

PHYLOGEOGRAPHY

Phylogeography of the ancient Eurasian medicinal plant genus *Bryonia* (Cucurbitaceae) inferred from nuclear and chloroplast sequences

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Medicinal uses of *Bryonia* (Cucurbitaceae) have been recorded for over two millennia, and even today there is a considerable market for *Bryonia* preparations in homeopathic medicine. The long use as a medicinal plant has led to anthropogenic range changes, followed by naturalization and invasiveness in disturbed habitats, for example, in the United States and New Zealand. Here we use phylogenetic and phylogeographic analyses of chloroplast and nuclear sequences to infer the major evolutionary units within *Bryonia* as well as their geographic history. Major clades in the gene trees fit with morphological differences and probably define ten biological species. Five species are endemic in the Irano-Turanian region, which also harbors the greatest chloroplast and nuclear haplotype diversity. Eurasia north of the southern permafrost border during the last glacial maximum has low species and haplotype diversity, fitting with relatively recent recolonization. The provenience of the *B. alba* genotype introduced to the United States could not be narrowed down; the *B. dioica* introduced to New Zealand and Georgia came from north-central, not southwestern Europe. In spite of anthropogenic range changes, *Bryonia* chloroplast haplotypes show a clear geographic pattern, and the role of interspecific hybridization appears to have been limited.

KEYWORDS: anthropogenic range change, Canary Islands, invasive weed, medicinal plants, nuclear *LEAFY* sequences, phylogeography

INTRODUCTION

Medicinal uses of *Bryonia* have been recorded for over two millennia. Probably the earliest references are in texts attributed to Hippocrates, who lived around 460–380 BC (text available at <http://etext.library.adelaide.edu.au/h/hippocrates/>). Other early mentions of bryonies are in Dioscorides's *De Materia Medica*, written in about 65 BC, and Pliny's *Historia Naturalis*, completed in 77 BC (Beck, 2005; Renner & al., 2008). The reason *Bryonia* is mentioned in these and other Egyptian, Greek, Roman, Medieval, and Renaissance sources is that bryony extracts contain numerous cucurbitacins that are biologically active (Oobayashi & al., 1992; Krauze-Baranowska & Ciskowski, 1995; Isaev, 2000; Sturm & Stuppner, 2000; Chen & al., 2005). In high doses, *Bryonia* extracts or fruits can be poisonous (Roth & al., 1994: 176; Bruneton, 1999: 243). Young shoots, however, are eaten as an asparagus substitute (Pieroni, 2000). Alcoholic extracts of the tubers, "Tinctura Bryoniae" or "Bryoniae Radix", in ancient times served to reduce the pain and cough of pleurisy and, in higher doses, as a diuretic or hydragogue cathartic for patients with dropsy. Today, there is a considerable market for *Bryonia* preparations, mostly for homeopathic medicine, although effectiveness remains contested (Konopa & al., 1974; Karageuzyan & al., 1998; Paris & al., 2008).

References to medicinal uses of *Bryonia* have remained ambiguous because of unclear species delimitations. For example, Tutin (1968) in *Flora Europaea* considered *B. dioica* Jacq. and *B. cretica* L. one and the same, while authors with narrower species concepts kept them separate and accepted as many as five bryony species in Europe and up to twelve over the entire range of the genus (Hayek, 1912; Jeffrey, 1969; Scholz, 2008). Phytochemical studies and general texts about medicinal plants usually follow *Flora Europaea* and include *B. dioica* in *B. cretica* (Roth & al., 1994; Bruneton, 1999; Sturm & Stuppner, 2000; Schönfelder & Schönfelder, 2004). An analysis that focused on polyploidy and sexual systems in *Bryonia* found that *B. cretica* is hexaploid and probably a hybrid between *B. dioica* and *B. syriaca* Boiss. and/or *B. multiflora* Boiss & Heldr. (Volz & Renner, 2008).

The several thousand-year long history of *Bryonia* as a medicinal plant raises the question of anthropogenic range change, for example, via escapes from gardens, followed by naturalization or even invasiveness in disturbed habitats (Hayek, 1912; Tutin, 1968; Jeffrey, 1969; Laferriere & al., 1993; Ludwig, 1995; Reynolds, 2002; Schönfelder & Schönfelder, 2004). Examples of anthropogenic range changes are the introduction of *B. alba* to the United States sometime after 1940 (at least since 1970) and the introduction of another species to New Zealand some time after 1991. In

less than 50 years, *B. alba* spread throughout Washington, Idaho, Montana, and Utah (Laferriere & al., 1993; Novak & Mack, 1995, 2000). Similarly, plants introduced to New Zealand and originally identified as *B. cretica* subsp. *dioica* are now invasive in the Rangitikei River area (Webb & al., 1995; T. Gilbertson, Department of Conservation, Mangaweka, New Zealand, pers. comm., March 2006). Within Eurasia, the ranges of *B. alba* and *B. dioica* may likewise have been expanded by man (Hayek, 1912; Jeffrey, 1969; Stokes & al., 2004; Scholz, 2008), and *B. multiflora*, a species native in Turkey, Syria, Iran, and Iraq, has been collected near Beijing in China (Jeffrey, 1969). The relevant collection (*Marcovich 17845*) was made on 2 July 1926, but could not be sampled for DNA (L. Bagmet, curator of the herbarium of the N.I. Vavilov Institute of Plant Industry in Saint Petersburg, pers. comm., 30 April 2008).

To study the ranges of genetically well-differentiated entities in *Bryonia* we carried out a phylogeographic analysis of chloroplast and nuclear DNA sequences from individuals collected throughout the area occupied by the genus, including the U.S. and New Zealand. Phylogeographic analysis (Avise & al., 1987) was initially devised to examine the geographical structuring of genealogical lineages within species. However, since hybridization and incomplete sorting of ancient polymorphisms during speciation are frequent events in plants, many botanical phylogeographic studies focus on clusters of species, rather than single species (Schaal & Olsen, 2000; Grivet & Petit, 2002; Cannon & Manos 2003; Dobes & al., 2004; Bänfer & al., 2006; Jakob & Blattner 2006; Dixon & al., 2007). Our expectations when we started this study were that there might be just four distinct entities in *Bryonia*, as suggested as a possibility in the only revision of the genus (Jeffrey, 1969), which nevertheless recognized twelve species; we also expected that there might be little geographic structure because of widespread introductions and naturalization, perhaps followed by hybridization.

MATERIALS AND METHODS

Taxon sampling. — Molecular studies of Cucurbitaceae have clarified the phylogenetic position of *Bryonia* as sister to *Ecballium* (Kocyan & al., 2007) and revealed that the closest relative of both is the Australian genus *Austrobryonia*, which has four species (Schaefer & al., 2008). Our sampling within *Bryonia* covers the geographic range of each of the species recognized by Jeffrey (1969). Field collecting was done in Bavaria, Schleswig-Holstein, Saxony-Anhalt (Germany), and Uzbekistan. Sampling in this study includes plants from Hungary, Georgia, and Armenia not analyzed in Volz & Renner (2008); an Appendix in the online version of this article lists all plants included in the present study, with Latin names and their

authors, locality data, herbarium vouchers, and GenBank accession numbers.

DNA isolation, amplification, and sequencing.

— Total DNA was isolated from silica gel-dried leaves or herbarium material using the NucleoSpin Plant Kit (Macherey-Nagel) according to the manufacturer's protocol. The *trnL* intron and *trnL-trnF* intergenic spacer (IGS) were amplified using the Taberlet & al. (1991) primers c, d, e, and f. The *psbA-trnH* spacer was amplified with the forward primer of Sang & al. (1997) and a *Bryonia*-adapted reverse primer 5' CGCGCATGGTGGATTCA CAATCC 3'. The *trnR-atpA* spacer was amplified with the forward and reverse primers of Chung & al. (2003). The second intron of the nuclear *Leafy* gene (referred to simply as *LFY* in the remainder of this paper) was amplified with the primers LFY CucF 5' TCTTCCACCTSTATGAR CAGTGTCGTAAT 3' and LFY CucR 5' CGAAATCA CAAA AGYTATTGSGYAKTYCA 3', using cloning to assess within individual diversity. Sequencing relied on Big Dye Terminator chemistry (ABI) and an ABI 3100 Avant capillary sequencer. Sequence assembly and editing were carried out in Sequencher 4.6 (GeneCodes, Ann Arbor, Michigan, U.S.A.). All sequences were BLAST-searched in GenBank.

