Annals of Botany **98**: 857–868, 2006
doi:10.1093/aob/mcl167, available online at www.aob.oxfordjournals.org

Karyotype Variation, Evolution and Phylogeny in *Borago* (Boraginaceae), with Emphasis on Subgenus *Buglossites* in the Corso-Sardinian System

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Received: 1 March 2006 Returned for revision: 11 April 2006 Accepted: 12 June 2006 Published electronically: 25 July 2006

• *Background and Aims* Karyological variation in the Mediterranean genus *Borago* and cytogeography of subgenus *Buglossites* in Corsica, Sardinia and the Tuscan Archipelago were investigated in combination with a molecular phylogenetic analysis aimed at elucidating relationships between subgenera and taxa with different chromosome features.
• *Methods* Karyotype analysis was performed on population samples of *B. pygmaea*, *B. morisiana*, *B. trabutii* and *B. officinalis*. Phylogenetic analyses were based on ITS1 nrDNA and *mat*K cpDNA sequences.
• *Key Results* Four base numbers were found, $x = 6$, 8, 9 and 15, and three ploidy levels based on $x = 8$. In subgenus *Buglossites* the Sardinian endemic *B. morisiana* is diploid with $2n = 18$, while *B. pygmaea* includes three allopatric cytotypes with $2n = 30$ (Sardinia), $2n = 32$ (southern Corsica) and $2n = 48$ (central northern Corsica and Capraia). In subgenus *Borago*, the Moroccan endemic *B. trabutii* and the widespread *B. officinalis* have $2n = 12$ and $2n = 16$, respectively. Molecular data support the monophyly of *Borago*, while relationships in subgenus *Borago* remain unclear. *Borago trabutii* appears as the earliest divergent lineage and is sister to a clade with *B. officinalis*, *B. morisiana* and *B. pygmaea*. Subgenus *Buglossites* is also monophyletic, but no correspondence between ITS1 phylogeny and *B. pygmaea* cytotypes occurs.
• *Conclusions* Chromosome variation in *Borago* is wider than previously known. Two base numbers may represent the ancestral condition in this small genus, $x = 6$ or $x = 8$. An increase in chromosome number and karyotype asymmetry, a decrease in chromosome size and heterochromatin content, and the appearance of polyploidy are the most significant karyological changes associated with the divergence of the *Buglossites* clade. High ITS1 variation in the tetra- and hypotetraploid races of *B. pygmaea* suggests a multiple origin, while the lower polymorphism of the hexaploid race and its allopatric distribution in the northernmost part of the range is better explained with a single origin via union of unreduced and reduced gametes.

Key words: Boraginaceae, *Borago*, chromosomes, cytogeography, ITS1, karyotype, *matK*, molecular phylogeny polyploidy.

INTRODUCTION

Borago L. is the 'type' genus of Boraginaceae tribe Boragineae Bercht. & J. Presl, a group of 16 genera native to the Old World and distributed in Asia, Africa and Europe (Hilger *et al*., 2005). A recent phylogenetic analysis based on plastid and nuclear molecular markers suggested that *Borago* represents a monophyletic clade sister to *Symphytum* L., within a most basal taxon-group of the tribe (Hilger *et al*., 2004). In contrast to *Symphytum*, however, *Borago* is a small genus with only five species, four of which are restricted to the south-western Mediterranean basin in north-west Africa, Corsica, Sardinia and the Tuscan Archipelago. Only the common borage, *B. officinalis* L., is widespread beyond the boundaries of the Euro-Mediterranean region as a wild weed or cultivated as a garden plant, crop vegetable or pharmaceutical herb.

According to Guşuleac (1928), *Borago* is split into two subgenera representing distinct evolutionary lineages, a continental one in North Africa and an insular one in Corsica and Sardinia. Subgenus *Borago* contains the type species *B. officinalis* plus *B. trabutii* Maire, endemic to the High and Anti-Atlas of Morocco, and *B. longifolia* Poir. endemic to northern Algeria and Tunisia. These three species share the erect habit and the showy cymes of blue flowers with rotate corollas and robust, exserted faucal scales and stamens, characters which represent adaptations for allogamic pollination by means of Hymenoptera. *Borago officinalis* is mainly an outbreeding species (Leach *et al*., 1990), although evidence of self-compatibility has also been established (Montaner *et al*., 2000). Subgenus *Buglossites* (Moris) Guşul. is restricted to Corsica and Sardinia, as well as a single locality in the Tuscan Archipelago (Capraia Island). It comprises *B. pygmaea* (DC.) Chater & Greuter, endemic to Sardinia, Corsica and Capraia, and *B. morisiana* Bigazzi & Ricceri, described from the small island of San Pietro in south-western Sardinia (Bigazzi and Ricceri, 1992). Both species are hemicryptophytic and have decumbent stems bearing small flowers with white (*B. morisiana*) or pale blue (*B. pygmaea*), campanulate corollas with stamens hidden inside and no clear adaptations for entomophilous pollination. There are no published data about reproductive systems in these two species. Ecologically they are linked to constantly humid, often shady habitats, such as wet rocks, permanent springs, drippings, margins of rivulets and small stretches of riparian *Alnus* woods, from sea level to approx. 1000 m a.s.l. (Arrigoni, 1980; Gamisans and Marzocchi,

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2005). Unlike species of subgenus *Borago*, which are exclusively myrmechocorus (cf. Hegi, 1975), seed dispersal in *Buglossites* is primarily by means of water and secondarily by means of ants, usually for short distances and within restricted biotopes (personal observation).

The narrow range, ecological specialization and the low competitive ability of these two species are in contrast with the condition of the ubiquist, ruderal, xerophytic and annual weed of *B. officinalis*, which suggested a 'derived' character for the latter and a relict character of paleoendemic for subgenus *Buglossites* (Guşuleac, 1928; Contandriopoulos, 1962; Pignatti, 1982; Bigazzi and Ricceri, 1992). Under this assumption and in the absence of more data, however, it was difficult to make hypotheses on the origin of the tetraploid chromosome complement reported for *B. pygmaea* (Strey, 1931; Contandriopoulos, 1962; Diana Corrias, 1980) compared with the diploid karyotype of *B. officinalis* (D'Amato and Marchi, 1983; Luque, 1989). The report $2n = 16$ in *B. morisiana* (here shown to be erroneous) indicated the existence of variation within *Buglossites*, at least in ploidy level, and suggested a possible derivation of the tetraploid *B. pygmaea* through polyploidization events not involving the taxa of subgenus *Borago* (Bigazzi and Ricceri, 1992). As in many angiosperm groups, polyploidy and other types of chromosomal processes are a major driving force of species formation in several Boragineae genera, among which *Symphytum*, *Cynoglottis*, *Pulmonaria, Nonea* and others (Selvi and Bigazzi, 2002; Hilger *et al*., 2004). In consideration of the lack of knowledge on this subject, a comparative karyological and cytogeographical investigation was undertaken to understand better the role of polyploidy and other types of chromosomal variation in the little-known evolutionary history of *Borago*. This survey brought to light an unexpected range of inter- and intraspecific variation in terms of ploidy levels and haploid numbers, suggesting the use of molecular tools to elucidate relationships between taxa with different base numbers, using the *matK* region of the chloroplast genome, and between species and infraspecific cytotypes, using the more variable ITS1 sequences of the nuclear ribosomal DNA. The usefulness of both markers in genus- and species-level systematics and phylogeny of Boraginales has been widely demonstrated in recent studies (Diane *et al.*, 2002; Winkworth *et al*., 2002; Hilger *et al.*, 2004; Selvi *et al.*, 2004). The results of this work are presented and discussed in this paper, as they may contribute to a better understanding of relationships in *Borago* and of the *Buglossites* phylogeography in the main insular system of the western Mediterranean.

