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Evolution of dioecy in the Cucurbitaceae genus *Bryonia* – a phylogenetic, phylogeographic, and SCAR-marker approach

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Erklärung:

Diese Dissertation wurde im Sinne von § 12 der Promotionsordnung der LMU von Frau Professor Dr. S. S. Renner betreut. Ich erkläre hiermit, dass die Dissertation nicht einer anderen Prüfungskommission vorgelegt worden ist und, dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

Ehrenwörtliche Versicherung:

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde.

Stefanie Volz

München, den 01.09.08

Note

In this dissertation, I present the results of my doctoral research, carried out from January 2004 until August 2007 at the University of Munich (Ludwig-Maximilians-Universität München), under the supervision of Professor Dr. S. S. Renner. It is organized in three chapters, each representing a submitted manuscript.

For the work presented in **chapter 1**, I generated, analyzed, and interpreted the data. The writing was done by myself and revised by S. S. Renner. chapter 1 is in press at the ***American Journal of Botany*** under the following title:

VOLZ, S. M. and S. S. RENNER. Hybridization, polyploidy, and evolutionary transitions between monoecy and dioecy in *Bryonia* (Cucurbitaceae).

For the work presented in **chapter 2**, I also generated, analyzed, and interpreted the data. The writing was done by myself and S. S. Renner. This chapter is in press at ***Taxon*** under the following title:

VOLZ, S. M. and S. S. RENNER. Phylogeography of the ancient Eurasian medicinal plant genus *Bryonia* (Cucurbitaceae) inferred from nuclear and chloroplast sequences.

For **chapter 3**, I performed the AFLP analyses and screened them for sex-linked markers. Dr. R. K. Oyama and I developed the SCAR markers. The manuscript was mostly written by R. K. Oyama and revised by S. S. Renner and myself. This chapter is in review at the ***Journal of Evolutionary Biology*** under the following title:

OYAMA, R. K., S. M. VOLZ, and S. S. RENNER. A sex-linked SCAR marker in *Bryonia dioica* (Cucurbitaceae), a dioecious species with XY sex-determination and homomorphic sex chromosomes.

Contents

Summary

Genetic crosses between the dioecious *Bryonia dioica* Jacq. (Cucurbitaceae) and the monoecious *B. alba* L. in 1903 provided the first clear evidence for Mendelian inheritance of dioecy and made *B. dioica* the classic case of XY sex determination in plants. We use chloroplast (cp) and nuclear (nr) DNA sequences from 129 individuals representing all morphological species to study species relationships and distribution, sexual system evolution, and association of ploidy-level with dioecy in *Bryonia*. Chloroplast and nuclear trees mostly fit morphological species concepts; there are seven dioecious and three monoecious species, together ranging from the Canary Islands to Central Asia. *Bryonia verrucosa,* the morphologically most differing species from the Canary Islands is sister to all other species. Our data argue for the inclusion of the narrowly endemic Central Asian species *B. lappifolia* and *B. melanocarpa* in *B. monoica*. Conflicts between cp and nr topologies imply that the dioecious hexaploid *B. cretica* arose from hybridization(s) involving the diploid species *B. dioica*, *B. syriaca*, and/or *B. multiflora*. The tetraploid *B. marmorata* likely originated via autopolyploidy. The nr phylogeny implies at least two transitions between dioecy and monoecy, but no correlation between change in sexual system and ploidy level. Fossil-calibrated molecular clocks using family-wide *rbc*L data with a *Bryonia*-centered sampling suggest that the deepest divergence in *Bryonia* occurred ca. ten million years ago and that monoecious and dioecious species crossed in the classic studies are separated by several million years of evolution. Traits, such as annual regrowth from a tuberous rootstock and other adaptations to a seasonal climate, as well as species and haplotype abundance, point to an origin of *Bryonia* in the Middle East. Species and haplotype poverty north of the Alps together suggest recolonization there after the last glacial maximum.

Most species of *Bryonia* have 10 chromosomes (as confirmed by my own counts), and there appears to be no morphologically distinct pair that would represent the sex chromosomes. However, we know from the crossings carried out by Correns and others that in *B. dioica*, sex shows monofactorial dominant inheritance, setting up the hypothesis that *B. dioica* may have a pair of chromosomes on which key sex-determining gene(s) and sex-linked genes have accumulated. To gain insight into the possible presence of such a pair of sex chromosomes in *B. dioica*, it is necessary to sequence a fairly long sex-linked region to study its substitution behavior and to eventually visualize its physical placement using FISH.

As a first step towards this goal, I developed a sex-linked SCAR marker for *B. dioica* from AFLP bands and sequenced it for individuals representing the full distribution range of the species from Scotland to North Africa. The region north of the Alps harbours distinct Y and X alleles that differ in a 197-bp indel, with the Y allele being perfectly linked to the male sex. In southern Europe, however, the XY system appears to break down (to an extent that is not clear), and there are signs of recombination between the Y and X homologues. Population genetic analyses suggest that the sex-linked region I amplified (i.e., the SCAR marker) experienced different evolutionary pressures in northern and southern Europe. These findings fit the evidence from my phylogenetic and phylogeographic analyses that the XY system in *Bryonia* is evolutionarily labile. Overall, my work suggests that *Bryonia* may be a good, but very complex, system in which to study the early steps of plant sex chromosome evolution.

General Introduction

In higher animals, including ourselves, having strictly separate sexes is the predominant condition. The situation in flowering plants, however, is very different: they mostly have hermaphroditic flowers that fulfill both, the male and the female function. Separate sexes (dioecy) evolved only in around six percent of the 240,000 flowering plant species (Renner and Ricklefs, 1995), usually from perfect-flowered or monoecious ancestors, as suggested by comparative evidence (Darwin, 1876; Lewis, 1942; Westergaard, 1958; Charlesworth and Charlesworth, 1978; Renner and Won, 2001; Dorken et al., 2002; Dorken and Barrett, 2004). Monoecious flowering plants have male and female organs (stamens and pistils) on every individual, while dioecious flowering plants either produce stamens or pistils, but never both. In addition to monoecy and dioecy, flowerings plants have at least 11 other sexual systems (andromonoecy, gynomonoecy, heterostyly, enantiostyly, androdioecy, gynodioecy, trioecy or polygamodioecy, in addition to the overlaid phenological strategies dichogamy, duodichogamy, and heterodichogamy). Unisexuality in land plants, i.e, embryophytes, which are characterized by the regular cycling between a gametophyte and a sporophyte generation, has evolved multiple times, implying that land plants may have many different forms of sex determination. As in animals, dioecy in plants sometimes results from the action of genes on distinct sex chromosomes (Westergaard, 1958; Vyskot and Hobza, 2004). The first visually distinct sex chromosomes found in a plant were those of the liverwort *Sphaerocarpus* (Allen, 1917), discovered shortly after the first sex chromosomes in an animal, namely those of the mealworm *Tenebrio* (Stevens, 1905). The sex determination that is most common in animals, heteromorphic XY or WZ sex chromosomes, evolved exceedingly rarely in plants and is only known from a dozen species in five genera: *Cannabis* L., *Humulus* L., *Rumex* L., *Silene* L., and *Coccinia* Wight in Arn. (Ming et al., 2007). However, both classic and modern molecular work on the evolution (and loss) of plant sex chromosomes has focused on very few model systems, such as *Silene*, papaya (*Carica papaya*), and *Rumex*, and we therefore know very little about broader patterns.

The main experimental system for early work on sex determination in plants was the Eurasian Cucurbitaceae genus *Bryonia* L. (Correns, 1903). This small cucurbit genus is among the relatively few groups of flowering plants in which monoecious and dioecious species are sufficiently closely related to permit crossing experiments and study of the

resulting hybrids. To infer the mode of sex determination in the dioecious species *B. dioica* Jacq., Correns performed reciprocal crosses between this species and the monoecious *B. alba* L. From the sex ratios obtained, he concluded that half the pollen grains of *B. dioica* carried a "female tendency," the other half a "male tendency." Dioecious species of *Bryonia* do not have morphologically distinct sex chromosomes.