Alignments and phylogenetic analyses.

— Alignments were created in MacClade 4.08 (Maddison & Maddison, 2003) and adjusted by eye. The chloroplast dataset included seven individuals of *Ecballium elaterium* and one of *Austrobryonia* as a more distant outgroup; *LFY* alignments did not include outgroups because *Ecballium* and *Austrobryonia* sequences were too divergent to be aligned with those of *Bryonia*. Indels in the chloroplast and *LFY* alignments were coded using simple indel-coding (Simmons & Ochoterena, 2000) as implemented in the SeqState software (Müller, 2005), and parsimony searches were conducted with and without indel characters. Maximum Likelihood and Bayesian searches both included the coded indels. All tree searches excluded the last nucleotides of a poly-A run of up to 16 nucleotides in the *psbA-trnH* spacer (last 7 nucleotides excluded), a poly-A run of up to 14 nucleotides in the *trnL* intron (5 excluded), and a poly-T run of up to 31 nucleotides (23 excluded) again in the *trnL* intron.

Bayesian analyses relied on the GTR+G model in MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003); maximum likelihood analyses relied on the same model in RAxML (Stamatakis, 2006). Likelihood searches were carried out following the RAxML manual. Convergence of Bayesian analyses was assessed by checking that final likelihoods and majority rule topologies in different runs were similar; that the standard deviations (SD) of split frequencies were <0.01; that the log probabilities of the data given the parameter values fluctuated within narrow limits; that the convergence diagnostic (the potential scale

reduction factor given by MrBayes) approached 1; and by examining the plot provided by MrBayes of the generation number versus the log probability of the data.

Parsimony analyses in PAUP* version 4.0b10 (Swofford, 2002) used heuristic searches with ten random-taxon-addition replicates, holding ten trees at each step, tree bisection-reconnection branch swapping, and the options MulTrees, Collapse zero-length branches, no Steepest Descent, and a limit of 100 trees held in memory.

Statistical support was measured by maximum likelihood-bootstrapping in RAxML with the same model and settings as used during tree searches, by posterior probabilities obtained from MrBayes, and by non-parametric bootstrapping in PAUP*, using the fast bootstrap option and one million replicates.

Network analyses. — Haplotype networks were constructed using statistical parsimony (Templeton & al., 1992) as implemented in TCS version 1.21 (Clement & al., 2000) and Network version 4.2.01 (Polzin & Daneschmand, 2003). In these networks, haplotypes separated by single substitution events (indels or single nucleotide polymorphisms [SNPs]) become neighbors, internal branching points represent extinct haplotypes or haplotypes not sampled, and the most derived haplotypes occur at tip positions. The alignments used for networks were the same as used in phylogenetic analyses, including the coded indels but excluding redundant sequences and outgroups because the latter differed in too many substitutions to fit in the same networks as the ingroup. The *LFY* sequences were too divergent from each other for meaningful network construction.

We geo-referenced all haplotypes, using geographic coordinates from labels or online gazetteers, and used GeoDis version 2.4 (Posada & al., 2000) to perform a permutational contingency analysis to test for a significant association between haplotype and location. For this analysis, the chloroplast DNA haplotypes were sorted into regional groups, based on geography.

RESULTS

Trees and networks from the chloroplast data.

— The *trnL* region in *Bryonia* comprises 928–939 bases, the *psbA-trnH* spacer 237–245, and the *trnR-atpA* spacer 517–537. A combined matrix of these loci, sequenced for 139 plants (including the outgroups *Ecballium* and *Austrobryonia*), had a length of 1,913 characters (including 49 coded indels). A maximum likelihood tree from these data (Fig. 1) shows that *B. verrucosa* from the Canary Islands is sister to all other species, but resolves few other species-level relationships. There were 31 chloroplast haplotypes that could be assigned to eleven major groups (labeled in large letters in Fig. 1).