MATERIALS AND METHODS

Plant material

The only two native populations of *B. morisiana* known to date and 12 populations of *B. pygmaea* were sampled in the field by transplanting three to five young plants for each population into small pots that were then transferred to the Botanical Garden of the University of Florence, Italy. A more intensive sampling was neither possible nor advisable in view of the rarity and often small population size of these endemics. In the case of *B. pygmaea*, the five Sardinian populations analysed were from the three main distribution sub-areas on the island, i.e. in the south, centre and north (Arrigoni, 1980). Likewise, the six Corsican populations were from distant sites in the southern, central and northern parts of the island. Finally, one (the only one existing today) was from Capraia Island in the Tuscan Archipelago. In addition, one accession of *B. officinalis* and one of *B. trabutii* from personal field collections obtained in central Italy and Morocco, respectively, were examined. The geographical origins of all the accessions are given in Table 1; voucher specimens were deposited in the Herbaria of Florence and Cagliari Universities (FI and CAG, respectively). Silica-gel dried samples of leaf tissue were prepared for each of these populations for molecular analyses.

Karyotype analysis

At least ten root tips were collected in early spring and/or autumn from each cultivated plant of *B. morisiana* and *B. pygmaea* (perennials), and from numerous germinating seeds in the case of the annuals *B. officinalis* and *B. trabutii*. In total, 30–50 root tips were examined for each accession. They were pretreated with 0·002 M 8-hydroxyquinoline or 0·05 % colchicine, 2·5 h at room temperature and then fixed overnight in ethanol : glacial acetic acid (3 : 1). When necessary, they were preserved in 70 % ethanol at 3–4 °C until preparation. The meristematic tissue was then thoroughly rinsed in distilled water, hydrolysed in 1 N HCl at 60 °C for 6–7 min, and stained in lacto-propionic orcein overnight (Dyer, 1979). The meristems were finally dissected and squashed on glass slides in a drop of 45 % acetic acid. The use of this staining technique (O-banding; Sharma and Sen, 2002) was useful for revealing heterochromatic segments which appeared as deeply stained regions in intercalary, telomeric or pericentromeric position. Metaphase plates were examined with a Zeiss Axioscop light microscope under oil immersion (×100), and photographed with a Canon digital system. Karyotype formulas were determined on selected enlarged prints by carefully measuring the length of chromosome arms and satellites. The centromeric index was calculated as the long : short arm ratio to classify chromosomes following the system of Levan *et al.* (1964): m = metacentric (r = 1·00–1·69), sm = submetacentric (r = 1·70–2·99), st = subtelocentric (r = 3·00–6·99). SAT chromosomes were recognized based on the occurrence and position of secondary constrictions corresponding to the nucleolar organizing region(s). The approximate heterochromatin content for each plate was estimated as a percentage of the length of heterochromatic bands with respect to the total karyotype length, measured on enlarged prints of selected micrographs (Bigazzi and Selvi, 2001). The intrachromosomal asymmetry index (A_1) was calculated according to Romero Zarco (1986), while the interchromosomal index (A_2) was measured as the ratio standard deviation of chromosome length/mean chromosome length. Final

TABLE 1. *Taxa, vouchers and accessions of the populations of* Borago *investigated*

Taxon	Code	Origin and vouchers (B & S: collection by M. Bigazzi & F. Selvi)	GenBank ITS1/*matK*
B. morisiana	ML	IT, Sardinia, Laconi, Funtanamela, *B & S* 03·02 (FI*, CAG†)	DQ657837/DQ657833
B. morisiana	MP	IT, Sardinia, Is. S. Pietro, Cala Vinagra, *B & S* 88·001 (FI, CAG)	DQ657838
B. pygmaea	CP	IT, Tuscany, Isola Capraia, Cala del Fondo, *B & S* 03·50 (FI)	DQ657839/DQ657834
B. pygmaea	BI	FR, Corsica, Bastia, Bigornu, *B & S* 04·50 (FI)	DQ657845
B. pygmaea	OL	FR, Corsica, Bastia, Olcani, *B & S* 88·002 (FI)	DQ657840
B. pygmaea	GT	FR, Corsica, Corte, Tavignano valley, *B & S* 88·003 (FI, CAG)	DQ657846
B. pygmaea	AL	FR, Corsica, Ajaccio, Alata, *B & S* 86·001 (FI)	DQ657841
B. pygmaea	CG	FR, Corsica, Sartene, Cargiaca, *B & S* s.n. (FI)	DQ657842
B. pygmaea	AU	FR, Corsica, Sartene, Aullene, *B & S* s.n. (FI)	–
B. pygmaea	CL	IT, Sardinia, Sassari, Calangianus, *B & S* 04·51 (FI)	DQ657844
B. pygmaea	SD	IT, Sardinia, Nuoro, Sadali, *Bacchetta et al.* s.n. (CAG)	–
B. pygmaea	LA	IT, Sardinia, Nuoro, Laconi, *Bacchetta et al.* s.n. (CAG)	DQ657847
B. pygmaea	SN	IT, Sardinia, Cagliari, Niu Crobu, *Bacchetta et al.* s.n. (CAG)	–
B. pygmaea	SI	IT, Sardinia, Cagliari, Sinnai, *Bacchetta et al.* s.n. (CAG)	DQ657843
B. officinalis	–	IT, Tuscany, Firenze, *B & S* 90·001 (FI)	See Materials and methods
B. trabutii	–	MO, Marrakech, Tizi-n-Ouzla, *B & S* 05·20 (FI)	DQ657848 / DQ657835

* FI, Herbarium Universitatis Florentinae, Firenze, Italy; † CAG, Herbarium, Dipartimento di Scienze Botaniche, Università di Cagliari, Italy.

karyotype formulas, chromosome size and asymmetry indexes for each accession resulted from the mean values of at least five individual karyotypes.

DNA extraction and amplification

Genomic DNA was extracted following a modified 2xCTAB protocol (Doyle and Doyle, 1990) using samples of tissue cut from leaves. Modification consisted of the purification of DNA using polyvinylpyrrolidone to eliminate the negative effects that the abundant polysaccharidic mucilage of *Borago* subgenus *Buglossites* had during subsequent amplification (Aljanabi *et al*., 1999). The extracted DNA was quantified after agarose gel electrophoresis (0·6 % w/v) in TAE buffer (1 mM EDTA, 40 mM Tris-acetate) containing 1 μg mL^{-1} of ethidium bromide by comparison with a known mass standard.

Analysis of the *matK* region was done for *B. trabutii* and for one accession of *B. morisiana*, *B. pygmaea* and *Trachystemon orientalis* (Table 1). The two primers matKF (5′-GAT TCG AAC CCG GAA CTA G-3′) and matKR (5′-CGA TCA ACA TCT TCT GGA ATC-3′) used in this study were obtained, respectively, from the two primers trnK3R and tK3MY2F used for *Myosotis* (Winkworth *et al*., 2002). The former was modified in the last four nucleotides and the latter in the second and eighth nucleotides, based on the published sequences of *Borago officinalis* (GenBank AJ429308) and *Echium candicans* L. f. (GenBank AF542610).

Analysis of ITS1 nuclear rDNA was done for *B. trabutii*, the two populations of *B. morisiana* and nine accessions of *B. pygmaea* representing the distribution range and the different cytotypes revealed by karyotype analysis (Table 1); the primers ITS4 and ITS5 of Baldwin (1992) were used.