The genus *Bryonia* (from greek *bryein*, to grow or sprawl) ranges from the Canary Islands to Central Asia. It comprises perennial herbs with tuberous roots that occur from extremely dry to nutrient-rich sites where they climb over and cover other vegetation with the help of long simple tendrils. The five-parted flowers of most species are small, greenish-white, and have five partially fused stamens or, in female flowers, staminodes. Fruits are black or red berries, up to one centimeter in diameter. The morphologically most distinct species is *B. verrucosa* Ait., endemic to the Canary Islands and the only bryony with greenish to orange striped fruits that are, like the flowers, bigger than in all other species. Also unique in the genus is the ejection of the *B. verrucosa* seeds from the ripe fruits. Jeffrey (1969), in the most recent revision, recognized 12 species, but indicated that he might as well have recognized only four. Flora Europaea recognized only two species in Europe (Tutin et al., 1968; also Scholz, 1979). My own research, based on DNA sequences from several chloroplast loci, two nuclear loci, chromosome numbers, phylogeographic analyses, and morphology, shows that there are probably only ten biologically distinct species of which three are predominantly monoecious and seven dioecious. Taxonomically important traits are colour and shape of the leaves, pubescence, and fruit colour and size. The two entities that should be included in another species (reducing the number of species from 12 to 10) were *B. lappifolia* Vass. and *B. melanocarpa* Nabiev, both only known from a few collections each. They are here considered mere variants of *B. monoica*.

Much information is available on the pollination of *Bryonia* (Kugler, 1981; Dukas, 1987; Westrich, 1989; Schröder and Lunau, 2001; Fahn and Shimony, 2001; Costich and Meagher, 2001; Rust et al., 2003). Pollen- and nectar- gathering bees, predominantly the oligolectic sandbee *Andrena florea* Fab., pollinate them. This bee depends on the pollen of *Bryonia* to feed its larvae and does not collect pollen from other species even if abundant alternative pollen sources are available (Schröder and Lunau, 2001). Male and female flowers provide nectar, albeit in different amounts, with female flowers producing abundant nectar.

Asexual reproduction is known in the genus only from *B. alba* (Bitter, 1905; Novak

and Mack, 2000, Volz, unpublished). This species was introduced to North America sometime post-1940 and is now invasive in the western United States (Washington, Oregon, Montana, Utah), where it reproduces predominantly by asexual seed formation and vegetative spread (Novak and Mack, 1995 and 2000). Experiments that I carried out on the second central European species, *B. dioica*, showed that this species is not capable of asexual seed formation (Volz, unpublished).

Bryonia dioica is like *B. alba* an invasive species in other temperate climate regions of the world, e. g. in New Zealand (Webb *et al.* 1995; T. Gilbertson, Department of Conservation, Mangaweka, New Zealand, personal communication). This and other changes in the distribution ranges of several *Bryonia* species have mostly been caused by man due to the plants' use as medicines and ornamental for over two millennia (Hippocrates; Renner and Schaefer, 2008). Even today *Bryonia* preparations are commonly used in homeopathic medicine to treat measels, scarlet fever, headache, and any kind of respiratory disease.

The sister group to *Bryonia* is the monotypic genus *Ecballium* A. Rich. (Kocyan et al., 2007). *Ecballium elaterium* (L.) A. Rich. has dioecious populations (*E. elaterium* subsp. *dioicum* (Batt.) Costich) and monoecious populations (*E. elaterium* subsp. *elaterium*). The former is restricted in distribution to northern Tunisia, Algeria, and Morocco and to southern Spain and Portugal, while the latter occurs throughout the Mediterranean region (Costich, 1995). Transplant and common garden experiments in Spain have shown that dioecious individuals are better able to cope with water stress than are monoecious ones (Costich and Galán, 1988; Costich and Meagher, 1992; Costich, 1995). The two forms are inter-fertile, and crosses between monoecious and dioecious *Ecballium* were also used in early investigations into the genetic basis of sex determination (Galán, 1946; Westergaard, 1958). As in *Bryonia*, the male is the heterogametic sex.

Phylogenetic relationships within Cucurbitaceae are now well known (Kocyan et al, 2007; Schaefer et al., in review). A molecular phylogeny that includes 95% of all Cucurbitaceae genera shows that *Bryonia* and *Ecballium* together form the sister clade to *Austrobryonia*, a genus of four monoecious species from Australia (Schaefer et al., 2008). Together, the three genera form the tribe Bryonieae (Jeffrey, 2005), which branches of near the base of Cucurbitoideae, the larger of the two subfamilies of Cucurbitaceae. The genera *Bryonia* and *Ecballium* are the only Cucurbitaceae clade centered in the Mediterranean, Irano-Turanian, and (in part) Holarctic floral kingdoms (Schaefer et. al, in review).

Understanding the evolution of sexual systems in *Bryonia* has been hindered by the

unclear species limits in *Bryonia* described above. Also, the ability of the monoecious and dioecious species in *Bryonia* to form fertile hybrids (Correns, 1903; Bateson, 1909; Heilbronn and Basarman, 1942; Heilbronn, 1953) raises the question of their phylogenetic proximity and the attendant question of the role of hybridization in the evolution of the genus, perhaps coupled with polyploidization. I therefore chose a combination of molecular markers to clarify species relationships, namely the chloroplast *trn*L intron and the *trn*L – *trn*F intergenic spacer, the *trn*R – *atp*A spacer and the *psb*A – *trn*H spacer. Each of these markers has been used successfully to infer species relationships in Cucurbitaceae (e.g., Chung et al., 2003; Renner and Schaefer, 2008). As a biparentally inherited marker, I used the second intron of the nuclear *LFY* gene. In most angiosperms, *LFY* is a single-copy gene, and recent allopolyploids may have one paralog from each ancestral diploid progenitor (Ahearn et al., 2001; Bomblies et al., 2003). This seemed especially relevant if species formation in *Bryonia* involved hybridization. I also cloned and sequenced the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. As did a previous study (Jobst et al., 1998), I found several (up to seven) paralogous copies per accession. I used the number of mutations (>1) and length variation in the 5.8S gene as well as the 18S and 25S exonic stretches to differentiate between functional and non-functional (pseudogenic) copies. Most of the 76 ITS alleles obtained from 14 accessions representing nine of the ten *Bryonia* species (all except *B. multiflora*) and *Ecballium elaterium* did not cluster according to the species boundaries found with the other markers and morphology, suggesting either ongoing gene flow or insufficient time for concerted evolution to homogenize copies within individuals and species. Since there is little evidence in the other nuclear data (the *LFY* data set) for widespread recent gene flow, I favor the explanation of incomplete concerted evolution. Slow concerted evolution and thus prolonged coexistence of ITS pseudogenes in individuals of *Bryonia* may be explained by many inactive 35S rDNA loci; there are at least five such loci in *B. dioica* (H. Weiss-Schneeweiss, University of Vienna, personal communication). A large number of these nucleolus organizing regions has also been found to correlate with high levels of ITS polymorphisms in other taxa (Razafimandimbison et al., 2004). The identical indels and SNPs in the rDNA shared between the pseudogenic ITS copies of different *Bryonia* species indicate that these ITS lineages most likely originated from duplication of the whole tandem repeat cluster before separation of the respective species.

Polyploidy is thought to sometimes correlate with a loss of dioecy, although there are

few phylogenetic studies testing this correlation (Westergaard, 1958; Pannell, 2004). Supporting evidence so far comes mainly from *Empetrum* L., in which tetraploid monoecious *E. hermaphroditum* Hagerup is derived from diploid dioecious *E. nigrum* L. (Richards, 1997). Further evidence comes from *Mercurialis annua* L., where polyploid populations of the otherwise diploid and dioecious species are monoecious (Pannell, 2004). The reverse effect is also possible: dioecy can be favored when self-incompatibility systems break down after polyploidization. Cucurbitaceae are usually self-compatible, and this also appears to be true of *Bryonia*, judging from self-pollinations carried out on *B. dioica* and *B. alba* (S. Volz, unpublished) Since *Bryonia* includes diploid and polyploid species, this study system permits addressing the question how polyploidy may relate to the evolution of monoecy or dioecy. Apart from polyploidization, I found that hybridization events also make it more difficult to disentangle the evolution of the *Bryonia* clades. Contradicting topologies of the chloroplast and the nuclear tree suggest at least one case of hybridization: the hexaploid *B. cretica* most likely originated via hybridization between *B. dioica* and *B. syriaca* and/or *B. multiflora* whose cp haplotypes it shares and whose distribution areas overlap. The tetraploid *B. marmorata*, a local endemic of Sardinia and Corse, appears to have originated from *B. dioica* through autopolyploidization (see chapter 1).