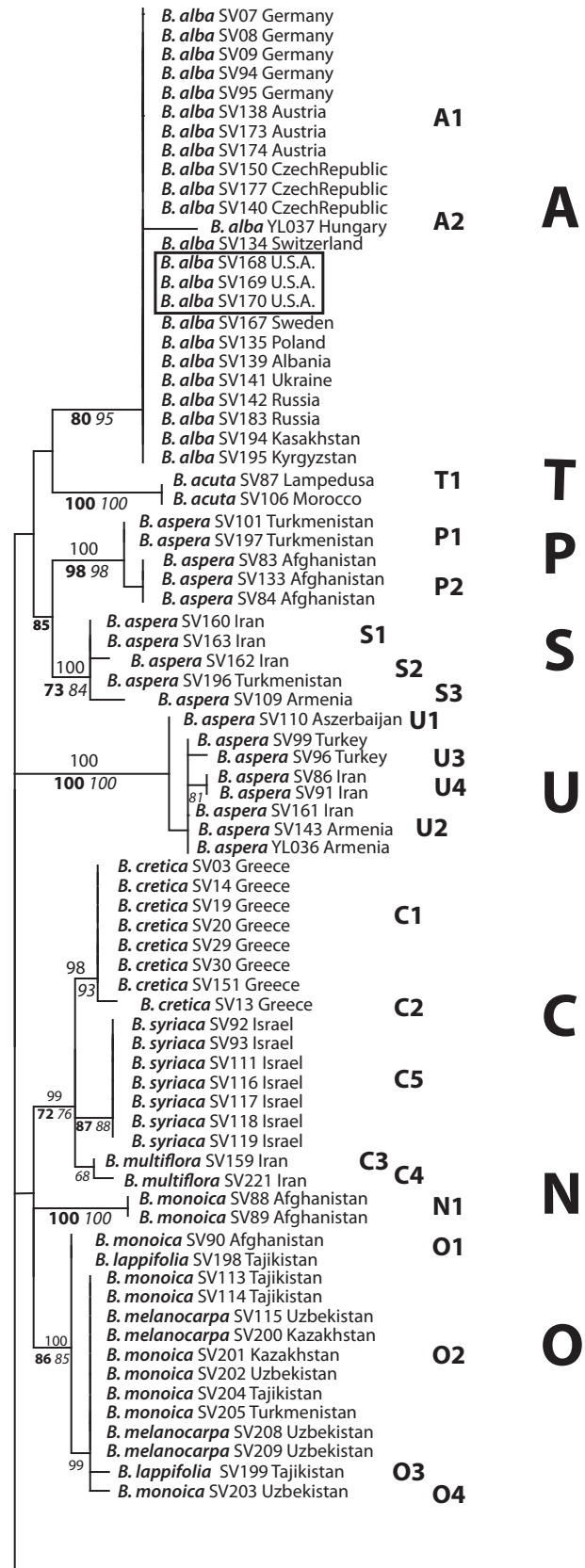


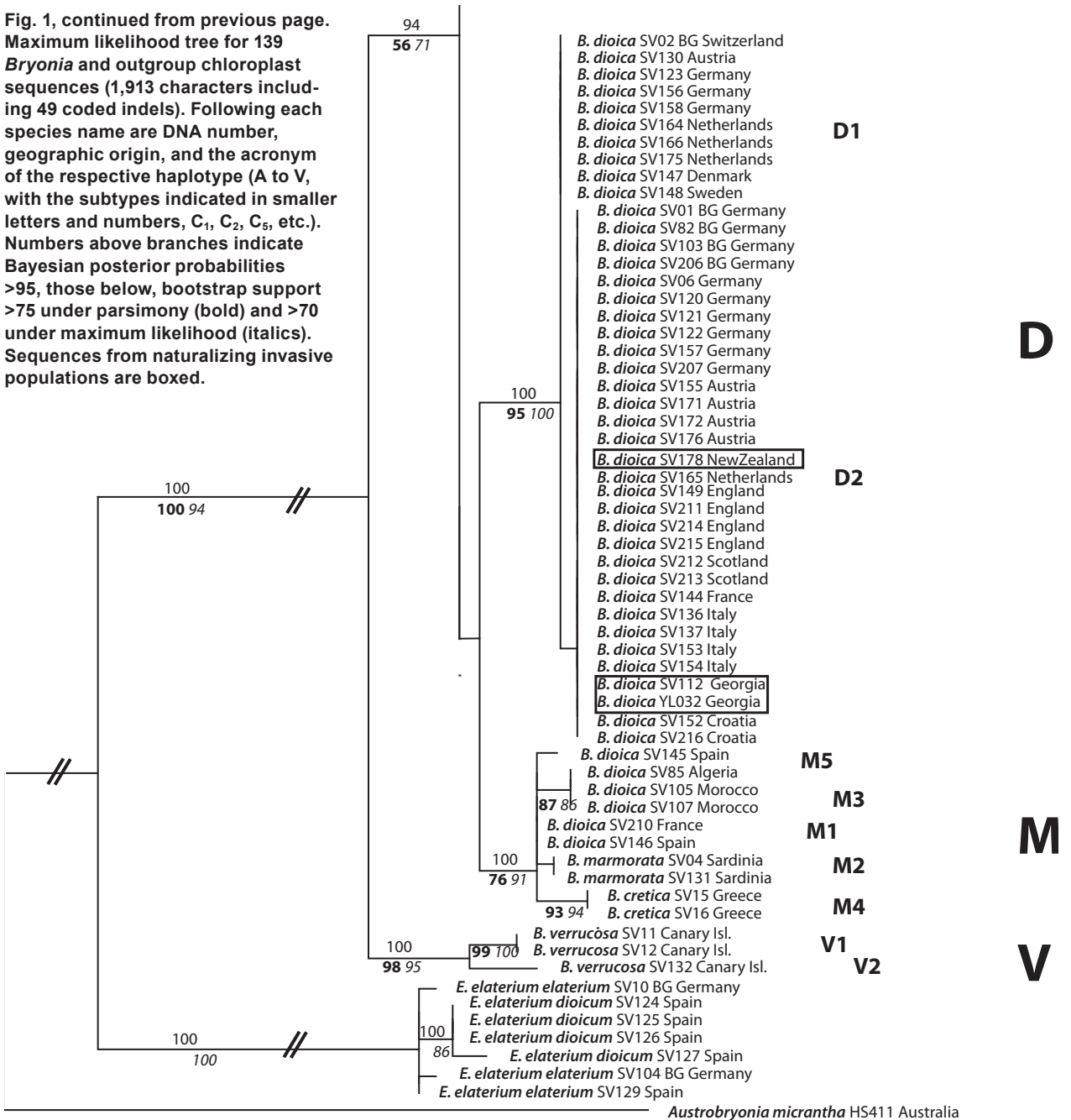
Fig. 1. Continued next page.

A permutational contingency test (10,000 resamples) on 81 accessions representing the eleven main haplotype groups rejected the null hypothesis of no clustering of haplotypes with geographic location ($\chi^2 = 75.3$, $P = 0.034$), and a minimum-spanning network of the chloroplast haplotypes is shown in Fig. 2. The figure also shows the number of individuals in which each haplotype was found. Most haplotypes are restricted to single morphological species; only haplotypes M and C occur in three species each. The M haplotype is found in southern individuals of *B. dioica* (Fig. 3), with special subtypes in Spain and France (M1,

M5), Algeria and Morocco (M3), Sardinia and Corsica (M2 in specimens identified as *B. marmorata*), and Kythera, an island just south of the Greek Peninsula (M4 in specimens identified as *B. cretica*). The C haplotype is found on the Peloponnesian Peninsula and Crete (C1 and C2 in *B. cretica*), in Israel (C5 in *B. syriaca*), and in Iran (C3 and C4 in *B. multiflora*). On Kythera, it was found in plants from the same population that also has the M haplotype.

Chloroplast sequence diversity north of the Alps is lower than in similarly sized regions south of the Alps: just two haplotypes, A and D, are found north of the southern

Fig. 1, continued from previous page. Maximum likelihood tree for 139 *Bryonia* and outgroup chloroplast sequences (1,913 characters including 49 coded indels). Following each species name are DNA number, geographic origin, and the acronym of the respective haplotype (A to V, with the subtypes indicated in smaller letters and numbers, C₁, C₂, C₅, etc.). Numbers above branches indicate Bayesian posterior probabilities >95, those below, bootstrap support >75 under parsimony (bold) and >70 under maximum likelihood (italics). Sequences from naturalizing invasive populations are boxed.



permafrost border during the Last Glacial Maximum (shown as the pale grey line in Fig. 3), and they contain a single SNP among 41 individuals of *B. dioica* and two SNPs plus one indel among 21 individuals of *B. alba*. Haplotype diversity is highest in the southeastern part of the genus range, where divergent haplotypes are separated by as many as 20 substitution events (Fig. 2). The morphological species with the greatest haplotype diversity is *B. aspera* (haplotypes P, S, and U), which also has a large distributional range (Fig. 3). The morphologically and geographically overlapping entities *B. melanocarpa*, *B. monoica*, and *B. lappifolia* share the N and O haplotypes (Figs. 1–3).

Material from apparently introduced and now naturalized bryonies was obtained from a population in Whitman County in the State of Washington; from the Rangitikei River in the foothills of the Ruahine Ranges on the North Island of New Zealand; and from near Tbilisi in Georgia. The *B. alba* plants from Whitman County have the A1 haplotype; the New Zealand material has the D2 haplotype only found in central and northern European *B. dioica*; and plants from Georgia have the D2 haplotype.

Analyses of the nuclear data. — We directly sequenced or cloned the *LFY* second intron from 47

individuals selected to represent the chloroplast haplotype diversity. Cloned sequences from the same plant mostly grouped together, and exclusion of all identical sequences left a dataset of 55 accessions (alignment length 523 characters, including 50 coded indels). A Maximum Likelihood tree from these data (Fig. 4) shows the species-level clades also seen in the chloroplast tree, namely *B. acuta*, *B. alba*, *B. aspera*, *B. monoica* plus embedded *B. lappifolia* and *B. melanocarpa*, *B. multiflora*, *B. syriaca*, and *B. verrucosa*. Sequences of *B. dioica*, *B. cretica*, and *B. marmorata*, however, did not form monophyla (Fig. 4). A topological difference between the nuclear tree and the chloroplast tree that is statistically supported is that *B. multiflora* groups with *B. cretica* and *B. syriaca* in the chloroplast tree, but with *B. alba* and *B. aspera* in the nuclear tree.

DISCUSSION

How many biological species of *Bryonia*? — A comparison of the morphology-based species circumscriptions of Jeffrey (1969) who distinguished twelve species of