For both *matK* and ITS1, PCR amplifications were performed in a total volume of 50 μL containing 5 μL of reaction buffer (Dynazyme II; Finnzyme, Espoo, Finland), 1·5 mM $MgCl_2$, 20 pmol of each primer, 200 μM of each dNTP, 1 U of *Taq* DNA polymerase (Dynazyme II; Finnzyme) and 10 ng of template DNA. Reactions were performed in a Perkin-Elmer 9600 thermocycler (Perkin Elmer, Norwalk, CT, USA). Subsequently, 5 μL of each amplification mixture was analysed by agarose gel (1·5 % w/v) electrophoresis in TAE buffer containing 1 μg mL^{-1} ethidium bromide. The PCR reactions were purified from excess salts and primer with the PCR Purification Kit (Roche, Mannheim, Germany).

Automated DNA sequencing was performed directly from the purified PCR products using BigDye Terminator v.2 chemistry and an ABI310 sequencer (PE-Applied Biosystems, Norwalk, CT, USA).

Sequence alignment and analysis

The ITS1 sequences were checked for orthology to the sequences of *Borago officinalis* (GenBank AY383283), *Anchusa officinalis* (AY045710) and *Trachystemon orientalis* (AY383287), all of the tribe Boragineae. The two latter species were then used as outgroups for cladogram construction based on their position in the phylogeny of the tribe (Hilger *et al*., 2004). The *matK* sequences were aligned with the sequences of *Borago officinalis* (AJ429308) and *Echium vulgare* L. (AY092893); the latter and *Trachystemon orientalis* (original *matK* sequence obtained from plants cultivated in the Botanical Garden of Florence University, voucher B & S 99·023—FI, GenBank no. DQ657836) were then used as outgroup representatives in the *matK* tree.

Multiple alignments were performed with Multalin (Corpet, 1988), and then further examined and slightly modified manually. All characters were weighted equally, and character state transitions were treated as unordered. Gaps were coded and added at the end of the sequences according to the 'simple gap' coding method of Simmons and Ochoterena (2000). Neighbour-joining (NJ) and maximum parsimony (MP) methods were used to analyse the aligned sequences. NJ trees (Saitou and Nei, 1987) were obtained on the basis of a Kimura-2 parameter distance matrix. MP trees were calculated with PAUP* ver.

4.0 b (Swofford, 1998) through a heuristic search, adding sequences at random, with tree-bisection-reconnection branch swapping, MULTREES option on, ADDSEQ = random, ten randomized replicates. Accelerated transformation (ACCTRAN) optimization was used to infer branch lengths. Internal support to the branches was estimated by means of 50 % majority-rule bootstrap analysis (Felsenstein, 1985), with 1000 random addition replicates for ITS1 and 100 for *matK*.

RESULTS

Karyotype analyses

The summary of somatic and haploid chromosome numbers, ploidy levels, karyotype formulas, mean chromosome lengths and approximate lengths of heterochromatic bands observed in the examined accessions is reported in Table 2.

Borago trabutii. All examined individuals (18) showed an invariable diploid complement of $2n = 2x = 12$, based on $x = 6$ (Fig. 1A). This is the first karyological analysis of a native accession of this rare endemic of the Moroccan Atlas, and the first finding of such a low number in *Borago*. This observation is in contrast with an old report of $2n = 16$ from non-native material (Contandriopoulos, 1962), suggesting that a wider population analysis may be useful to ascertain the existence of infraspecific chromosomal races. However, occurrence of cytotypes with different base numbers in such a well-characterized endemism with little morphological variation, restricted range and isolated position is unlikely. The karyotype has a very low intrachromosomal asymmetry (Fig. 2) due to the presence of only metacentric chromosomes, two of which are provided with satellites (Fig. 3A). Chromosomes are distinctly larger than in *B. morisiana* and *B. pygmaea* but slightly smaller than in *B. officinalis* (see below). With respect to the latter species, a larger variation in size accounts for a higher interchromosomal asymmetry. Each homologous pair shows a distinct banding pattern visibile as deeply stained heterochromatic regions in pericentromeric, intercalary and telomeric positions. Representing approx. 25 % of the total karyotype length, heterochromatin is more abundant than in the two *Buglossites* species, but less than in *B. officinalis* (see below).

Borago officinalis. The accession examined confirmed that this widespread herb is diploid with $2n = 2x = 16$ (Fig. 1B), as already known from previous reports from Italy and Spain (e.g. D'Amato and Marchi, 1983; Luque, 1989). The base number is therefore $x = 8$, as in *B. pygmaea* (see below). However, the karyotype of this species is clearly closer to that of *B. trabutii* in terms of chromosome size class, asymmetry (Fig. 2), and heterochromatin content. In addition to the higher number of chromosomes, it differs from *B. trabutii* also by a higher A_1 value due to the presence of two pairs of submetacentrics and six pairs of metacentrics, one of which is satellited (Fig. 3B). Interchromosomal asymmetry is instead lower. *Borago officinalis* shows the largest chromosomes in the genus (approx. 4·5 µm) and the highest content of constitutive heterochromatin (about 38 % of the total karyotype length). However, the banding pattern in this species is different from that observed in *B. trabutii*. In all the homologue pairs, heterochromatin is organized in large and compact regions in the pericentromeric position, while no telomeric and intercalary bands could be detected.

Borago morisiana. The two populations examined (MP and ML, the only ones known to date) are geographically isolated from each other, as one is from a coastal habitat in the Island of San Pietro (south-western Sardinia) and the other from the rugged and mountainous Laconi region in the central part of the island (Fig. 4). The discovery of this rare endemic in the latter locality is recent and still not published.

Both populations showed an identical chromosome complement consisting of $2n = 2x = 18$ based on $x = 9$ (Fig. 1D). The karyotype is formed by four pairs of metacentrics, three of submetacentrics and two pairs of subtelocentrics, one of which is distinctly smaller and provided with satellites on the short arms (Fig. 3C). The intrachromosomal asymmetry is considerable, due to the prevalence of subtelo- and submetacentric chromosomes with respect to metacentric chromosomes (Fig. 2). Interchromosomal asymmetry is also relatively high due to the variation in chromosome length (1·9–2·5 µm). Heterochromatin was visible as deeply stained regions mainly in intercalary and pericentromeric positions, but in its content is always <20 % of the total karyotype length.

Borago pygmaea. The populations examined revealed remarkable differences in somatic karyotypes and even base chromosome numbers. Three allopatric cytotypes were recognized, and their geographical distribution is shown in Fig. 4.

The five Sardinian accessions (SI, SN, LA, SD and CL) are all characterized by a complement with $2n = x = 30$ based on $x = 15$ (Fig. 1E). The karyotype consists of nine pairs of metacentrics, one of which was provided with satellites, and six pairs of submetacentrics (Fig. 3D). No subtelocentrics are present. The A_1 asymmetry is lower than in *B. morisiana* (Fig. 2), whereas mean chromosome size, A_2 asymmetry and mean length of heterochromatic bands are comparable.

The three populations from south Corsica (AU, CG and AL) possess an invariable tetraploid complement of $2n = 4x = 32$, based on $x = 8$ (Fig. 1F). This differs from that of the Sardinian accessions by the presence of eight pairs of metacentrics, seven pairs of submetacentrics, one of which is satellited, and one pair of subtelocentrics (Fig. 3E). Consequently, the average A_1 asymmetry is slightly higher, whereas for the A_2 value, mean chromosome size and heterochromatic bands are comparable with the Sardinian populations.