Given that sexual systems in *Ecballium* correlate with habitat and geographic distribution (dioecy predominates in drier areas; Costich, 1995), I decided to also investigate the spatial context of the distribution of monoecy and dioecy in *Bryonia*, using a phylogeographic approach (Avise, 2000). I constructed a chloroplast haplotype network that shows six main geographic groups, with the greatest haplotype diversity in Southwest Asia. Genetic diversity is lowest in Central Europe, fitting with a bottleneck after the last glacial maximum (see chapter 2).

A fossil-calibrated molecular clock applied to 198 sequences of Cucurbitaceae *rbc*L suggests that *Bryonia* diverged from its sister group *Ecballium* c. 41 my ago, while the deepest split within *Bryonia* occurred some 8.4 my ago. Similar estimates were obtained with a relaxed clock constrained with Miocene (15-13 my) seeds of *Bryonia*. These seeds, reported from Middle Miocene fossil beds in the Tambov district in Western Siberia, were assigned to *Bryonia* (Dorofeev, 1963, 1988). The seeds are 1.8-2.3 x 2.5-4 mm, while extant *Bryonia* seeds typically measure 3 x 5 mm, a size difference that appears to be normal, however (Tertiary seed specialist M. Pingen, Hürtgenwald, Germany, personal communication). The oval, laterally flattened shape of the fossil seeds corresponds to most

Bryonia seeds, except those of *B. verrucosa*, which are larger and more oblong.

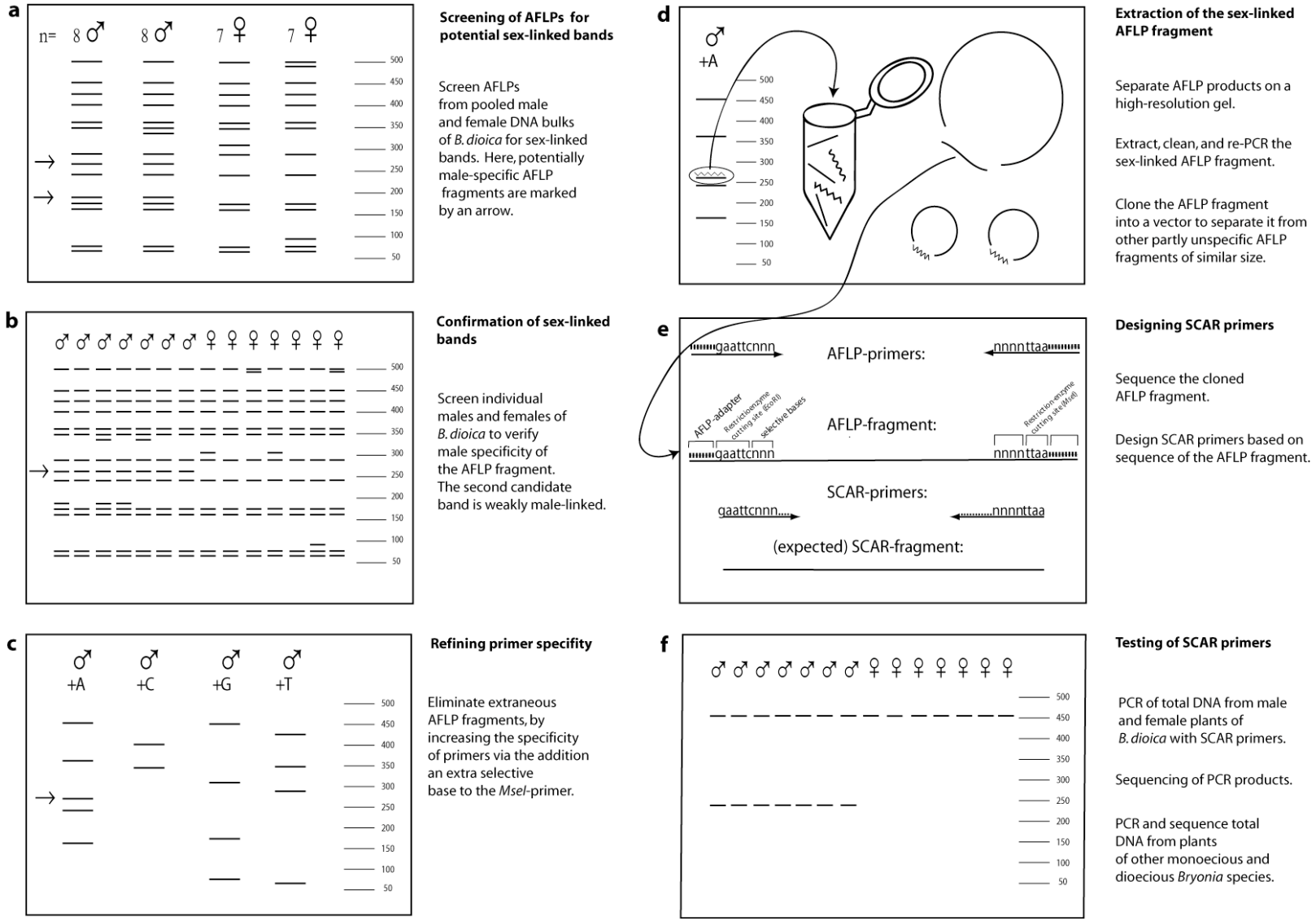
By comparing chloroplast and nuclear phylogenies, and the distribution of geo-referenced chloroplast haplotypes, I was able to examine the junction between the intra- and inter-specific levels and clarify species limits and relationships. A phylogenetic framework is thus provided for a cluster of species that may become another plant system for the study of sex chromosome evolution. Genetic sex determination requires the suppression of recombination between the sex-determining loci that code for male- and female-sterility (Muller, 1918). The lack of recombination may lead to an accumulation of mutations, insertion of transposable elements, and inversions on chromosomes housing the sex-determining genes (Bull, 1983; Charlesworth et al., 2005; Bachtrog, 2006). As recombination suppression spreads along these chromosomes the homo- and the heterozygous sex chromosomes will gradually diversify and only a limited pseudoautosomal region (PAR) will be able to recombine during meiosis. Whether this has happened in *Bryonia dioica*, we do not yet know. However, the crossings carried out by Correns and others (Bateson 1909; Correns, 1903; Heilbronn 1948; Westergaard, 1958) demonstrate that in *B. dioica*, sex shows monofactorial dominant inheritance, leading to the hypothesis that *B. dioica* may already have a pair of chromosomes on which key sex-determining gene(s) and sex-linked genes have accumulated. To gain insight into the possible presence of such a pair of sex chromosomes in *B. dioica*, it will be necessary to sequence a fairly long sex-linked region to study its substitution behavior and to eventually visualize its physical placement using fluorescent-in-situ-hybridization (FISH). As a first step towards this goal, I developed a sex-linked sequence-characterized amplified region (SCAR) marker for *B. dioica*.

Using 32 AFLP primer combinations, I screened two male and two female bulks of central European *B. dioica* for sex-linked fragments. Primer combinations that yielded putative sex-linked bands in the bulk assay were tested with single individuals (see flowchart at the end of this introduction). Repeating the selective amplification with more specific primers that contained one or two additional bases on the 3'end reduced the overall number of AFLP fragments and facilitated separating the target fragments on high-resolution electrophoresis gels from neighboring, non sex-linked bands. The target fragments were re-PCRed and cloned into T/A vectors for amplification and separation of unspecific DNA fragments of similar length. Positive clones were sequenced, and sequences with the appropriate length were manually aligned to design sequence-characterized amplified region (SCAR) primers. These primers comprised the restriction-enzyme recognition site, the three

to five selective bases and 12 to 20 additional, sequence specific bases. As the SCAR primers do not comprise the artificial adapter sequences of the AFLP primers, the SCAR fragment is 21 bp shorter than the AFLP fragment it amplifies. I tested the sex specificity of the SCAR primers with genomic DNA of male and female *B. dioica* with the touchdown PCR mentioned earlier.