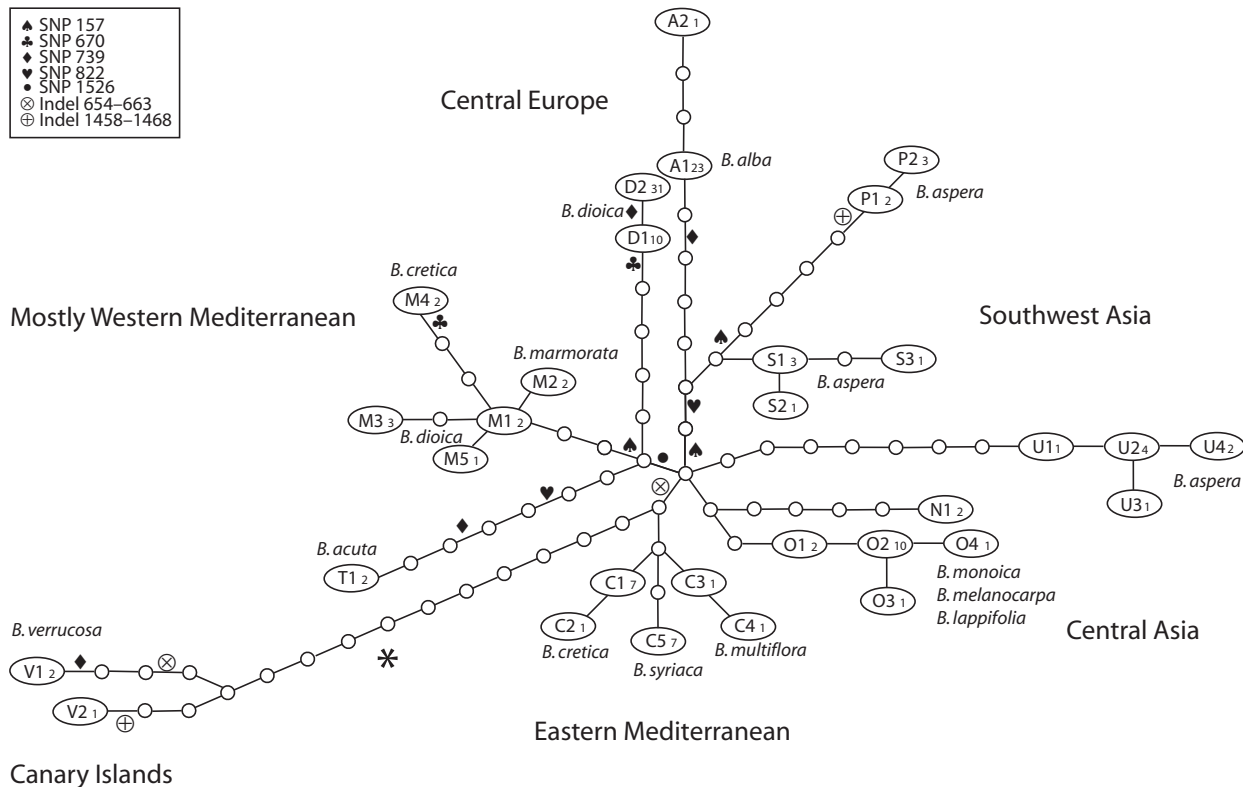


Fig. 2. Minimum-spanning network of the 31 chloroplast haplotypes found among 131 individuals of *Bryonia* from throughout the range of the genus. Haplotypes are labeled as in Fig. 1; numbers in subscript refer to the number of individuals in which each haplotype was found. Small open circles indicate inferred intermediate haplotypes. Five point mutations and two indels (listed in the inset) due to homoplastic mutations were assigned by hand to eliminate reticulations in the network. The asterisk marks the position of the root as inferred from analyses that included outgroups (Fig. 1).

Bryonia with the clusters seen in the nuclear and chloroplast trees shows a large extent of agreement. The molecularly supported entities are *B. acuta*, *B. alba*, *B. aspera*, *B. monoica* (as long as *B. lappifolia* and *B. melanocarpa* are included; below), *B. multiflora*, *B. syriaca*, and *B. verrucosa* (statistical support is usually higher in the nuclear tree than in the chloroplast tree, Figs. 1, 4). This suggests that the combination of subtle morphological characters and geographic occurrence used by Jeffrey for the most part correlates with reproductively isolated gene pools.

Species that Jeffery (1969) considered problematic were *B. lappifolia* and *B. melanocarpa*. *Bryonia lappifolia* was described from fruiting specimens (Vassilczenko, 1957) and the name appears not to have been used since. Based on leaf morphology, Jeffrey (1969: 450) suggested that *B. lappifolia* was but a variant of *B. monoica*, and this is supported by the nuclear and chloroplast data (Figs. 1, 4). Likewise, *B. melanocarpa*, which Jeffrey (1969: 452) suspected to “eventually [will prove] no more than a local variant of *B. monoica*”, is embedded in the *B. monoica* clade. The geographic range of *B. monoica* completely encloses those of *B. lappifolia* and *B. melanocarpa* (Fig. 3),

and based on these data, both names are best treated as synonyms of *Bryonia monoica*.

The conflicts between the chloroplast and nuclear trees, and the non-monophyly of *B. dioica*, can be resolved by two assumptions. First, the nesting of *B. marmorata* within *B. dioica* in both the chloroplast and the nuclear tree would fit with an autopolyploid origin of *B. marmorata* from *B. dioica*; the former is tetraploid, the latter diploid (Volz & Renner, 2008). Second, the contrasting placements of *B. dioica* and *B. cretica* relative to each other and to *B. syriaca* and *B. multiflora* in the chloroplast and nuclear trees would fit with a hybrid origin of the hexaploid *B. cretica*. The latter has multiple *LFY* haplotypes, some resembling those of *B. dioica*, others those of *B. syriaca* and *B. multiflora* (Fig. 4) and also two chloroplast haplotypes (Fig. 2), one resembling *B. dioica* (the M haplotype), the other *B. syriaca* and *B. multiflora* (the C haplotype).

Biogeography and likely area of origin. — All species of *Bryonia* occur on well-drained soils, such as sand dunes, dry riverbeds, or rocky slopes in mountainous areas, and all have water-storing underground tubers. In some species, such as *B. monoica* in the Kyzyl Kum in

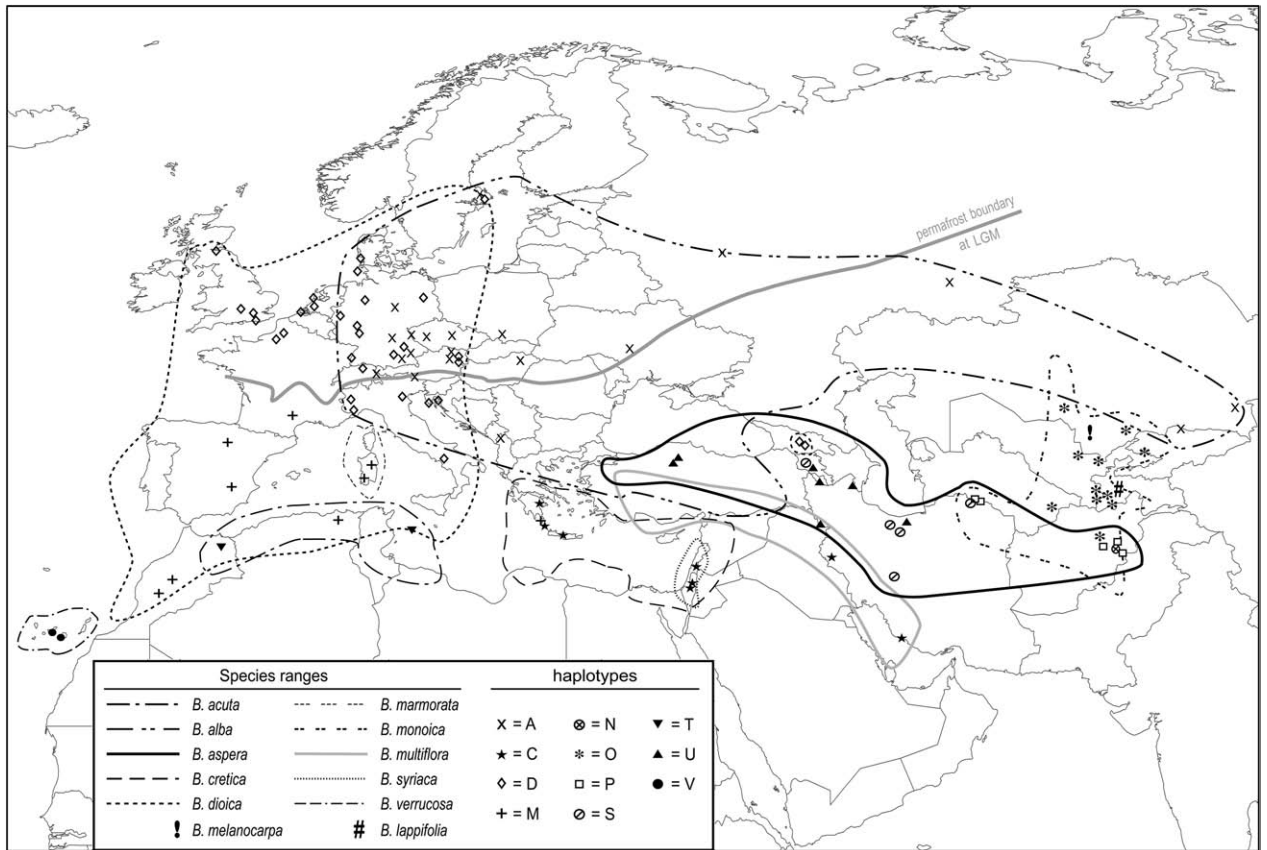


Fig. 3. Geographic distribution of the major chloroplast haplotypes found in *Bryonia*. Species boundaries after Jeffrey (1969), but including newly discovered occurrences of *B. acuta* in Morocco and *B. dioica* in Georgia. The labeling of haplotypes corresponds to that in Figs. 1 and 2.