The three accessions from central and northern Corsica (GT, BI and OL) and the one from the western coast of Capraia (CP) are characterized by an invariable hexaploid complement of $2n = 6x = 48$, based on $x = 8$ (Fig. 1C). Their karyotype consists of 12 pairs of metacentrics and ten

TABLE 2. *Somatic chromosome number (*2n*), base number (*n*), ploidy level (*x*), karyotype formula, mean chromosome length (*L*) and mean length of heterochromatic bands (Het. cr.) of the populations of* Borago *investigated*

Taxa and accessions	2*n*	*n* (*x*)	Formula	L (μm)	Het. cr. (%)
B. officinalis	16	8 (2*x*)	10 m + 2 m^{SAT} + 4 sm	3·9	38·3
B. trabutii	12	6 (2*x*)	10 m + 2 m^{SAT}	3·0	25·4
B. morisiana MP	18	9 (2*x*)	8 m + 6 sm + 2 st + 2 st^{SAT}	2·2	19·2
B. morisiana ML	18	9 (2*x*)	8 m + 6 sm + 2 st + 2 st^{SAT}	2·2	19·4
B. pygmaea LA	30	15 (2*x*)	16 m + 2 m^{SAT} + 12 sm	2·3	19·1
B. pygmaea SD	30	15 (2*x*)	16 m + 2 m^{SAT} + 12 sm	2·3	19·8
B. pygmaea CL	30	15 (2*x*)	16 m + 2 m^{SAT} + 12 sm	2·3	19·7
B. pygmaea SI	30	15 (2*x*)	16 m + 2 m^{SAT} + 12 sm	2·2	19·5
B. pygmaea SN	30	15 (2*x*)	16 m + 2 m^{SAT} + 12 sm	2·1	19·2
B. pygmaea AL	32	8 (4*x*)	16 m + 12 sm + 2 sm^{SAT} + 2 st	2·5	20·3
B. pygmaea AU	32	8 (4*x*)	16 m + 12 sm + 2 sm^{SAT} + 2st	2·5	20·1
B. pygmaea CG	32	8 (4*x*)	16 m + 12 sm + 2 sm^{SAT} + 2st	2·4	19·5
B. pygmaea BI	48	8 (6*x*)	24 m + 18 sm + 2 sm^{SAT} + 4 st	2·2	18·3
B. pygmaea OL	48	8 (6*x*)	24 + 18 sm + 2 sm^{SAT} + 4 st	2·2	18·3
B. pygmaea GT	48	8 (6*x*)	24 m + 18 sm + 2 sm^{SAT} + 4 st	2·1	19·1
B. pygmaea CP	48	8 (6*x*)	24 m + 18 sm + 2 sm^{SAT} + 4 st	2·1	18·5

The codes follow Table 1.

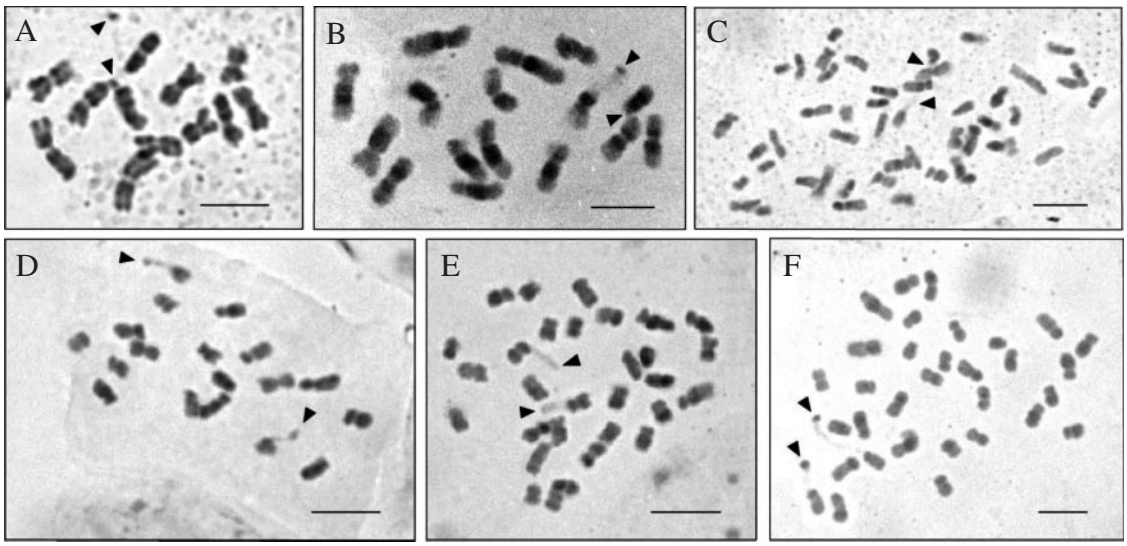

FIG. 1. Micrographs of metaphase chromosome plates: (A) *B. trabutii*, 2*n* = 12; (B) *B. officinalis*, 2*n* = 16; (C) *B. pygmaea*, 2*n* = 48 (accession BI), (D) *B. morisiana*, 2*n* = 18 (accession MP); (E) *B. pygmaea*, 2*n* = 30 (accession CL); (F) *B. pygmaea*, 2*n* = 32 (accession AU). Arrowheads indicate satellites. Scale bars: A, B = 4 μm; C–F = 5 μm.

pairs of submetacentric chromosomes, one of which with satellites on the short arms, and two pairs of subtelocentrics. Average intra- and interchromosomal asymmetry values are higher than in the other accessions (Fig. 2). Mean chromosome size is comparable, whereas the percentage of heterochromatin is slightly lower.

Phylogenetic analyses

Region matK. Sequences have been deposited in GenBank-EMBL-DDB and can be retrieved using the numbers in Table 1 (except for *Trachystemon orientalis*, accession in Materials and methods). The aligned sequences (available from the authors in NEXUS format upon request) are 531 bp long, including codified gaps (16 positions). Ingroup variation is low, with only 28 variable positions in at least one accession. In the phylogenetic analysis, 461 positions are constant, 52 are variable but parsimony non-informative and 18 are parsimony-informative.

The single most-parsimonious tree (length = 77; consistency index = 0·95; retention index = 0·80), topologically identical to the neighbour-joining tree, is shown in Fig. 5. *Borago* forms a strongly supported clade (94 % BS), with *B. trabutii* appearing as the earliest divergent lineage and sister to the other members of the genus. This species does not cluster with *B. officinalis*, which differs in eight 1-bp substitutions and 12 1-bp deletions. *Borago officinalis* is, in turn, sister to *B. morisiana* and *B. pygmaea* with which it forms a well-supported clade (90 % BS). The monophyly of subgenus *Buglossites* is also strongly supported (91 % BS). *Borago*