Following this approach, I successfully developed a sex-linked SCAR marker for *B. dioica* and sequenced it for individuals representing the full distribution range of the species from Scotland to North Africa. North of the Alps, I found distinct Y and X alleles differing in a 197-bp indel, with the Y allele being perfectly linked to the male sex (see chapter 3).

In southern Europe, however, the XY system appears to break down, and recombination was detected between the Y and X homologues. Population genetic analyses suggest that the sex-linked region I amplified (i.e., the SCAR marker) experienced different evolutionary pressures in northern and southern Europe. These findings fit with the evidence from my phylogenetic and phylogeographic analyses that the XY system in *Bryonia* is evolutionarily labile. Overall, my work suggests that *Bryonia* may be a good, but very complex, system in which to study the early steps of plant sex chromosome evolution.



FLOWCHART Process of developing a SCAR-marker from AFLP fragments.

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Chapter 1

Hybridization, polyploidy, and evolutionary transitions between monoecy and dioecy in *Bryonia* (Cucurbitaceae)

STEFANIE M. VOLZ AND SUSANNE S. RENNER

Abstract

Correns's 1903 (*Berichte der Deutschen Botanischen Gesellschaft* 21: 133–147) crosses between a monoecious and a dioecious species of *Bryonia* revealed the simple Mendelian inheritance of dioecy and provided the first instance of an XY sex determination system in any organism. *Bryonia* ranges from the Canary Islands to Central Asia and comprises seven dioecious and three monoecious species; its closest relative, *Ecballium elaterium*, has dioecious and monoecious populations. We used chloroplast (cp) and nuclear (nr) gene phylogenies to infer sexual system evolution in *Bryonia*. We also tested for associations between sexual system and ploidy level, based on published and original chromosome counts. Conflicts between cp and nr topologies imply that the dioecious hexaploid *B. cretica* arose from hybridization(s), probably involving the dioecious diploids *B. dioica*, *B. syriaca*, and/or *B. multiflora*. A tetraploid dioecious endemic on Corsica and Sardinia probably originated from *B. dioica* via autopolyploidy. While the cp phylogeny resolves few species relationships, the nr tree implies at least two evolutionary changes in sexual system. There is no correlation between sexual system and ploidy level. Molecular clocks suggest that the deepest divergence, between a species on the Canary Islands and the ancestor of all remaining species, occurred ca. 10 million years ago.

Key words: *Bryonia*; chloroplast DNA haplotypes; chromosome counts; Cucurbitaceae; dioecy; hybridization; monoecy; nuclear *LEAFY* intron; polyploidy.

Introduction

The first experimental system for the genetics of sex determination was the Cucurbitaceae genus *Bryonia* (Correns, 1903, 1907; Rheinberger, 2000). From the sex ratios of almost 1000 offspring from reciprocal pollinations between dioecious *B. dioica* and monoecious *B. alba*, Correns inferred that half the pollen grains of *B. dioica* carried a "female tendency," the other half a "male tendency." Because of the importance of this discovery, Bateson (1909) repeated Correns's experiments, with the same results. Later studies used *B. dioica* and *B. aspera* as an additional dioecious/monoecious pair for reciprocal crossing (Heilbronn and Basarman, 1942; Heilbronn, 1953; summarized in Westergaard, 1958). These experiments confirmed XY sex determination, with the male the heterogametic sex. The sister group to *Bryonia* is the Mediterranean genus *Ecballium* (Kocyan et al., 2007), which comprises a single species that can be dioecious or monoecious (*E. elaterium* subsp. *dioicum* and subsp. *elaterium*). The dioecious subspecies of *Ecballium* has XY sex determination, with the males again the heterogametic sex (Galán, 1946; Westergaard, 1958). Transplant and common garden experiments in Spain have shown that dioecious populations are better adapted to water stress than monoecious ones (Costich and Galán, 1988; Costich and Meagher, 1992; Costich, 1995). Together, *Bryonia* and *Ecballium* provide an exceptional system in which to study the evolution of XY sex determination and the evolution of dioecy and monoecy.

A taxonomic treatment of *Bryonia* accepts 10–12 species (Jeffrey, 1969), two of them with considerable doubt, and a phylogeographic analysis of chloroplast and nuclear data suggests that there are 10 biological species (S. Volz and S. Renner, unpublished manuscript). Of these, seven are dioecious, three monoecious (references for each species' sexual system are provided in Materials and Methods). On the basis of herbarium specimens, Jeffrey (1969) suggested that two normally monoecious species might be dioecious in parts of their range; they are *B. alba*, which ranges from Central Europe to Kazakhstan and *B. monoica*, which occurs in Afghanistan, Tajikistan, Kazakhstan, and Uzbekistan. Jeffrey found unisexual specimens from Macedonia, Turkey, Belarus, and the Ukraine. However, distinguishing dioecy from monoecy based on herbarium specimens alone is problematic because incomplete collections of this climbing species may easily suggest unisexuality.

Because experimental interspecific crosses result in fertile progeny (as shown by the Mendelian studies described), the question arises if natural hybridization is an important factor in the evolution of *Bryonia*. Hybridization might explain why some species of

Bryonia can be difficult to distinguish from each other. For example, individuals of *B. cretica* and *B. multiflora* from Turkey are difficult to separate and so are some specimens of *B. cretica* and *B. dioica* (Jeffrey, 1969; S. Volz, personal observation). However, difficulties in species identification in *Bryonia* might also be an artifact because herbarium material may not show features, such as fruit color and stigma pubescence, that are important species characters in this genus (Jeffrey, 1969). Molecular sequences can be a powerful tool to assign individuals to natural gene pools and to identify hybrid individuals, provided that within-species sampling is sufficiently dense and that the nuclear and organelle loci chosen evolve at suitable speeds (Rieseberg and Wendel, 1993; Sang et al., 1997).

Transitions between monoecy and dioecy may correlate with polyploidy. For example, dioecy appears to break down in polyploid populations of *Empetrum*: The diploid *E. nigrum* var. *nigrum* is dioecious, while the polyploid var. *hermaphroditum* is hermaphroditic (Westergaard, 1958). Also in *Mercurialis annua*, the diploid forms are dioecious, while polyploid forms are monoecious or androdioecious (Durand and Durand, 1992; Pannell et al., 2004, 2008; but see Obbard et al., 2006 for exceptions). A possible mechanism could be that some forms of nuclear-determined dioecy may break down in polyploids because of dosage imbalances (Westergaard, 1958; Smith, 1969; Pannell et al., 2004). However, there are polyploid dioecious species of *Rumex* (Polygonaceae; Smith, 1969), *Lycium* (Solanaceae; Miller and Venable, 2000; Yeung et al., 2005), and *Fragaria* (Rosaceae; Westergaard, 1958). Indeed, a positive association between polyploidy and dioecy might be favored by the frequent breakdown of self-incompatibility in recent polyploids, which in turn might select for dioecy as an alternative outcrossing mechanism (Miller and Venable, 2000). This proposed explanation does not fully match the observed pattern, however, because *Mercurialis* and *Rumex* are self-compatible, while *Empetrum nigrum*, *Lycium*, and *Fragaria* are self-incompatible. As far as known, Cucurbitaceae do not possess genetic self-incompatibility (Rubino and Wehner, 1986).

Here we employ nuclear and chloroplast sequences from a dense sample of individuals representing all accepted species of *Bryonia* (with multiple accessions) to examine species relationships, hybridization, and the evolution of monoecy and dioecy. To determine if ploidy levels correlate with monoecy or dioecy, we augmented published chromosome counts for the genus by new counts from wild-collected material.