Central Asia, the tubers can reach a length of 75 cm and a weight of 27 kg (Nabiev, 1961; our Fig. 5). Given the tuberous roots, it is unlikely that any bryonies could have survived permafrost conditions. The presence of *B. dioica* and *B. alba* in northern Europe therefore likely postdates the last glacial maximum some 10,000 years ago (the southern permafrost border is shown in Fig. 3). Relatively recent northwards expansion fits with the reduced chloroplast haplotype diversity in these species compared with that found in more southern species with similar sized ranges, such as *B. aspera*. Reduced haplotype diversity paralleling post-Pleistocene range expansion has been reported in numerous phylogeographic studies of both plants and animals (Hewitt, 2000; Dobes & al., 2004; Dorken & Barrett, 2004; for *B. dioica*: Oyama & al., 2009).

Adaptations, such as water storing root tubers and seasonal growth with aboveground parts dying back

completely during the unfavorable season, point to an origin of *Bryonia* in a region with prolonged seasonal droughts. Such climates characterize the Irano-Turanian biogeographic region, which extends eastwards from Anatolia to include most of Syria, Iran and northeast Afghanistan, south to northern Iraq and parts of Lebanon, Jordan, and Israel, and northwards into Central Asia (including most of Kazakhstan). This is precisely the region with the peak haplotype diversity and highest density of species of *Bryonia* (Fig. 3). The closest living relative of *Bryonia*, *Ecballium elaterium*, also occurs in Turkey, Lebanon, Jordan, Israel, and into Georgia as well as in the western Mediterranean.

Following divergence from *Ecballium* somewhere in the Irano-Turanian region, *Bryonia* clearly expanded its range along the Tethys shores and must have reached the Canary Islands early during its evolution, given that the

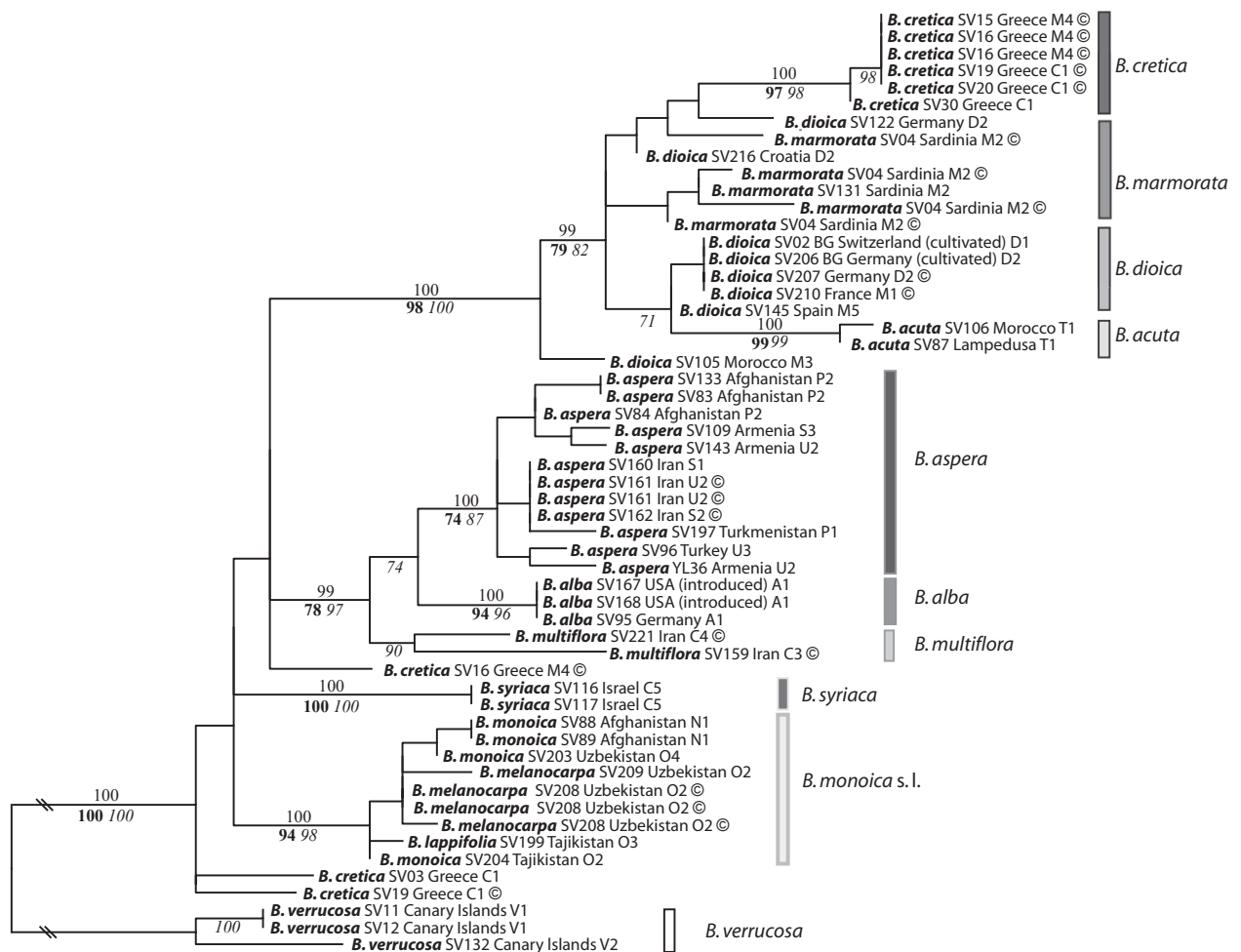


Fig. 4. Maximum likelihood tree from 55 nuclear *LFY* sequences of *Bryonia*, rooted on *B. verrucosa*. Following the species names are DNA number, geographic origin, and chloroplast haplotype labeled as in Figs. 1 and 2. Cloned sequences are marked by a ©. Numbers above branches indicate Bayesian posterior probabilities >95, those below, bootstrap support >75 under parsimony (bold) and >70 under maximum likelihood (italics). The non-monophyly of *B. dioica*, *B. cretica*, and *B. marmorata* is discussed in the text.

Canary Island endemic *B. verrucosa* is sister to all other species. Similar sister relationships between species from the Eastern Mediterranean and the Macaronesian Islands have been noted in *Convolvulus* (Carine & al., 2004) and *Hypochaeris* (Cerbah & al., 1998). Bird or ocean currents may both have played a role during the range expansion of *Bryonia*. The size and color of the berries of most species suggest bird-dispersal, although floating in water also occurs (Praeger, 1913; Ridley, 1930), perhaps especially in thick-skinned berries such as those of *B. verrucosa*. Oceanic dispersal may also explain the disjunction between the European/Irano-Turanian *Bryonia/Ecballium* clade and its Australian sister clade, *Austrobryonia*. A molecular clock places the divergence between these clades in the Middle Eocene (Schaefer & al., 2008).

Most species of *Bryonia* have red or black berries that reach <1 cm in diameter and are bird-dispersed (Ridley, 1930; Laferriere & al., 1993; Ludwig, 1995; Fig. 5a). Only *B. verrucosa*, the species endemic to the Canary Islands, has berries that reach 2–5 cm in diameter and that at maturity are orange yellow. Since the bird fauna of the Canary Islands comprises numerous species of migrants, including warblers, fruit doves, and other relatively large birds that may occasionally travel without having voided all undigested seeds, introduction by a migrant bird is conceivable, although water dispersal of an occasional floating berry may be equally plausible. *Bryonia alba* forms a trichotomy with *B. aspera* and *B. multiflora* from the Caucasus and Asia minor (Turkey, Syria, Iran, Iraq), and thus probably extended into northern Europe from the



Fig. 5. Habit and habitats of *Bryonia*. a, male flower and fruit of two plants of *B. dioica* growing next to each other; b, the first author holding a tuber of *B. alba* dug up near a train track in Bavaria, Germany; c, tuber of *B. melanocarpa* dug up in the Kyzyl Kum desert in Uzbekistan; d, a second tuber of *B. melanocarpa* from the same locality as c.

southeast. By contrast, *B. dioica* is closest to *B. acuta* from western North Africa (Morocco, Tunisia, Algeria, Libya), suggesting that it may have reached northern Europe via Spain and France.