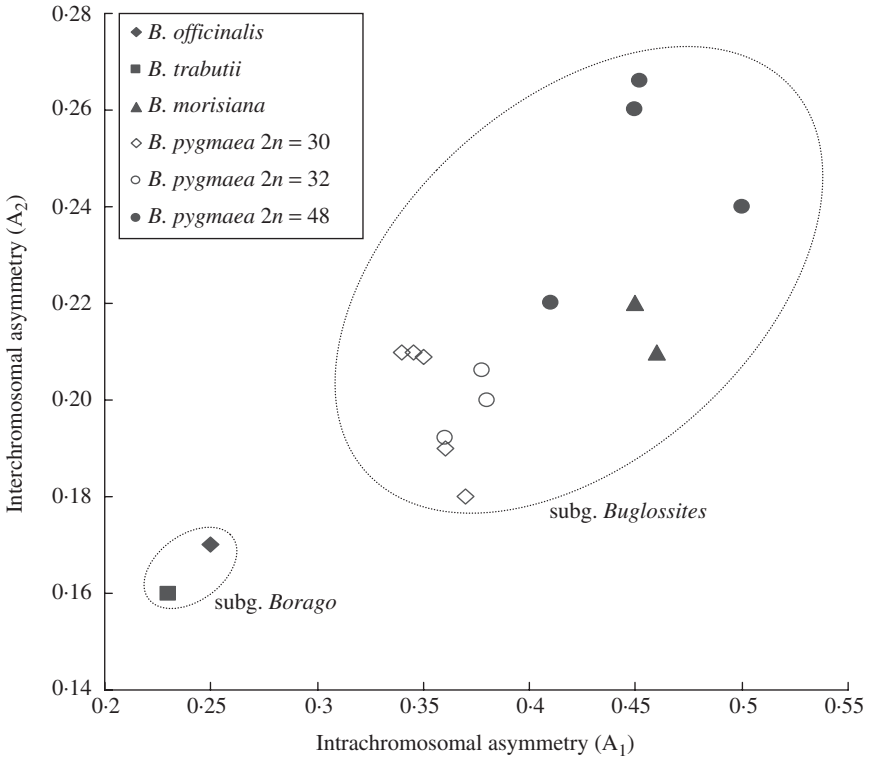

FIG. 2. Bidimensional ordination of the accessions of *Borago* examined as a function of the intrachromosomal (A_1) and interchromosomal (A_2) karyotype asymmetry.

pygmaea differs from *B. morisiana* in only two deletions (one of 2 bp and one of 1 bp) and one 1-bp insertion.

Region ITS1. Sequences have been deposited in GenBank-EMBL-DDB (accession numbers in Table 1). The aligned sequence data set (available in NEXUS format) is 282 bp long. Ingroup variation is remarkable, with 115 positions (approx. 41 %) being variable in at least one accession. Adding the two outgroups for the purposes of the phylogenetic analysis, 131 characters are constant, 83 parsimony-uninformative and 68 (= 24.1 %) parsimony-informative. The most-parsimonious phylogeny (L = 208, CI = 0·89, RI = 0·82), which resulted in being topologically identical to the strict consensus and neighbour-joining trees, is shown in Fig. 6. Optimized branch lengths and bootstrap values >50 % are shown, and somatic chromosome numbers are superimposed for each accession examined.

With respect to the outgroups, *Borago* is monophyletic and strongly supported (100 % BS). *Borago trabutii* is again the early divergent lineage and sister to the rest of the ingroup. However, there is no evidence for the monophyly of subgenus *Borago* due to the position of *B. officinalis* which does not cluster with *B. trabutii* but is sister to the group of *B. morisiana*/*B. pygmaea.* Together with the latter, *B. officinalis* forms a well-supported clade (91 % BS), but the monophyly of subgenus *Buglossites* is even more strongly corroborated (98 %).

Within *Buglossites*, no clear correspondence between the clades and cytotypes of *B. pygmaea* can be detected. The hypotetraploid ($2n$ = 30) accession of this species from northern Sardinia (CL) shows several autapomorphic indels in the sequence and appears sister to the rest of *Buglossites*, failing to cluster with the two other accessions from southern and central Sardinia with the same chromosome complement (LA and SI, $2n$ = 30). These form a small group with one of the two Corsican tetraploid accessions (CG), though this relationship is not supported by bootstrap values. The other Corsican tetraploid population (AL) is external to this group, confirming the lack of relationship with either chromosome complement or geographical location in the group of $2n$ = 32 and 30 races. The mixed Corsican/Sardinian accessions of tetra- and hypotetraploid *B. pygmaea* (CG, SI and LA) form a moderately supported lineage (69 %), together with a monophyletic, sister clade (79 %) consisting of the two accessions of *B. morisiana* (51 %) and the hexaploid populations of *B. pygmaea*. The two northernmost accessions of the latter from Capraia Island (CP) and the Corsican 'finger' (OL) cluster together with 60 % BS support, but their relationships to the two more southern hexaploids (GT and BI) remain unresolved.

DISCUSSION

According to the scanty literature available, *Borago* is characterized by the base x = 8, with *B. officinalis*, *B. trabutii*, *B. longifolia* and *B. morisiana* being diploid

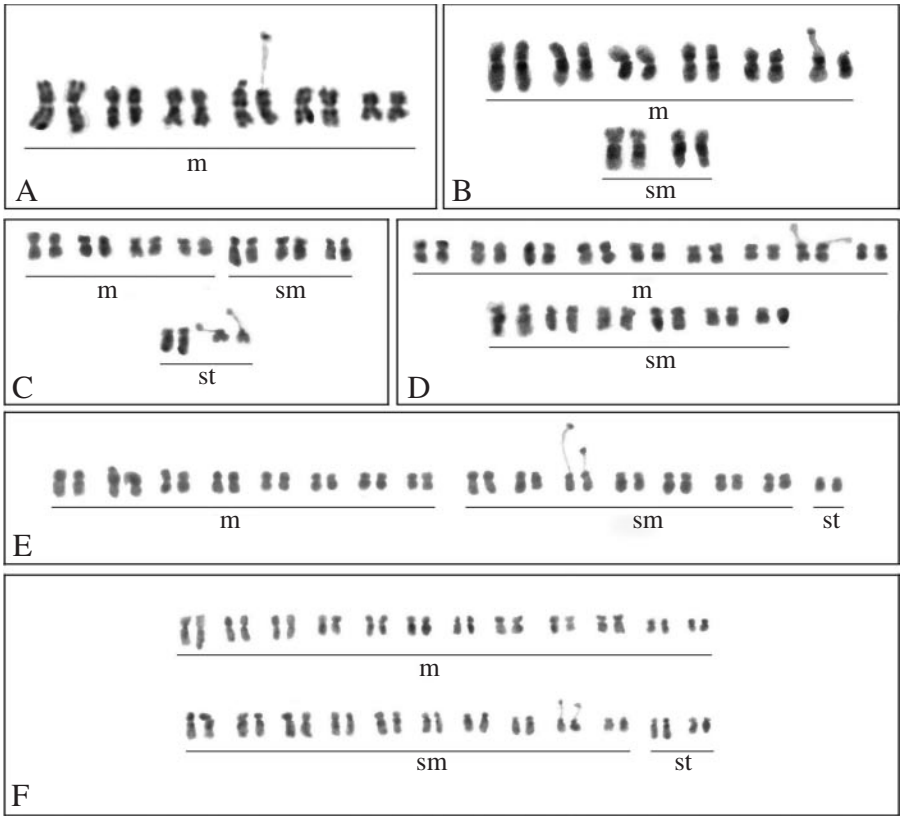

FIG. 3. Karyotypes of (A) *B. trabutii*, $2n = 12$; (B) *B. officinalis*, $2n = 16$; (C) *B. morisiana*, $2n = 18$ (MP); (D) *B. pygmaea*, $2n = 30$ (CL); (E) *B. pygmaea*, $2n = 32$ (AU); (F) *B. pygmaea*, $2n = 48$ (BI). The magnifications are the same as in Fig. 1. m, metacentric; sm, submetacentric; st, subtelocentric.