Materials and Methods

Taxon sampling, range mapping, and sexual systems

The phylogenetic relationships of *Bryonia* and *Ecballium* within Cucurbitaceae have been tested with sequence data for 236 species from most of the family's genera (Kocyan et al., 2007; Schaefer et al., 2008). In these studies, *Bryonia* was represented by three species spanning the root of the genus: *B. alba*, *B. dioica*, and *B. verrucosa*. The closest relative of *Bryonia* and *Ecballium* turned out to be the Australian genus *Austrobryonia*, all four species of which are monoecious (Schaefer et al., 2008). Biogeographically, the *Bryonia-Ecballium* clade results from a dispersal event from Asia into the Mediterranean sometime in the Eocene (H. Schaefer unpublished manuscript). On the basis of these findings, we rooted the *Bryonia* trees on *Ecballium* and one species of *Austrobryonia*.

Our sampling of *Bryonia* covers the geographic range and morphological diversity of the species; 123 of the 135 total DNA extracts generated came from herbarium specimens (up to 124 yr old). Information on sexual systems comes from the observation of living plants of *B. alba*, *B. cretica*, *B. dioica*, and *B. verrucosa*, and from the following references: Dioecious species are *B. acuta* (Jeffrey, 1969), *B. cretica* (Jeffrey, 1969; Feinbrun, 1978), *B. dioica* (Correns, 1903, 1907), *B. marmorata* (Jeffrey, 1969; Chiappini, 1985), *B. multiflora* (Heilbronn and Basarman, 1942; Heilbronn, 1953), *B. syriaca* (Rottenberg, 2000, three populations and 134 individuals were studied), and *B. verrucosa* (Jeffrey, 1969). Monoecious species are *B. alba* (Correns, 1903, 1907; Heilbronn and Basarman, 1942; Jeffrey, 1969), *B. aspera* (Bilge, 1955, under the synonymous name *B. macrostylis* Heilbronn and Bilge, 1954), and *B. monoica* (Vassilczenko, 1957; Jeffrey, 1969).

DNA isolation, amplification, and sequencing

Total DNA was isolated from silica-gel-dried leaves or herbarium material using the NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. DNA concentration and quality were checked on 1% agarose gels by electrophoresis using lambda DNA as a reference. Polymerase chain reactions (PCR) were performed with 10 µM of primers, 25 µM $MgCl_2$, 1.25 µM of each dNTP, 1´ PCR buffer, 0.5 units of *Taq* DNA polymerase (New England Biolabs, Frankfurt am Main, Germany), and 10–50 ng of template DNA per 25 µl reaction. PCR reactions were set at an initial 5 min at 95°C, followed by 35 cycles of 95°C for 30 s for DNA denaturation, 30 s for primer annealing at a temperature appropriate for the specific primer pair, and 72°C for 1 min for

primer extension; with a final elongation period at 72°C for 7 min to complete all primer extensions.

The *trnL* intron and *trnL-trnF* intergenic spacer (IGS) were amplified using the Taberlet et al., (1991) primers c, d, e, and f, and an annealing temperature of 55°C. The *psbA-trnH* spacer was amplified at the same annealing temperature with the forward primer of Sang et al. (1997) and the *trnH*2 reverse primer 5 CGCGCATGGTGGATTCACAATCC 3 (developed by C. Heibl). The *trnR-atpA* spacer was amplified with the forward and reverse primers of Chung et al. (2003). For *Austrobryonia*, we used the primer pair *ccSSr4* F2 (5 AAATTTCTATATCATGTCAAGAGG 3) and *ccSSr4* R2 (5 GAACGTTTT CTACTTCAAGACC 3), developed by S. Volz. For recalcitrant DNAs, we reduced annealing temperatures to 48°C or used Phusion High-Fidelity polymerase (Finnzymes Oy, Espoo, Finland) or KOD Hot Start DNA polymerase (Novagen, Houston, Texas, USA) according to the manufacturers' protocols. PCR products were purified with the Wizard SV Gel and PCR clean up system (Promega, Madison, Wisconsin, USA). Sequencing reactions used the same primers as PCR amplifications except for the *psb*A forward primer, which was replaced by *psb*A-5, 5 AYAACTTYCCTCTAGAYYTAGC 3 (developed by A. Kocyan, University of Munich).

Nuclear loci amplified were the internal transcribed spacer (ITS) of the ribosomal DNA and the second intron of the nuclear *LFY* gene (referred to herein simply as *LFY*). The ITS region, including 130 nucleotides of the 3 end of the 18S gene, the 5.8S gene, and 92 nucleotides of the 5 end of the 25S gene, was amplified using the primers of Balthazar et al. (2000). The *LFY* intron was amplified with the primers *LFY* CucF 5 TCTTCCACCTSTATGARCAGTGTCGTGAAT 3 and *LFY* CucR 5 CGAAATCACAAA AGYTATTGSGYAKTYCA 3 (developed by H. Schaefer, University of Munich). For both nuclear markers, we used cloning to assess within-plant sequence divergence. For cloning, we pooled three parallel PCR products, obtained with high-fidelity polymerase, and purified and ligated them into plasmids of the Promega pGEM-T Vector system (Promega). Plasmids were transformed in ultracompetent *E. coli* DH5alpha strains or JM109 competent cells (Promega). Positive (white) plasmid colonies were picked from the ampicillin blue/white selection agar plates, dissolved in H_2O and directly amplified with primer oligonucleotides and settings as mentioned. PCR products were purified and sequenced using the same primers.

Sequencing relied on Big Dye Terminator kits (Applied Biosystems, Foster City,

California, USA) and an ABI 3100 Avant capillary sequencer (Applied Biosystems). Sequence assembly and editing were carried out in the program Sequencher (version 4.6; Gene Codes, Ann Arbor, Michigan, USA). All sequences were BLAST-searched in GenBank.

Alignments and phylogenetic analyses

Sequences were aligned in MacClade version 4.08 (Maddison and Maddison, 2003) and adjusted by eye. The chloroplast data matrix included sequences from both subspecies of *Ecballium elaterium* (three monoecious and four dioecious individuals were sequenced), plus an *Austrobryonia* as a more distant outgroups. We excluded the last seven nucleotides of a poly A run of up to 16 nucleotides in the *psbA-trnH* spacer, the last five of a poly A run of up to 14 nucleotides in the *trnL* intron, and the last 23 of a poly T run of up to 31 nucleotides again in the *trnL* intron. *LFY* alignments did not include outgroups because *Ecballium* and *Austrobryonia* sequences were too divergent to be aligned with those of *Bryonia*. We prepared two *LFY* matrices, one with all sequences and one that excluded identical sequences from the same individual. Indels in the chloroplast and *LFY* alignments were coded using simple gap coding (Simmons and Ochoterena, 2000) as implemented in the SeqState software (Müller, 2005), and parsimony searches were conducted with and without gap characters. Maximum likelihood (ML) and Bayesian searches included the coded gaps.

We used the programs Modeltest version 3.7 (Posada and Crandall, 1998) and DTModSel (Minin et al., 2003) to find best-fitting substitution models, applying both the Akaike and Bayesian information criteria. For the chloroplast (cp) data (with indels excluded), both softwares and criteria favored the $K81_{uf}$ + G model, while for the *LFY* data, Modeltest chose the HKY + G model and DTModSel the HKY + I model. We nevertheless had to rely on the slightly more parameter-rich GTR + G and GTR + G + I models because of limited model choice in the programs RAxML version 2.2.3 (Stamatakis, 2006) and MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Bayesian searches involved four chains and two runs, using the default priors in MrBayes. Convergence of Bayesian analyses was assessed by checking that final likelihoods and majority rule topologies in different runs were similar, that the standard deviations (SDs) of split frequencies were <0.01, and that the convergence diagnostic (given by MrBayes) approached 1 and by examining the plot provided by MrBayes of the generation number vs. the log probability of the data. Likelihood searches were carried out according to the RAxML manual. Parsimony analyses

in the program PAUP* version 4.0b10 (Swofford, 2002) used heuristic searches with 10 random-taxon-addition replicates, holding 10 trees at each step, tree-bisection-reconnection (TBR) 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 (1) ML bootstrapping with the same model and settings as used during tree searches, (2) posterior probabilities (PP) as obtained from MrBayes, and (3) nonparametric bootstrapping in PAUP*, using the fast bootstrap option and 1 million replicates.

