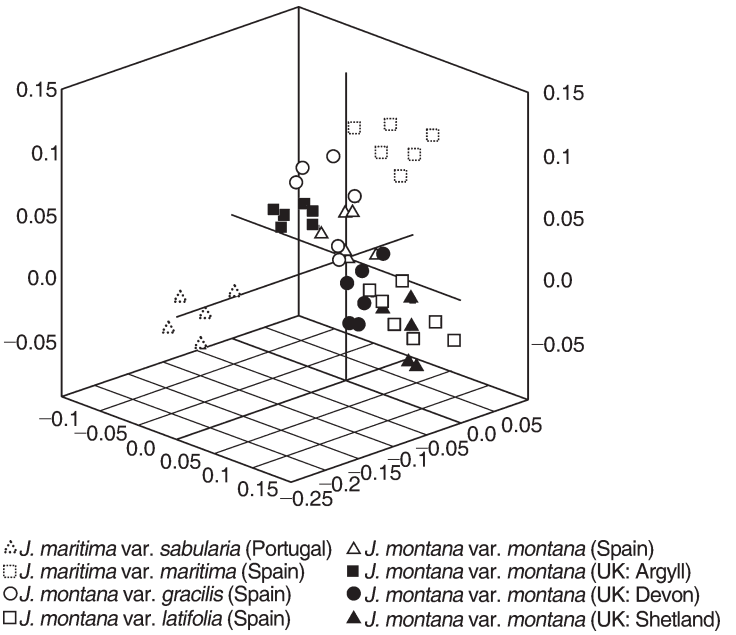

FIG. 5. Three-dimensional PCO plot of 47 samples of populations of *Jasione montana* and *J. maritima* based on Jaccard's coefficient of the community of the AFLP data.

those of *J. maritima* var. *maritima*, although forming a separate cluster opposite and most distant from *J. maritima* var. *sabularia*, were closer to the *J. montana* cluster, in particular to *J. montana* var. *gracilis*.

The *Jasione montana* varieties formed a loose aggregation of subclusters positioned between *J. maritima* var. *sabularia* and var. *maritima*. Genetic distances between samples of *J. montana* var. *gracilis* were greater than in any of the other groups, and overlapped greatly with *J. montana* var. *montana* from Spain. As in the UPGMA tree, the British samples from Argyll, Devon and the Shetland Islands fell across the *J. montana* cluster, but did not overlap each other. The other *J. montana* variety, *J. montana* var. *latifolia* collected in Spain, overlapped greatly with samples of *J. montana* var. *montana* from the Shetland Islands but not with those from Spain.

DISCUSSION

ITS evolution and phylogeny

Several DNA substitutions and insertion–deletion events in the ITS region have occurred in the *Jasione* species under study. Maximum sequence divergence within *Jasione* was 10%, which is at the lower end of infrageneric levels found in angiosperms. In genera of *Asteraceae* subtribe *Madiinae* and in the *Polemoniaceae*, the figures are 17.55% and 21.7%, respectively (Baldwin, 1992), and in *Streptocarpus*

(*Gesneriaceae*) 23.5% (Möller & Cronk, 1997a). Thus ITS was only partially successful in determining interspecific relationships in 15 out of the 25 *Jasione* taxa studied.

Two clades, A and B, were resolved, each with just a few taxa, while the majority of taxa resided in a large polytomy, clade C (Fig. 2). ITS sequence analysis has been used successfully for infrageneric classification in many taxonomic groups (Baldwin *et al*., 1995; Möller & Cronk, 2001) but the presence of unresolved groups in ITS phylogenies is also known from other angiosperm studies (Sang *et al*., 1994; Zhang *et al*., 2001), and has been associated with rapid radiation and a relatively recent origin of the taxa involved (Möller & Cronk, 1997b). The polytomy in *Jasione*, and the short branch lengths of the many taxa included there, indicate a lack of molecular divergence that could suggest recent speciation. This interpretation is supported by the fact that several samples of different *Jasione* taxa had identical ITS sequences (e.g. *J. crispa* subsp. *mariana* SP10 from Spain and *J. montana* var. *montana* from Scotland). A speciation rate higher than a molecular ITS evolution rate would explain the small degree of divergence in the ITS sequences (Möller & Cronk, 1997b), an example of punctuated equilibrium *sensu* Bateman (1999), where morphological changes outpace molecular change. Despite the many taxa with unresolved relationships involved in the polytomy, some variation within this group was observed in terms of branch lengths on phylograms (Fig. 3). The accession of *J. crispa* subsp. *tristis* had the longest terminal branch with five base changes, making it the most distinct taxon in the polytomy.