(Contandriopoulos, 1962; D'Amato and Marchi, 1983; Luque, 1989; Bigazzi and Ricceri, 1992) and *B. pygmaea* tetraploid (Strey, 1931; Contandriopoulos, 1962; Diana Corrias, 1980). However, this study shows that the reports for *B. morisiana* and probably *B. trabutii* are wrong, while it reveals the existence of four base numbers, $x = 6$, $x = 8$, $x = 9$, $x = 15$, and three ploidy levels (di-, tetra- and hexaploid) based on $x = 8$. Chromosomal variation in this small genus is therefore much wider than previously known (Fig. 7), suggesting that karyological rearrangements have been a major driving force for the evolution and radiation of the taxa into present-day ranges. This is not surprising, as comparable levels of variation are known to occur in related Boragineae groups, such as *Symphytum* (Gadella *et al.*, 1983), *Pulmonaria* (Sauer, 1975) and *Nonea* (Selvi and Bigazzi, 2002; Bigazzi and Selvi, 2003).

Relationships between primary base numbers

As in other plant groups, a major issue of evolutionary significance concerns the relationships between the base numbers (Grif, 2000; Stace, 2000). Two possible scenarios can be considered in the light of the present karyological data and molecular cladograms, the first of which assumes the number $2n = 12$ of the early divergent lineage of *B. trabutii* as the ancestral condition in *Borago*. The base $x = 6$ is the lowest in Boraginaeae, and is known only for a few other taxa with plesiomorphic morphological characters like the species of *Brunnera* (Britton, 1951; Al-Shehbaz, 1991; Bigazzi and Selvi, 2001). Under this scenario, the backcrossing of unreduced ($n = 12$) and regular ($n = 6$) gametes may have resulted in the formation of a triploid 'bridge' with $2n = 18$ and in the origin of the new base $x = 9$ found in *B. morisiana*. Descending aneuploidy by tandem centric fusion between two homologous pairs in $2n = 18$ *Buglossites* populations could explain the origin of $2n = 16$ and $x = 8$, at least in the Corso-Sardinian system.

More plausible, however, is the hypothesis of $x = 8$ as the ancestral base conserved in both subgenera *Borago* (*B. officinalis*) and *Buglossites* (*B. pygmaea*), as well as in most taxa of the outgroup genera *Anchusa*, *Echium* (Strey, 1931; Britton, 1951) and, possibly, the paleopolyploid *Trachystemon orientalis* ($2n = 56$; Markova and Ivanova, 1970; Coppi *et al.*, 2006). This hypothesis would imply a dysploid origin for both $x = 9$ in the *Buglossites* clade, possibly by chromosome fission, and $x = 6$ in the *B. trabutii* clade, by progressive descending aneuploidy. At the diploid level, the latter type of rearrangement does not imply a loss of the requisite genetic material but simply a decrease in the number of chromosomes as separate linkage groups (Jackson and Casey, 1980; Levin, 2002). Although no karyological evidence for the origin of $x = 6$ by descending aneuploidy can be provided here, it is known that this mechanism has played an important role in speciation processes of other Boragineae genera such as *Nonea* (Selvi and Bigazzi, 2002; Bigazzi

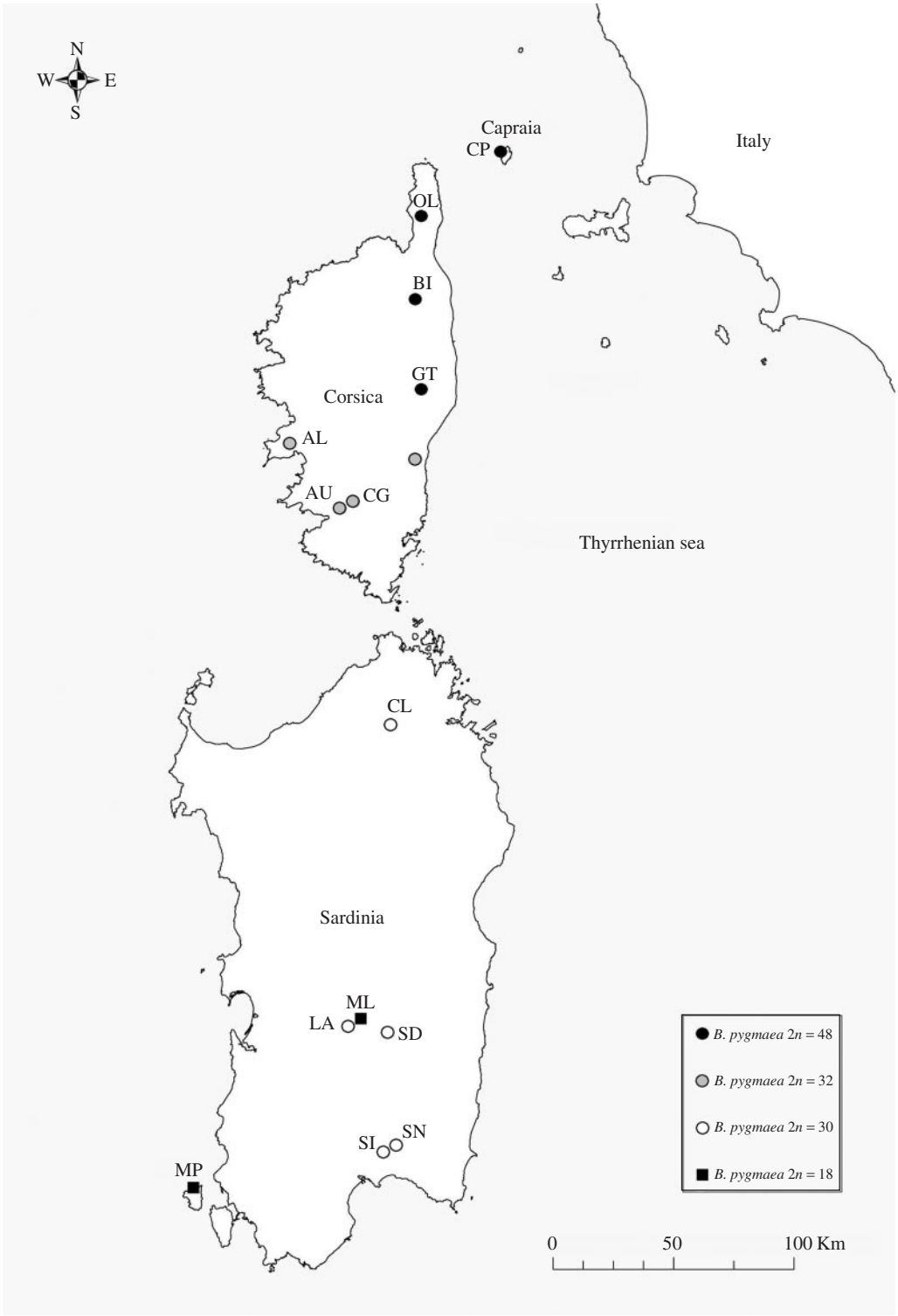

FIG. 4. Geographic location of the populations of *B. morisiana* and B. *pygmaea* investigated and distribution of the tetra-, hypotetra- and hexaploid cytotypes of *B. pygmaea* in Corsica, Sardinia and the island of Capraia (Tuscan Archipelago). The dot of tetraploid *B. pygmaea* without a population code refers to the report by Contandriopoulos (1962) from the Solenzara river, eastern Corsica.

and Selvi, 2003) and *Pulmonaria* (Sauer, 1975), as well as in other dicot groups [i.e. *Vicia* (Hollings and Stace, 1974), *Genista* (Sañudo, 1979), *Minuartia* (Favarger, 1999) and *Houstonia* (Church, 2003)]. The hypothesis of $x = 8$ as the ancestral condition in *Borago* and related groups would be supported by a confirmation of $2n = 16$ in *B. longifolia*, the other North African endemic of subgenus *Borago* (Contandriopoulos, 1962).