Anthropogenic range changes. — From the literature one may gain the impression that the northern European presence of *B. alba* and *B. dioica* is largely anthropogenic (Tutin, 1968; Jeffrey, 1969; Reynolda, 2002; Schönfelder & Schönfelder, 2004; but see Ludwig, 1995 for a different view). This is indeed known for certain well-studied areas. For example, *B. dioica* was first recorded in Ireland in 1803, and it was also introduced in Scotland, Northwest England, and Northwest Wales (Stokes & al., 2004; J. Parnell, Trinity College, pers. comm., Feb. 2008). The chloroplast spacer regions and the intron in the nuclear *LFY* gene sequenced here are insufficiently variable to differentiate population expansions at time scales of hundreds or thousands of years, and it is therefore difficult to distinguish man-made from natural expansions. Only disjunctions separated by oceans or by well-collected areas in which the absence of a particular species is known can serve to infer anthropogenic dispersal of bryonies. An example is *B. dioica*, the range of which includes a disjunction of ca. 2,300 km between its occurrence in Tbilisi (in Georgia at 44°47'E) and the eastern border of its main range (Fig. 3) in western Hungary, Slovakia, and the extreme western parts of Bulgaria. The easternmost collections cited by Jeffrey (1969) are a collection from Győr, 17°38'E, and one from Trencin, 18°02'E. Floras of Bulgaria and Romania do not include *B. dioica* as a native plant (Velenovsky, 1891; Savulescu, 1964), and the herbaria in Vienna, Bucharest, and Sofia also do not harbor bryony collections from these countries. However, while an older Romanian flora lists *B. dioica* records as doubtful (Savulescu, 1964: 30), a more recent flora states that *B. dioica* may occur at Arad, Radna, and Bucharest (Ciocarlan, 2000). It is not known whether the species may have escaped from the Bucharest Botanical Garden (P. Anastasiu, University of Bucharest, Department of Botany, pers. comm., March 2008).

In Georgia, *B. dioica* has been collected near the botanical garden of Tbilisi in 1999 and near the city's old fortress in 2002. The garden may date back to 1625 and like other botanical gardens in Europe may have harbored *Bryonia alba* and/or *B. dioica* in its medicinal plant section. It is therefore plausible that the presence of *B. dioica* in Georgia results from human introduction, perhaps through the botanical garden, perhaps through ancient medicinal plant gardens. Seeds must have come from northern or central Europe, because the Georgia plants have the D2 haplotype. This haplotype is also found in the *B. dioica* material introduced to New Zealand and originally identified as *B. cretica* subsp. *dioica* (Webb & al., 1995).

CONCLUSIONS

Findings of this study mostly support Jeffrey's (1969) species of *Bryonia*, with the exception of *B. lappifolia* and *B. melanocarpa*, which are part of *B. monoica*. Over the past 40–100 years, two species, *B. alba* and *B. dioica*, have been introduced to New Zealand and the United States, and we found new evidence of anthropogenic range expansion of *B. dioica* from central Europe to Georgia, fitting with the hypothesis that this species may be spreading east (Ludwig, 1995). Bryonies are currently naturalized or becoming invasive in several countries, and this study provides a baseline from which to assess the ongoing range changes of the ten distinct species.

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Appendix. *Bryonia*, *Ecballium*, and *Austrobryonia* species and their authors, voucher information, collecting locality, DNA number, chloroplast haplotype, and GenBank accession numbers for all sequences used in this study. When no collection number has been assigned by the collector, the collection date is given instead. BG stands for botanical garden.

Species, vouchers, collecting locality, DNA, chloroplast haplotype, *trnL*, *atpA-trnR*, *psbA-trnH*, *LFY* intron, *rbcL*