Jasione foliosa, *J. corymbosa* and *J. montana* var. *bracteosa* formed a sister clade to all the other taxa. These three grow in the southern Iberian Peninsula and N Africa, suggesting possible survival in refugia in these areas during Pleistocene glaciations. Although there is no fossil evidence in *Jasione*, the southern Iberian Peninsula has been considered a glacial refugium for many other species, including herbs (Comes & Abbott, 1998; Tollefsrud *et al*. 1998; Zhang *et al*., 2001; Kropf *et al*., 2002).

Jasione foliosa, *J. corymbosa* and *J. montana* var. *bracteosa* share the absence of true trichoids, expansions on the leaf surface of uncertain origin and function (Bokhari & Sales, 2001), which are present in all the other taxa analysed here, indeed in the whole genus. Of these three putatively more ancestral taxa, *J. foliosa* is morphologically the most distinct (together with its closest relative *J. mansanetiana*, which was not available for this study), because of its loose inflorescence and total absence of trichoids on leaf margins (Sales & Hedge, 2001b). The loose inflorescence is often considered primitive, yet in our ITS phylogeny it could also be interpreted as derived. From our results it is equally parsimonious to consider the loose inflorescence as an autapomorphy and the compact inflorescence as primitive, or to regard it as ancestral with repeated evolution of compact inflorescences.

In addition to the characteristic inflorescence, the leaf anatomy of *Jasione foliosa* is very distinct. *Jasione corymbosa* and *J. montana* var. *bracteosa* seem to occupy an intermediate position between taxa with and without true trichoids. *Jasione*

corymbosa and *J. montana* var. *bracteosa* both lack true trichoids but possess tooth-like processes that seem to be vascularized as the true trichoids are. The presence and development of leaf trichoids may be of more importance than is indicated in the *Flora iberica* account (Sales & Hedge, 2001b). Such structures should be fully investigated to assess their function and adaptive value. *Jasione corymbosa* has sometimes (e.g. in Greuter *et al*., 1984) been regarded as a subspecies of *J. montana* although, anatomically at least, it is quite distinct (Bokhari & Sales, 2001). Our ITS phylogeny clearly supports these anatomical findings and the status of *J. corymbosa* as a species in *Flora iberica* (Sales & Hedge, 2001b). The distant phylogenetic position of *J. montana* var. *bracteosa* from the rest of the *J. montana* samples clearly illustrates the conflicting data that can emerge from morphological and molecular evidence. It is true, though, that *J. montana* var. *bracteosa* is the most distinct Iberian variety of this species and it has been related to *J. corymbosa* subsp. *cornuta* (Sales & Hedge, 2001a). In the field it can be easily confused with *J. penicillata*, unfortunately not available for this study. Based on its molecular distinctness, and if reinforced by a better understanding of the evolutionary significance of the leaf trichoids, a higher taxonomic status for *J. montana* var. *bracteosa* might be justified.

Sister to the polytomy, clade B containing the Turkish *Jasione supina* and the Spanish accession of *J. sessiliflora* (also in N Africa) is difficult to interpret in terms of the present geographical distribution. However, similar east–west Mediterranean disjunctions have been found in other *Campanulaceae* genera: for example *Campanula primulifolia* Brot. occurs in the SW of the Iberian Peninsula, *C. alata* Desf. in N Africa and *C. peregrina* L. in Turkey (Trias Blasi, 2005). *Jasione supina* and *J. sessiliflora* are more or less caespitose perennials with sessile flowers and can grow in rocky places, *J. supina* at higher altitudes around 2000m (Damboldt, 1978) and *J. sessiliflora* at lower altitudes around 600m (Sales & Hedge, 2001b). The two species have different chromosome numbers, *J. supina* having $2n = 12$ and *J. sessiliflora* $2n = 24$ (Damboldt, 1978), suggesting that the latter is of polyploid origin. Studies of plants growing in the central Mediterranean, for example *J. sphaerocephala* in Italy, halfway between *J. sessiliflora* and *J. supina*, might yield important information.