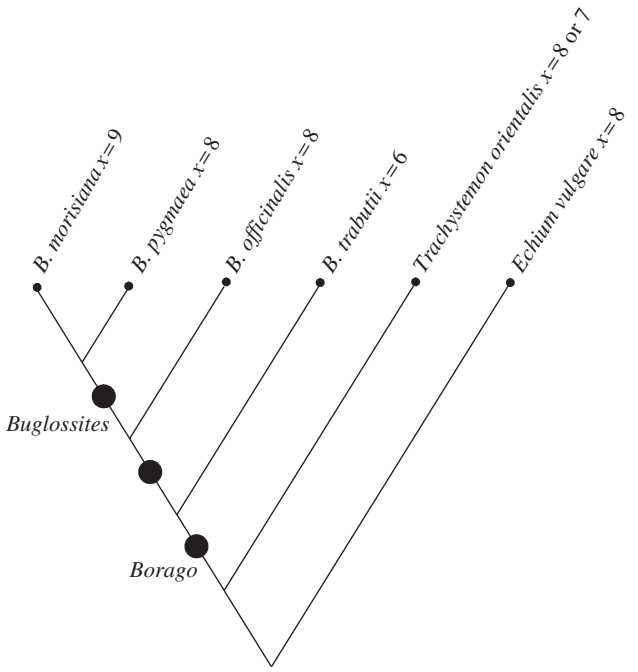

FIG. 5. The single most-parsimonious phylogeny generated by *matK* sequences (L = 77, CI = 0·95, RI = 0·80). Black dots on branches indicate strong boostrap support (>90 %). Base chromosome numbers are indicated for ingroup and outgroup taxa.

The tetra- and hypotetraploid races of B. pygmaea

In *B. pygmaea*, the apparent extinction of diploid progenitors with $2n = 16$, already hypothesized by Contandriopoulos (1962) and here corroborated, may have been concomitant with ancestral autopolyploidization events which have triggered the formation of the tetraploid race via sexual union of unreduced gametes. This is a prime mechanism of increase in ploidy level and chromosome number in many angiosperm groups (Thompson and Lumaret, 1992; Bretagnolle and Thompson, 1995; Leitch and Bennett, 1997; Ramsey and Schemske, 1998; Burton and Husband, 2001; Levin, 2002), not least in other taxa of Boragineae (Bigazzi and Selvi, 2001; Selvi and Bigazzi, 2002). The achievement of the polyploid condition in *B. pygmaea* has possibly enhanced its competitive ability, for example, in allowing the high rate of selfing that was observed in cultivated plants (personal observation). The breakdown of mechanisms that reduce inbreeding in diploids is believed to be a prime biological feature that conveys a competitive advantage to natural polyploids over diploid progenitors (Thompson and Lumaret, 1992). Furthermore, the increased heterozygosity and allelic diversity which occur in sexual autopolyploids as a consequence of genome multiplication is acknowledged as another factor leading to the formation of new adaptive gene combinations (Brochmann *et al*., 1992; Soltis and Soltis, 1993, 1995, 2000; Otto and Whitton, 2000; Wendel, 2000; Levin, 2002). Finally, the successful establishment, persistence and spread of autotetraploid *B. pygmaea* has possibly been enabled by the small size of the populations, in which genetic and environmental effects, as well as effects due to the increased mating that occurs among closely related individuals, may cause a marked increase in the frequency of $2n$ gamete formation (Thompson and Lumaret, 1992). Under these conditions the diploid progenitors may have become extinct as a consequence of 'the minority cytotype exclusion' rule which is believed to occur in local plant populations (Levin, 1975).

In contrast to the diploid *B. morisiana*, the newly formed tetraploid *B. pygmaea* may have easily radiated across the Corso-Sardinian system because of the land connections between the two islands which occurred for the best part of the last 30 Myrs (Cherchi and Montadert, 1982; Speranza *et al*., 2002).

The origin and establishment of $x = 15$ has probably represented a second step in the karyological evolution of *B. pygmaea*. Aneuploid reduction again provides a plausible explanation for the formation of this hypotetraploid race, although the reasons for its current distribution restricted to Sardinia are obscure. Karyotype analysis suggests a secondary origin via a tandem centric fusion between the pair of SAT submetacentrics and the pair of subtelocentrics of the $2n = 32$ race, with formation of the two SAT metacentrics and loss of subtelocentrics in the $2n = 30$ complement. This kind of rearrangement by descending aneuploidy leading to the secondary formation of higher base numbers is common in polyploid complexes, e.g. *Artemisia* (James *et al*., 2000; Stace, 2000).

The hexaploid race of B. pygmaea

Backcrossing between $2n$ and regular n gametes in $2n = 32$ populations followed by minor rearrangements in the SAT submetacentric and subtelocentric units and an increase in intrachromosomal asymmetry is a likely mechanism for the cryptic formation of the hexaploid cytotype (32 unreduced: $2n = 16m + 12sm + 2sm^{SAT} + 2st$; 32 reduced: $n = 8m + 6sm + 1sm^{SAT} + 1st \rightarrow$ hexaploid: $24m + 18sm + 2sm^{SAT} + 4st$). In this case, there is evidence that SAT chromosomes are frequently involved in karyotype changes of *Buglossites*, possibly in relation to the necessity of maintaining only one functional nucleolar organizing region per genome. Several polyploid plants have only one nucleolar organizing region because those resulting from genome multiplication that are in excess and not functional are 'suppressed' or silenced in some way (Stace, 2000).

The apparent lack of hybrid zones between the tetraploid and hexaploid races in Corsica is likely to depend on historical and ecological causes, as in other cases of geographical separation between cytotypes with different ploidy levels (Thompson and Lumaret, 1992; Levin, 2002; Thompson, 2005). Also in the light of the monophyly of the $2n = 48$ accessions suggested by the phylogenetic analysis, a model of single-event origin along the northern limit of the tetraploid range is, in this case, more plausible than an alternative one of multiple events. The latter often results in mixed polyploid complexes with no clear distributional patterns of the cytotypes as a consequence of directional or balanced selection (Petit *et al*., 1999; Soltis and Soltis, 1999; Weiss *et al*., 2002). In *B. pygmaea*, the geographical