Molecular clock analyses

Likelihood ratio tests of the *Bryonia* chloroplast data (including *Ecballium* and *Austrobryonia*) under the GTR + G model with and without the assumption of clock-like substitution accumulation rejected the strict clock model. We therefore used a relaxed clock model, specifically the Bayesian autocorrelated-rates approach implemented in the program multidivtime (Thorne and Kishino, 2002). Prior gamma distributions on parameters of the clock model were as follows. The mean of the prior for the root age was set to 45.5 million years (Myr), based on the age of this node obtained in a strict clock analysis that used a Cucurbitaceae family-wide data set (described later); the SD of this prior was also set to 45. The mean (and SD) of the prior for the ingroup root rate were set to 0.0004 substitutions/site/Myr by dividing the median of the distances between the ingroup root and the tips by 45.5 Myr. The prior (and SD) for the Brownian motion parameter were set to 0.022, based on the manual's recommendation that the time between root and tips multiplied by this parameter should be about 1. As an internal constraint, we used the age of *Bryonia*-like seeds from the Middle Miocene fossil beds of Tambov, Western Siberia (Dorofeev, 1963, 1988). The layer where the seeds were found is part of the Ternovskie stratum, which dates to the Lower Sarmat, 15–13 Myr ago (Nalivkin and Sokolov, 1986). The seeds measure 1.8–2.3 × 2.5–4 mm, while extant *Bryonia* seeds typically measure 3–4 × 4–5 mm. Their oval, laterally flattened shape corresponds to most *Bryonia* seeds except those of *B. verrucosa*, which are larger and more oblong. On the basis of these fossils, we assigned a minimal age of 14 Myr to the split of *B. verrucosa* from the remaining species (this being the deepest split in the genus; see Results). Markov chains were run for 10 million generations, sampling every 100th generation for a total of 100c000 trees, with a burn-in of 10c000 trees before the first sampling of the Markov chain. The analysis was repeated twice from different random initial seed numbers to approximate posteriors and check for convergence.

In an alternative approach, we applied a strict clock model to an *rbcL* data set of 198 Cucurbitaceae, including *Austrobryonia* (all four species), *Ecballium elaterium*, *B. alba*, *B. dioica*, and *B. verrucosa*, plus Corynocarpaceae and Coriariaceae for rooting purposes. The *rbcL* branch lengths were optimized under the GTR + G + I + clock model on the maximum likelihood topology obtained for these 198 taxa with five combined chloroplast loci, including *rbcL* (sequences from Kocyan et al., 2007; Schaefer et al., 2008; this study). The resulting branch length table from PAUP* was saved, and a substitution rate calculated by dividing the genetic distance between a calibration node and the present by the age of the calibration node. The calibration node used was the stem lineage of *Trichosanthes*, which is about 60 Myr old, based on *Trichosanthes* seeds from the uppermost Paleocene (Collinson et al., 2003). The resulting substitution rate was used to calculate the divergence ages of interest. Following Renner and Meyer (2001), we used binomial probability theory to estimate the SD of the distance from the fixed calibration node to the tips and then used this value to obtain the SDs of the estimated ages of interest.

Chromosome counts and experimental interspecies crosses

For chromosome counting, wild plants of *Bryonia alba*, *B. aspera*, *B. cretica*, *B. dioica*, *B. monoica*, *B. syriaca*, and *B. verrucosa* were brought into cultivation at the Botanical Garden Munich. No living material could be obtained of the North African *B. acuta*. Root tips were harvested, pretreated with 2 mM 8-hydroxyquinoline solution, fixed in 3c:c1 ethanolc:cacetic acid, and stored until use at -20°C. Chromosome spreads were obtained after hydrolysis in 0.5 N hydrochloric acid at 60°C for 10 min and staining with a saturated orcein-glacial acetic acid solution.

Results

Phylogenetic and phylogeographic analyses of the chloroplast data

The sequenced individuals with species names and authors, collecting locality, place of voucher deposition, and GenBank accession numbers are listed in Appendix S1. Alignments and trees have been submitted to TreeBASE (http://www.treebase.org, submission name Stefanie M. Volz, PIN 25754). Because these chloroplast loci represent a single linkage group, the data were combined into a combined matrix of 1906 aligned characters, including 48 coded indels. This matrix comprised 135 sequences, 127 of *Bryonia*, seven of *Ecballium*, and one of *Austrobryonia,* and contained little homoplasy as indicated by a parsimony consistency index of 0.9. A maximum likelihood tree from these

data strongly supports the monophyly of *Bryonia* (Fig. 1). *Bryonia verrucosa* from the Canary Islands is sister to all other species, and the remaining sequences fall into 10 clades that correspond to the morphological species recognized by Jeffrey (1969).

There were 30 chloroplast (cp) haplotypes that could be assigned to 11 main groups (Fig. 1, labeled with capital letters, A, C, …V, followed by smaller letters and numbers indicating subtypes). Nine haplotypes are exclusive to single morphological species, while two are shared by three species each (Fig. 1); haplotype C is shared by *B. cretica*, *B. multiflora*, and *B. syriaca*, and haplotype M is shared by *B. cretica*, *B. dioica*, and *B. marmorata*. Figure 2 shows the distribution of haplotypes with respect to species range boundaries as assessed from morphology (Jeffrey, 1969). The C haplotype is widespread in the southeastern part of the range of *Bryonia* (where it occurs in *B. cretica*, *B. multiflora*, and *B. syriaca*), while the M haplotype is widespread in the southwestern part of the range (occurring mostly in the species *B. dioica* and *B. marmorata*, but also in the westernmost part of the range of *B. cretica*).

The species with the greatest haplotype diversity is *B. aspera*, which harbors the P, S, and U haplotypes (see Figs. 1 and 2).

Nuclear phylogenies, differences between chloroplast and nuclear data, and sexual system evolution

Nuclear *LFY* sequences were obtained from 46 plants, selected to represent all cp haplotypes (Appendix S1). Extensive cloning resulted in 111 sequences. The alignment comprised 524 characters, including 51 coded indels. Cloned sequences from the same plant usually fell together, and exclusion of identical colonies and recombinant sequences left a data set of 54 sequences. A ML tree from these sequences is shown in Fig. 3. Sequences grouped by species except for those of *B. cretica*, *B. dioica*, and *B. marmorata*. *Bryonia cretica* sequences from different individuals as well as cloned sequences from the same individual appear in three places, namely near the base of the tree (plants SV03 and SV19), in the "center" of the tree (an allele from SV16), and near its top (other alleles of plants SV16 and SV19 plus sequences from plants SV15, SV20, SV30). Haplotypes of *B. dioica* form a polytomy with those of *B. cretica*, *B. acuta*, and *B. marmorata* (Fig. 3). Table 1 shows interallelic genetic distances among *LFY* alleles found in the different species. As expected from the tree, genetic distances among *B. cretica* alleles are by far the largest