B. acuta Desf., *H. Ross 431* (M), Italy, Lampedusa, SV 87, T1, EU102403, EU102538, EU096433, EU102560, –; *D. Podlech 42293* (MSB), Morocco, Oujda, SV 106, T1, EU102404, EU102539, EU096434, EU102559, –; *B. alba* L., *M. Kropf 24* Aug. 2006 (M), Hungary, Tokaj, YL 37, A2, EU0683744, EU0683741, EU0683738, –, –; *E. Dörr 28* Feb. 1987 (M), Germany, Bavaria, Erlangen, SV 07, A1, EU102289, EU102422, EU096317, –, –; *G. Cocora-Tietz & S. Tietz 98/39* (M), Germany, Bavaria, Munich-Feldmoching, SV 08, A1, EU102290, EU102423, EU096318, –, –; *F. Gierster 11* Aug. 1898 (M), Germany, Bavaria, Dingolfing, SV 09, A1, EU102291, EU102424, EU096319, –, –; *S. Volz 5* (MSB), Germany, Saxony-Anhalt, Wegeleben, SV 94, A1, EU102292, EU102425, EU096320, –, –; *S. Volz 6* (MSB), Germany, Saxony-Anhalt, Neinstedt, SV 95, A1, DQ533867, EU102426, EU096321, EU102563, DQ535744; *F.-O. Wolf 22* July 1897 (M), Switzerland, Gresson, SV 134, A1, EU102293, EU102427, EU096322, –, –; *H. Merxmüller 16103* (M), Poland, Myslenice, SV 135, A1, EU102294, EU102428, EU096323, –, –; *O. Angerer 21* June 1984 (M), Austria, Reinthal, SV 138, A1, EU102295, EU102429, EU096324, –, –; *A. Baldacci 316* (M), Albania, Broja, SV 139, A1, EU102296, EU102430, EU096325, –, –; *F. Urban 24* Aug. 1910 (M), Czech Republic, Tachov, SV 140, A1, EU102297, EU102431, EU096326, –, –; *Karwowski s.n.*, 1897 (M), Ukraine, Kiev, SV 141, A1, EU102298, EU102432, EU096327, –, –; *A. Skvortsov 661* (M), Russia, Moscow, SV 142, A1, EU102299, EU102433, EU096328, –, –; *M. Deyl & B. Deylova 312* (M), Czech Republic, Praha, SV 150, A1, EU102300, EU102434, EU096329, –, –; *L. Wanntorp 7* Oct. 2004 (MSB), Sweden, Vallentuna, SV 167, A1, EU102301, EU102435, EU096330, EU102561, –; *J. Brokaw 138* (M), U.S.A., Washington, Whitman Co., SV 168, A1, EU102302, EU102436, EU096331, EU102562, –; *J. Brokaw 139* (M), U.S.A., Washington, Whitman Co., SV 169, A1, EU102303, EU102437, EU096332, –, –; *J. Brokaw 140* (MSB), U.S.A., Washington, Whitman Co., SV 170, A1, EU102304, EU102438, EU096333, –, –; *M. Kropf 15* July 2005b (M), Austria, Kogelsteine, SV 173, A1, EU102305, EU102439, EU096334, –, –; *M. Kropf 15* July 2005a (M), Austria, Haugsdorf, SV 174, A1, EU102306, EU102440, EU096335, –, –; *T. Hájek* Sep. 2005 (M), Czech Republic, Distr. Mělník, SV 177, A1, EU102307, EU102441, EU096336, –, –; *C. Kuierovskaj 537* (MHA), Republic of Bashkortostan, Sterlitamak, SV 183, A1, EU102308, EU102442, EU096337, –, –; *N. Sipcinsno 210* (MHA), Kazakhstan, Alatau Zhotasy, SV 194, A1, EU102309, EU102443, EU096338, –, –; *Gorburnova 23* Aug. 1966 (MHA), Kyrgyzstan, Bishkek, SV 195, A1, EU102310, EU102444, EU096339, –, –; *B. aspera* Stev. ex Ledeb., *D. Kurbanov 657* (MO), Turkmenistan, western Kopet Dag, SV 101, P1, EU102393, EU102528, EU096423, –, –; *N. Androsow 2547* (MHA), Turkmenistan, Ak-Gaudan, SV 197, P1, EU102394, EU102529, EU096424, EU102526, –, –; *K. Rechingher 18076* (M), Afghanistan, Kabul, SV 83, P2, EU102395, EU102530, EU096425, EU102577, –, –; *K. Rechingher 32214* (M), Afghanistan, Jaji, SV 84, P2, EU102396, EU102531, EU096426, EU102578, –, –; *D. Podlech 12286* (MSB), type of *B. afghanica* Podlech, Afghanistan, Kapisa, SV 133, P2, EU102397, EU102532, EU096427, EU102565, –, –; *S. Zarre 35280* (MSB), Iran, Tehran, SV 160, S1, EU102398, EU102533, EU096428, EU102567, –, –; *M. Parishani 14209* (M), Iran, Isfahan, SV 163, S1, EU102399, EU102534, EU096429, –, –; *A. Jarmolenko 913* (MHA), Turkmenistan, central Kopet Dag, SV 196, S1, EU102400, EU102535, EU096430, –, –; *S. Zarre 35818* (MSB), Iran, Mazandaran, SV 162, S2, EU102401, EU102536, EU096431, EU102571–75, –, –; *D. McNeal 475* (MO), Armenia, Gora, SV 109, S3, EU102402, EU102537, EU096432, EU102564, –, –; *E. Vitek 03-0273*, Armenia, Lori province, SV 179, S3, FJ009170, –, –, –; *E. Vitek 03-0668*, Armenia, Kotayk province, SV 180, S3, FJ009171, –, –, –; *T. Heideman 5323-3000* (MO), Azerbaijan, Naxcivan, SV 110, U1, EU102405, EU102540, EU096435, –, –; *E. Vitek 04-0526* (MSB), Armenia, Kotayk Province, YL 36, U2, EU0683746, EU0683743, EU0683740, EU0683747, –, –; *G. Fayvush & al. 1201* (M), Armenia, Kotayk Province, SV 143, U2, EU102408, EU102543, EU096438, EU102566, –, –; *S. Zarre 35812* (MSB), Iran, Mazandaran, SV 161, U2, EU102409, EU102544, EU096439, EU102568–70, –, –; *J. Bornmüller 14581* (Z), Turkey, Paphlagonia, Ilgas, SV 99, U2, EU102407, EU102542, EU096437, EU102665, –, –; *P. Sintenis 4746* (Z), Turkey, Paphlagonia, Tosya, SV 96, U3, EU102410, EU102545, EU096440, EU102663, –, –; *K. Rechingher 49111* (M), Azerbaijan, Sardasht, SV 86, U4, EU102411, EU102546, EU096441, –, –; *K. Rechingher 40362* (M), Azerbaijan, Germi, SV 91, U4, EU102412, EU102547, EU096442, –, –; *B. cretica* L., *A. Kocyan 030509/3/01* (M), Greece, Kythera, SV 03, C1, EU102311, EU102445, EU096340, EU102579, –, –; *H. Tillich 4728* (M), Greece, Crete, SV 14, C1, EU102312, EU102446, EU096341, –, –; *A. Kocyan 122c* (M), Greece, Kythera, SV 19, C1, EU102313, EU102447, EU096342, EU102611–16, –, –; *A. Kocyan 122d* (M), Greece, Kythera, SV 20, C1, EU102314, EU102448, EU096343, EU102617–21, –, –; *A. Kocyan 120e* (M), Greece, Kythera, SV 29, C1, EU102315, EU102449, EU096344, –, –; *A. Kocyan 120f* (M), Greece, Kythera, SV 30, C1, EU102316, EU102450, EU096345, EU102622, –, –; *TU-Exkursion 7* Apr. 1989 (M), Greece, Nafplion, SV 151, C1, EU102317, EU102451, EU096346, –, –; *H. Tillich 4724* (M), Greece, Crete, SV 13, C2, EU102318, EU102452, EU096347, –, –; *A. Kocyan 121a* (M), Greece, Kythera, SV 15, M4, EU102375, EU102510, EU096405, EU102580–85, –, –; *A. Kocyan 121b* (M), Greece, Kythera, SV 16, M4, EU102376, EU102511, EU096406, EU102586–610, –, –; *B. dioica* Jacq., *S. Renner 2187* (M), Switzerland, BG Zurich, SV 2, D1, DQ536791, EU102462, EU096357, EU102626, DQ535786; *F. Schuhwerk 05/224* (M), Germany, Bavaria, SV 123, D1, EU102328, EU102463, EU096358, –, –; *M. Kropf*, no voucher, Germany, Trollbachtal, SV 130, D1, EU102329, EU102464, EU096359, –, –; *L. Holm-Nielsen 374* (M), Denmark, Fanø, SV 147, D1, EU102330, EU102465, EU096360, –, –; *K. Thedenius* Aug. 1884 (M), Sweden, Stockholm, SV 148, D1, EU102331, EU102466, EU096361, –, –; *H. Förther 8307* (MSB), Germany, North Rhine-Westphalia, SV 156, D1, EU102332, EU102467, EU096362, –, –; *F. Schuhwerk* June 1968 (M), Germany, Kaiserstuhl, SV 158, D1, EU102333, EU102468, EU096363, –, –; *B. Gravendeel 2609* (L), Netherlands, Berheide, SV 164, D1, EU102334, EU102469, EU096364, –, –; *W. de Wilde 22297* (MSB), Netherlands, Beverwyk, SV 166, D1, EU102335, EU102470, EU096365, –, –; *B. Schlumpberger 250* (M), Netherlands, Zeeland, Middelburg, SV 175, D1, EU102336, EU102471, EU096366, –, –; *S. Renner & al. 2779* (M), Germany, BG Mainz, SV 01, D2, EU102337, EU102472, EU096367, –, –; *H. Förther 5811* (M), Germany, Bavaria, Regensburg, SV 06, D2, EU102338, EU102473, EU096368, –, –; *S. Renner 2728* (M), Germany, BG Berlin, SV 82, D2, EU102339, EU102474, EU096369, –, –; *M. Kropf*, no voucher, Germany, Minden, SV 103, D2, EU102340, EU102475, EU096370, –, –; *S. Volz 17a* (MSB), Germany, Berlin, SV 120, D2, EU102342, EU102477, EU096372, –, –; *S. Volz 17b* (MSB), Germany, Berlin, SV 121, D2, EU102343, EU102478, EU096373, –, –; *F. Schuhwerk 05/222b* (M), Germany, Bavaria, SV 122, D2, EU102344, EU102479, EU096374, EU102624, –, –; *S. Volz 4c* (MSB), Germany, Saxony-Anhalt, SV 74, D2, FJ174449, FJ009140, FJ009154, –, –; *S. Volz 4e* (MSB), Germany, Saxony-Anhalt, SV 76, D2, FJ174448, FJ009141, FJ009155, –, –; *W. Lippert 04* May 1966 (M), Italy, Padua, SV 136, D2, EU102345, EU102480, EU096375, –, –; *T. Schauer 01* June 1963 (M), Italy, Fontane, SV 137, D2, EU102346, EU102481, EU096376, –, –; *B. de Retz 93102* (MSB), France, Merval, SV 144, D2, EU102347, EU102482, EU096377, –, –; *P. Ball 22* June 1960 (M), Great Britain, Sussex, SV 149, D2, EU102348, EU102483, EU096378, –, –; *H. Dihm 08* July 1912 (M), Croatia, Istria, Pula, SV 152, D2, EU102349, EU102484, EU096379, –, –; *R. Camoletto 2089* (M), Italy, Torino, SV 153, D2, EU102350, EU102485, EU096380, –, –; *J. Pfadenhauer 03* June 1968 (M), Italy, Napoli, SV 154, D2, EU102351, EU102486, EU096381, –, –; *I. & H. Hertel 6671* (M), Austria, Burgenland, SV 155, D2, EU102352, EU102487, EU096382, –, –; *H. Kalheber 78-415* (M), Germany, Hesse, SV 157, D2, EU102353, EU102488, EU096383, –, –; *B. Gravendeel 2610* (L), Netherlands, Berheide, SV 165, D2, EU102354, EU102489, EU096384, –, –; *M. Kropf 15* Sep. 2005a, female (M), Austria, Vienna, SV 171, D2, EU102355, EU102490, EU096385, –, –; *M. Kropf 15* Sep. 2005b, male (M), Austria, Vienna, SV 172, D2, EU102356, EU102491, EU096386, –, –; *G. Schneeweiß 10886* (MSB), Austria, Vienna, SV 176, D2, EU102357, EU102492, EU096387, –, –; *T. Gilbertson 20116* (WELTU), New Zealand, North Island, Rangitikei River in foothills of Ruahine Ranges, SV 178, D2, EU102358, EU102493, EU096388, –, –; *S. Volz 12* (MSB), Germany, Bavaria, BG Andechs, SV 206, D2, EU102359, EU102494, EU096389, EU102627, –, –; *S. Volz 23* (MSB), Germany, Helgoland, SV 207, D2, EU102360, EU102495, EU096390, EU102628–29, –, –; *R. Milne 2006-BL* (E), England, Barnes, SV 211, D2, EU102361, EU102496, EU096391, –, –; *R. Milne 2006-BEa* (E), Scotland, Edinburgh, SV 212, D2, EU102362, EU102497, EU096392, –, –; *R. Milne 2006-BEb* (E), Scotland, Edinburgh, SV 213, D2, EU102363, EU102498, EU096393, –, –; *M. Dorken*, no voucher, England, Oxford, SV 214, D2, EU102364, EU102499, EU096394, –, –; *M. Kropf*, no voucher, Croatia, Vrbinik, –

Appendix. Continued.