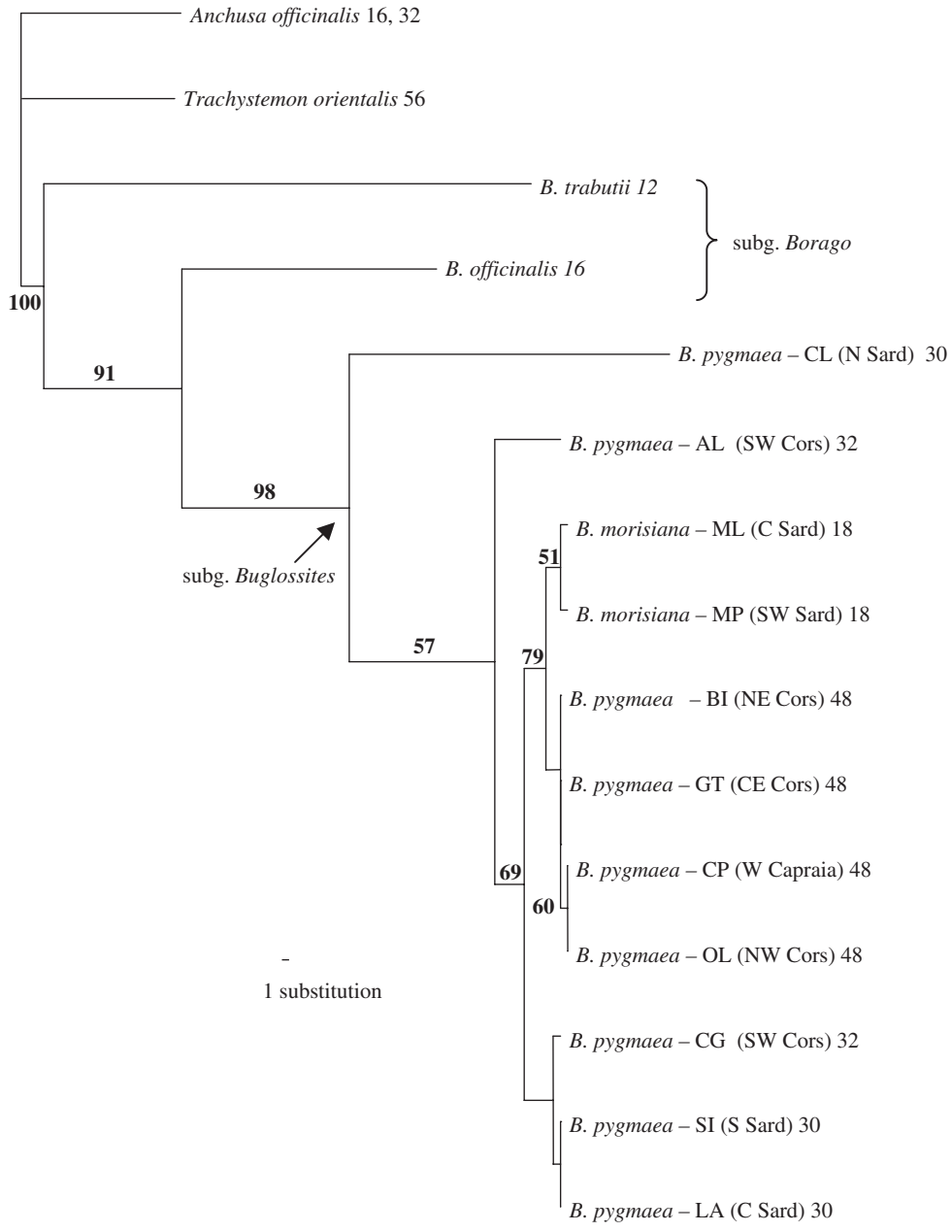

FIG. 6. One of the most-parsimonious trees of *Borago* with branch lengths (topologically identical to the strict consensus and neighbour-joining trees, L = 208, CI = 0·89, RI = 0·82). Bootstrap values are shown above branches when >50 %. Abbreviations of *Buglossites* accessions follow Table 1. Chromosome numbers and geographic origin are indicated for each accession.

segregation may be the result of a northward, unidirectional spread of the hexaploid race successively maintained by ecological factors limiting the range of the tetraploid to the north but not that of the more tolerant hexaploid. Ecological differentiation is acknowledged as a key factor for maintaining cytotypes in an allopatric condition (Thompson and Lumaret, 1992; Levin, 2002; Husband, 2004). Comparative autoecological analyses of the tetra- and hexaploid races could be useful in understanding the role of edaphic factors in the substantial correspondence between the range of the hexaploid and the so-called 'schistose sector' of north-eastern Corsica formed mainly by schistose and volcanic rocks (Contandriopoulos, 1962; Thompson, 2005).

The occurrence of *B. pygmaea* on the wild, western coast of Capraia Island is also worth mentioning. In both karyotype and ITS1 sequence, this small population is identical to the northernmost accession examined from Corsica (OL), which strongly supports a relationship with the populations in northern Corsica and a relatively recent event of disjunction. The lack of adaptative traits for long-distance seed dispersal in *B. pygmaea* and the volcanic origin of Capraia, which emerged from the sea in the Upper Miocene (7·3–6·0 Myrs ago; Borelli and Groppelli, 2002)

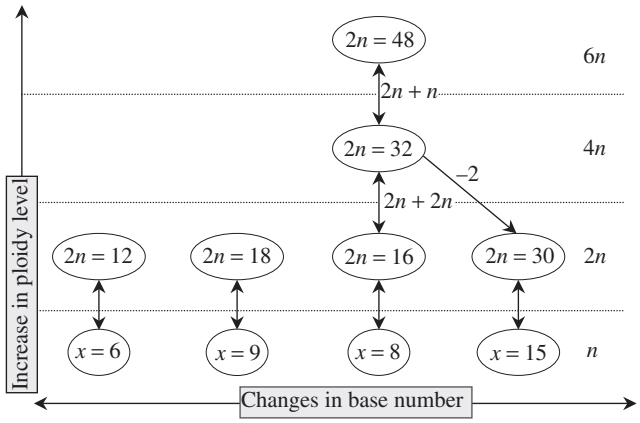

FIG. 7. Diagram of chromosome variation in *Borago* showing base numbers and possible mechanisms of increase in ploidy level in *B. pygmaea*.

about 31 km at the north-east of the Corsican cape, pose the question of how and when the hexaploid race successfully reached the island.

Phylogeny of Borago

Sequence variation in the *matK* and ITS1 regions indicate that the genus *Borago* and subgenus *Buglossites* are both monophyletic lineages, whereas relationships in subgenus *Borago* still remain unclear. To this purpose, further analyses should make use of additional markers and also include the North African endemic *B. longifolia*. Nonetheless, the present reconstruction indicates that the split of *Buglossites* from *Borago* has been karyologically paralleled by an increase in chromosome number and karyotype asymmetry, a decrease in chromosome size and heterochromatin content, and the appearance of polyploidy.

Relationships between the two endemic *Buglossites* species are obscured by the high infraspecific ITS1 variation in the polyploid *B. pygmaea* compared with the diploid *B. morisiana*. The identical sequences and chromosome complements found in the two isolated populations of the latter species support the hypothesis of an ancient origin and a condition of relict paleo-endemic with no morphological variation and extremely reduced, fragmented distribution (Bigazzi and Ricceri, 1992). On the other hand, chromosomal and ITS1 variation indicate that active evolutionary forces have worked in the case of *B. pygmaea*. Polymorphism in the ITS1 region is especially found in the $2n = 32$ and $2n = 30$ accessions (12·8 % of variable positions) possibly due to the occurrence of paralogue sequences in the multiple copies of the genome, a well-known feature of several polyploid complexes (Mansion *et al*., 2005; Stace, 2005). In particular, different paralogues may be the result of recurrent events of polyploidization, as in other autopolyploids with multiple origins (Soltis and Soltis, 1999). The lower ITS1 variation in the hexaploid cytotype may appear therefore unexpected, although this is likely to be related to its more recent, monophyletic origin in central northern Corsica as discussed above. The sister position that this race takes with respect to *B. morisiana* in the cladogram appears difficult to explain, and parallel evolution of the ITS1 repeats in the hexaploid cytotype and *B. morisiana* cannot be ruled out as a possible cause of this unexpected relationship.

ACKNOWLEDGEMENTS

This paper is dedicated to the memory of our beloved friend and colleague Massimo Bigazzi, who started this work with enthusiasm but passed away prematurely on 19 April, 2006. F.S. and A.C. wish to thank Gianluigi Bacchetta (Cagliari) for his help during field work and invaluable information on the distribution of *B. morisiana* and *B. pygmaea*. Two anonymous referees provided useful suggestions on the first version of the manuscript. Research grants from the Italian Ministry of Scientific Research (MIUR) and University of Firenze are acknowledged.

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