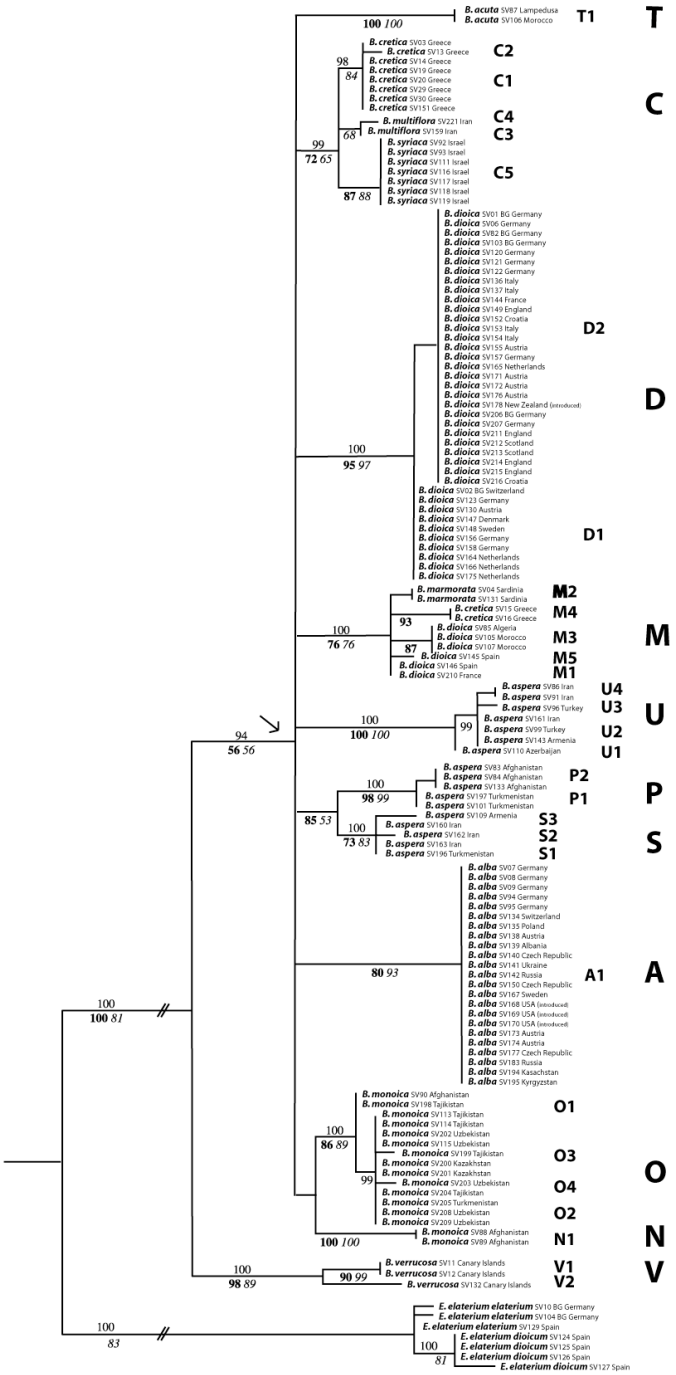

FIGURE 1-1 Maximum likelihood bootstrap consensus tree from chloroplast sequences of *Bryonia* and *Ecballium* (1906 characters, including 48 coded indels; 1000 replicates). The tree is rooted on *Austrobryonia* (not shown; the leftmost branches have also been shortened by 50% to save space). Because this is a consensus tree, branch lengths are slightly distorted. After the species names are plant number (e.g., SV16) and geographic origin. Letters A to V on the right indicate the major haplotype groups, with the subtypes labeled by combinations of letters and numbers. Numbers above branches indicate Bayesian posterior probabilities >95; those below indicate bootstrap support >50 under parsimony (bold) and ML (italics). The node marked by an arrow in a relaxed molecular clock analysis was estimated as 9.8 (95% CI 5.8–15.9) Myr old.

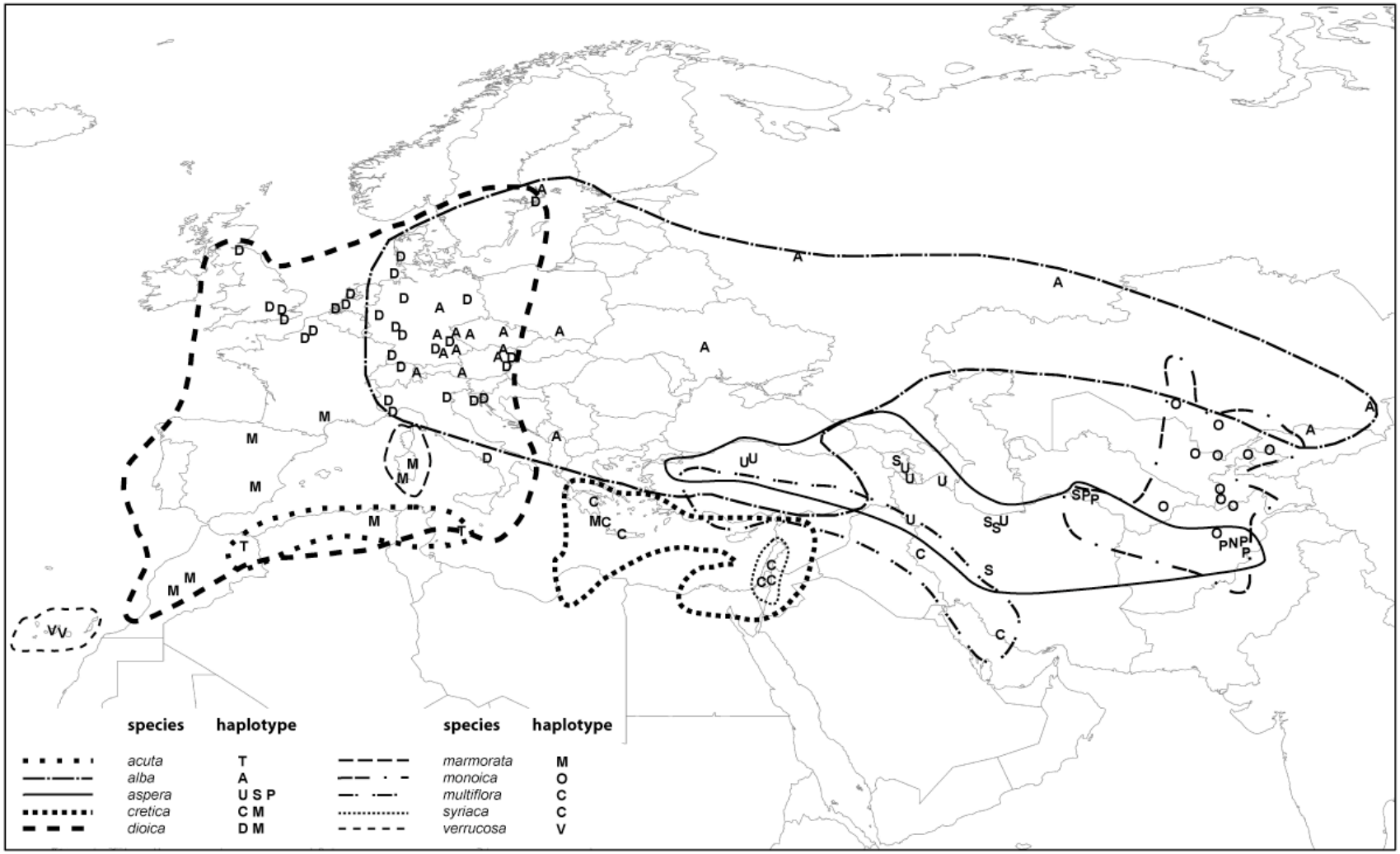

FIGURE 1-2 Geographic distribution of the 10 major chloroplast haplotypes found in *Bryonia*. The labeling of haplotypes corresponds to that in Figs. 1 and 3. Species ranges modified from Jeffrey (1969).