In the polytomy, clade C, the genetic closeness of varieties of *Jasione montana* and *J. maritima* was not a total surprise. The latter has often been considered a coastal sand-dune form of the inland *J. montana* (Parnell, 1980, 1982). It was however surprising that *J. laevis* was also part of the polytomy. Though it has many times been confused with *J. montana* on inadequate herbarium specimens, it is perennial and, apparently, always stoloniferous (Sales & Hedge, 2001b). The Turkish accessions of *J. heldreichii* Boiss. & Orph. also fell in the *montana*-complex polytomy. *Jasione heldreichii* is morphologically similar to *J. montana* and in older classifications was considered a variety of it (*J. montana* var. *dentata* A.DC.; De Candolle, 1839). *Jasione crispa* subsp. *tristis* is the most distinct taxon in the ITS polytomy. It grows at altitudes from 1700 to c.3500m and is endemic to the Sierra Nevada in southern Spain (Sales & Hedge, 2001b). Interestingly, chromosome counts of $n = 9$ (Baltisberger & Charpin, 1989) and of tetraploids with $2n = 36$ (Küpfer & Favarger,

1967) have been obtained for this taxon. A haploid number of $n=9$ would be unique in *Jasione* and may be linked to its relatively distinct ITS sequences. However, further confirmation of these chromosome counts is needed.

To understand better the evolution of the genus, further morphological and molecular studies, using material of *Jasione* throughout its total range, are necessary. Inclusion of the taxa not included here would provide better understanding of interspecific relationships. Furthermore, the use of additional molecular markers, which evolve faster than ITS, to increase the resolution of the phylogenetic tree would be desirable. However, a gene faster than ITS might increase the difficulties of alignment with potential outgroups; such difficulties were already encountered using the ITS sequences here.

Despite the limitations of this study, the fact that various *Jasione* species fell in unresolved polytomy reflects the taxonomic complexity of *Jasione* and the need to review the taxonomical rank of some of the taxa considered here.

AFLPs

In contrast with the very low ITS sequence variation, the AFLP data revealed a high level of genetic variation between the species and varieties, and also within the small population samples.

The significantly greater genetic distances in *Jasione montana* var. *montana* samples from Spain compared with that of populations from the UK can be explained by the wider sampling area covered for the Spanish group; these basically represented samples from three different populations. The same holds true for the samples of *J. montana* var. *gracilis*. Both sample groups included taxa from the same localities in La Coruña (Lariño & Lira and Cabo Finisterre). It was however significant that the level of variation in groups arranged by locality was slightly lower than in those arranged by taxon. This may be an artefact of the low sampling number, but may also indicate that genetic distance reflects geography rather than taxonomy (see below).

Three main clusters were found in the UPGMA trees and the PCO plots (Figs 4 and 5). In order of decreasing similarity these were:

1 All the accessions of *J. maritima* var. *sabularia*
2 All the accessions of *J. maritima* var. *maritima*
3 The three varieties of *J. montana*.

The most distinct group revealed by AFLPs, in both UPGMA trees and PCO plots, consisted of all the accessions of *Jasione maritima* var. *sabularia*. Morphological characters that differentiate *J. maritima* var. *sabularia* from var. *maritima* are very few, such as leaf shape and the presence of a denser indumentum (Sales & Hedge, 2001b). The two varieties grow on maritime sand-dunes and do not show any stem or leaf anatomical differences (Bokhari & Sales, 2001). Nevertheless, *J. maritima* var. *sabularia* has, with current knowledge, a distinct distribution, restricted to the

shores of NW Portugal (Sales & Hedge, 2001a,b), while *J. maritima* var. *maritima* is in NW Spain, on few beaches in N Spain and in W France as far north as Gironde. Based on the AFLP data, the taxonomic rank of *J. maritima* var. *sabularia* merits reconsideration. Any taxonomic change might involve conservation issues as it is a vulnerable endemic Portuguese taxon growing in seaside resorts.

The accessions of *Jasione maritima* var. *maritima* also formed a clearly separate cluster in both the UPGMA tree (Fig. 4) and the PCO plot (Fig. 5), but were closer to *J. montana* than to *J. maritima* var. *sabularia*. Although *J. maritima* has often been linked with *J. crispa* and *J. montana*, our results indicated that *J. maritima* var. *sabularia* is genetically distinct from *J. maritima* var. *maritima* and *J. montana*. Further morphological work is required to assess whether it deserves recognition as a species.