SV 215, D2, EU102365, EU102500, EU096395, –, –, *M. Kropf*, no voucher, Croatia, Vrbnik, SV 216, D2, EU102366, EU102501, EU096396, EU102635, –, *R. Gagnidze & al.* 269 20 June 2002 (MO), Georgia, Kartli, Tbilisi, Narikala, YL 32, D2, EU683745, EU683742, EU683739, –, –, *M. Merello & al.* 2220 8 June 1999 (MO), Georgia, Kartli, SV 112, D2, EU102341, EU102476, EU096371, –, –, *I. Granzow & J.P. Zaballos* 384 (M), Spain, Soria, SV 146, M1, EU102368, EU102503, EU096398, –, –, *G. Gerlach*, no voucher, France, Aveyron, St. Affrique, SV 210, M1, EU102369, EU102504, EU096399, EU102630–34, –, *A. Faure* 17 Jun. 1923 (M), Algeria, Lamoriciere, SV 85, M3, EU102372, EU102507, EU096402, –, –, *D. Podlech* 40924 (MSB), Morocco, Taroudan, SV 105, M3, EU102373, EU102508, EU096403, EU102623, –, *D. Podlech* 47692 (MSB), Morocco, De Beni-Mellal, SV 107, M3, EU102374, EU102509, EU096404, –, –, *A. Garcia-Villarraco* Aug. 1982 (M), Spain, Albacete, SV 145, M5, EU102367, EU102502, EU096397, EU102625, –, ***B. marmorata*** Petit, *H. Merxmüller* 21007 (M), Italy, Sardinia, SV 131, M2, EU102371, EU102506, EU096401, EU102644, –, *M. Erben & L. Klingenberg* 2 June 2003 (MSB), Italy, Sardinia, SV 04, M2, EU102370, EU102505, EU096400, EU102637–43, –, ***B. monoica*** Aitch. & Hemsl., *D. Podlech* 17761 (MSB), Afghanistan, Kabul, SV 88, N1, EU102377, EU102512, EU096407, EU102653, –, *K. Rechinger* 34470 (M), Afghanistan, Kabul, SV 89, N1, EU102378, EU102513, EU096408, EU102654, –, *D. Podlech* 6418 (MSB), Afghanistan, Bamian, SV 90, O1, EU102379, EU102514, EU096409, –, –, *Y. Soskov* 924 (MO), Tajikistan, Tizmansi, Bobotogh, SV 113, O2, EU102381, EU102516, EU096411, –, –, *P. Obchinnikab* 164 (MO), Tajikistan, Mogoltau Mts., SV 114, O2, EU102382, EU102517, EU096412, –, –, *V. Smirnov*, 123 (MHA), Kazakhstan, Kzyl Orda, SV 201, O2, EU102385, EU102520, EU096415, –, –, *Petaeva* 103 (MHA), Uzbekistan, near Karatau City, SV 202, O2, EU102386, EU102521, EU096416, –, –, *R.V. Kamelin* 1786 (MHA), Tajikistan, Babatag Mts., eastern slopes, SV 204, O2, EU102387, EU102522, EU096417, EU102652, –, *Nikolayev* 24 Apr. 1956 (MHA), Turkmenistan, SE Kara Kum desert, SV 205, O2, EU102388, EU102523, EU096418, –, –, *R.V. Kamelin & Machmebow* 253 (MHA), Uzbekistan, Babatag Mts., near Garmobulak, SV 203, O4, EU102392, EU102527, EU096422, EU102651, –, ***B. monoica*** (***B. lappifolia*** Vass.), *E. Varivtseva* 208 (MHA), Tajikistan, Kara-Tau Mts., Khrebet, SV 198, O1, EU102380, EU102515, EU096410, –, –, *E. Varivtseva* 7 June 1948 (MHA), Tajikistan, Gardaim-Ushin, Pereval, SV 199, O3, EU102391, EU102526, EU096421, EU102636, –, ***B. monoica*** (***B. melanocarpa*** Nabiev), *M. Pimenov & al.* (MHA), Uzbekistan, Kyzylkum, Baymurat, SV 115, O2, EU102383, EU102518, EU096413, –, –, *Krasovskaja* 17 Apr. 1986 (MHA), Kazakhstan, Shardara, SV 200, O2, EU102384, EU102519, EU096414, –, –, *S. Volz* 21 (MSB), Uzbekistan, Kyzylkum, Baymurat, SV 208, O2, EU102389, EU102524, EU096419, EU102645–49, –, *S. Volz* 22 (MSB), Uzbekistan, Kyzylkum, Baymurat, SV 209, O2, EU102390, EU102525, EU096420, EU102650, –, ***B. multiflora*** Boiss & Heldr., *S. Zarre* 35811 (MSB), Iran, Bushehr, SV 159, C3, EU102319, EU102453, EU096348, EU102655–61, –, *V. Mozaffarian*, 88359 (TARI), Iran, Ilam, SV 221, C4, EU102320, EU102454, EU096349, EU102662, –, ***B. syriaca*** Boiss., *A. Liston* 9-4-1982 (M), Israel, Jerusalem, Judean Mts., SV 92, C5, EU102321, EU102455, EU096350, –, –, *A. Liston*, 7-85-245/4 (M), Israel, Hebron, Judean Mts., SV 93, C5, EU102322, EU102456, EU096351, –, –, *A. Liston* 1-85-245/4 (MO), Israel, Hebron, Judean Mts., SV 111, C5, EU102323, EU102457, EU096352, –, –, *A. Dafni* 1, female (M), Israel, Horshat Tal National Park, SV 116, C5, EU102324, EU102458, EU096353, EU102666, –, *A. Dafni* 2, female (M), Israel, Horshat Tal National Park, SV 117, C5, EU102325, EU102459, EU096354, EU102667, –, *A. Dafni* 1, male (M), Israel, Horshat Tal National Park, SV 118, C5, EU102326, EU102460, EU096355, –, –, *A. Dafni* 2, male (M), Israel, Horshat Tal National Park, SV 119, C5, EU102327, EU102461, EU096356, –, –, ***B. verrucosa*** Ait., *M. Erben* 20 March 2004 (MSB), Canary Islands, Gran Canaria, SV 11, V1, EU102413, EU102548, EU096443, EU102668, EU102671; *M. Erben*, no voucher, Canary Islands, Gran Canaria, SV 12, V1, EU102414, EU102549, EU096444, EU102669, –, *Müller*, 29 March 64 (M), Canary Islands, Tenerife, SV 132, V2, EU102415, EU102550, EU096445, EU102670, –, ***E. elaterium*** subsp. ***elaterium*** (L.) A. Rich., *S. Renner & al.* 2768 (M), Germany, BG Mainz, SV 10, –, AY973006, EU102551, EU096446, –, AY973023; *S. Volz* 14 (MSB), Germany, BG Munich, SV 104, –, EU102416, EU102552, EU096447, –, –, *J. Laborde* 116921 (MSB), Spain, Madrid, SV 129, –, EU102417, EU102553, EU096448, –, –, ***E. elaterium*** subsp. ***dioicum*** (Batt.) Costich, *J. Laborde* 116922m (MSB), Spain, Madrid, SV 124, –, EU102418, EU102554, EU096449, –, –, *J. Laborde* s.n. (MSB), Spain, Madrid, SV 125, –, EU102419, EU102555, EU096450, –, –, *J. Laborde* 116922f (MSB), Spain, Madrid, SV 126, –, EU102420, EU102556, EU096451, –, –, *J. Laborde* s.n. (MSB), Spain, Madrid, SV 127, –, EU102421, EU102557, EU096452, –, –, ***Austrobryonia micrantha*** (F. Muell.) I. Telford, *I. Telford* 8173 (CANB), Australia, HS 411, –, EF487575, EU102558, EU096453, –, EF487552.