In the third cluster, *Jasione montana*, there was an interesting partial grouping of the three varieties studied in both the UPGMA tree and the PCO plot. This may reflect lower genetic differentiation between the samples or is possibly an artefact due to the small scale of the study. However, some patterns may be of note. The fact that all British samples of *J. montana* var. *montana* appeared very close to samples of the same variety from Spain may indicate that this region is the original source of the British *J. montana* stock. During the last glaciation (~ 18,000 years ago), when sea levels dropped, migrations of *Jasione* from an Iberian Peninsula refugium into Britain probably occurred. This has been demonstrated for other plants such as *Alnus glutinosa* (L.) Gaertn. (King & Ferris, 1998), *Pinus sylvestris* L. (Sinclair *et al*., 1999) and *Asplenium ceterach* L. (Trewick *et al*., 2002). The scattered genetic patterning of *J. montana* var. *montana* from Devon, Argyll and the Shetland Islands may be explained by a common gene pool becoming fragmented, but with insufficient time for the British groups to have differentiated from the Iberian progenitors. The abundant colonization of *Jasione* on the Shetland Islands is more difficult to explain, especially because the plant is relatively uncommon in the nearby Orkney Islands to the south.

The intermixing of *Jasione montana* var. *gracilis* with var. *montana* in the UPGMA tree and the PCO plot indicates that both come from the same genetic stock and that *J. montana* var. *gracilis* may be just a repeating segregant of *J. montana*. The fact that var. *gracilis* can be found among var. *montana* populations (e.g. DNA samples gracilis-2, 3, 4 with montana-7, 8, 9, and gracilis-5, 6 with montana-10, 11) and is only distinguished by its weaker habit and late flowering time (Sales & Hedge, 2001b) supports this idea. Var. *gracilis* could be interpreted as an ecological adaptation of *J. montana*, extending the seed production and dispersal period into higher summer.

Jasione montana var. *latifolia* is recorded from the N and NW of the Iberian Peninsula and the British Isles, especially the Shetland Islands. It is usually characterized by broad flat leaves and many lateral fertile stems. In our study *J. montana* var. *latifolia*, from Spain, appeared surprisingly scattered and close to *J. montana* var. *montana* from Devon and the Shetland Islands. These results may

indicate that *J. montana* var. *latifolia* is a segregate from a *J. montana* var. *montana* stock, but with morphological differences. Parnell's multivariate analyses based on morphological data also clearly distinguished var. *latifolia* from var. *montana*, even when both were cultivated under controlled environmental conditions (Parnell, 1980, 1987).

Conclusions

This study was mainly concerned with Iberian taxa of *Jasione*, but the results proved to be of broader interest for the genus as a whole, especially in throwing new light on the problems of interrelationships of species and infraspecific taxa. Much of the molecular evidence supported the recent classification in *Flora iberica* (Sales & Hedge, 2001b), though some contradicted it, and some gave surprising insights into possible alliances. An example of the latter was the linking of *Jasione corymbosa* with *J. foliosa*, forming with *J. montana* var. *bracteosa* one of the main clades in the phylogeny. All previous classifications based on morphology placed *J. foliosa* far distant from the other two species, indeed even in different subgenera due its distinct inflorescence. Our results suggest that the taxonomic rank of *J. montana* var. *bracteosa* needs to be reassessed. Further, the AFLP data strongly suggested that *J. maritima* var. *sabularia* is distinct enough from var. *maritima* to merit species rank, but more morphological work is required before any taxonomic decision is made. Of particular interest was the fact that the three British samples named *J. montana* var. *montana* (from Devon in SW England, Argyll in W Scotland and the Shetland Islands) were genetically distant from each other but close to Spanish samples so-named.

Although caution is needed when interpreting these results due to the limitations of the small data set, particularly those of the AFLP analysis, our study has proved useful in the assessment of inter- and infraspecific variation of *Jasione* and provided useful information for adjustments in the classification of the genus.

In future studies, an increased number of individuals per population and number of populations, together with more primer combinations and thus markers, should provide a clearer and more reliable picture of the genetic variation within and between *Jasione* taxa.

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