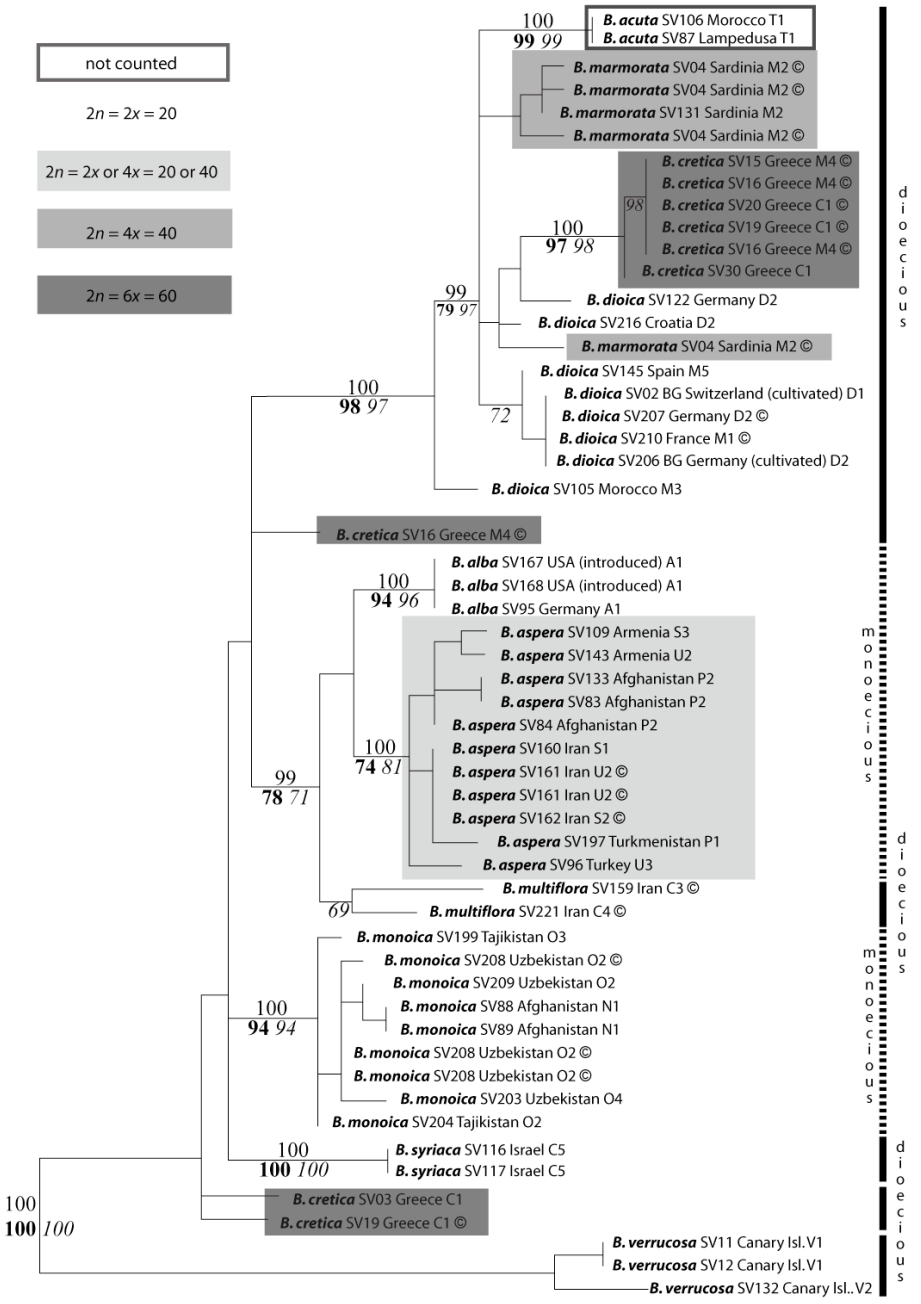

FIGURE 1-3 Maximum likelihood tree from 54 nuclear LFY sequences of *Bryonia*, rooted on *B. verrucosa* (see Materials and Methods). Following the species names are plant number, geographic origin, and chloroplast haplotype labeled as in Fig. 1. Cloned sequences are marked by a ©. Different ploidy levels are indicated by the shaded boxes; compare with Table 2 for chromosome counts. Numbers above branches indicate Bayesian posterior probabilities >95, those below, bootstrap support >65 under parsimony (bold) and ML (italics).

The only statistically supported conflict between the chloroplast and nuclear trees concerns the placement of *B. multiflora*. In the cp tree, this species groups with *B. cretica* and *B. syriaca* (99% ML bootstrap support), while in the nr tree, it groups with *B. alba* and *B. aspera* (99% ML bootstrap support). In an attempt to increase resolution, we combined the nuclear and chloroplast data, using sequences from the same individuals and excluding *B. multiflora* and *B. cretica*. However, this did not result in better-supported species relationships.

TABLE 1. Intraspecific allele diversity in *Bryonia* nuclear *LFY* sequences under the HKY model of molecular substitution.

				Interallelic HKY distances		
Species	Alleles	Sequences	Individuals	Mean	Maximal	Minimal
B. acuta	2	2	2	0.003	0.003	0.003
B. alba	1	3	3	n.a.	n.a.	n.a.
B. aspera	9	16	10	0.007	0.019	0
B. cretica	16	44	6	0.019	0.04	0
B. dioica	5	13	8	0.004	0.009	0
B. marmorata	5	8	2	0.007	0.015	0.003
B. monoica	10	17	9	0.006	0.010	0
B. multiflora	3	8	2	0.001	0.018	0.003
B. syriaca	1	2	2	n.a.	n.a.	n.a.
B. verrucosa	2	3	3	0.008	0.008	0.008

Notes: n.a. = not applicable

A ML tree from the nuclear ITS sequences (Appendix S2; 76 sequences from 14 plants representing all species; alignment length 883 characters, including 48 coded indels) yielded almost no resolution and showed much intermixing of sequences from different species, albeit without statistical support (Appendix S1). Thirty-one of these sequences had ≥1 indels or substitutions in the 5.8S gene, the 3′ region of the 18S gene, or the 5′ region of the 25S gene and were therefore probably pseudogenes. Appendix S3 also gives their G/C contents, a measure of RNA-folding stability.

The distribution of monoecy and dioecy on the nuclear tree (Fig. 3) implies at least two transitions between these two systems, irrespective of whether the ancestral sexual system was dioecy or monoecy (see Discussion).

Chromosome numbers and sexual system

Table 2 lists chromosome counts for *Bryonia* and *Ecballium*. Most *Bryonia* species have *2n* = *2x* = 20, but *B. cretica* is hexaploid (*2n* = *6x* = 60; two individuals counted), *B. marmorata* tetraploid (*2n* = *4x* = 40; one individual counted), and *B. aspera* diploid or tetraploid (three individuals counted). Plotting of the chromosome numbers on the nuclear tree (Fig. 3) reveals no consistent relationship between polyploidy and sexual system; there are diploid and polyploid dioecious and monoecious species of *Bryonia*. Molecular clock dating and indirect evidence for the age of *Bryonia* from nonsilent substitutions in the *rbcL* gene.

A strict clock model applied to 198 *rbcL* sequences of Cucurbitaceae (see Materials and Methods) yielded an age of 45.5 ± 7.6 Myr for the split between *Austrobryonia* and *Ecballium*/*Bryonia*, 41.2 ± 7.2 Myr for the split between *Ecballium* and *Bryonia*, and 8.4 ± 1.8 Myr for the split between *B. verrucosa* and *B. alba*/*B. dioica*.

A relaxed clock model applied to combined intron and spacer sequences of *Bryonia* and *Ecballium* with the root (the split from *Austrobryonia*) constrained to maximally 45.5 Myr old (based on the result from the strict clock) and the split between *B. verrucosa* and the remaining species constrained to minimally 14 Myr old based on *Bryonia* fossil seeds (see Materials and Methods), yielded an age of 9.8 (95% CI 5.8–15.9) Myr for the most recent common ancestor of the remaining haplotypes of *Bryonia* (the node marked by an arrow in Fig. 1). Because the relaxed clock model was poorly constrained and species relationships are not solidly resolved by the cp data, we refrained from estimating node ages higher up in the tree. As an alternative means to gain a sense of the absolute age of *Bryonia*, we examined silent and nonsilent substitutions in the conservative *rbcL* gene. The three species sequenced for *rbcL* (*B. alba*, *B. dioica*, and *B. verrucosa*) when compared to 195 other Cucurbitaceae sequences share two synapomorphic silent substitutions (CTA instead of TTA at position 270 and TTA instead of TTG at position 280; numbered according to spinach *rbcL* for which protein crystal structure is available (Knight et al., 1990)). *Bryonia alba* furthermore differs in two silent substitutions from the other two (CCC instead of CCT at position 168 and CTG instead of CTT at position 335), and *B. verrucosa* differs in three nonsilent substitutions from *B. alba* and *B. dioica* (TAT (tyrosine) instead of TTT (phenylalanine) at position 226; TCT (serine) instead of GCT (alanine) at position 281; TCT (serine) instead of GCT (alanine) at position 328).

TABLE 2. Sexual system and chromosome number (meiotic/mitotic) in *Ecballium* and *Bryonia*. For complete voucher localities, see Appendix 1-1. No living material could be obtained for the North African *B. acuta*.

Species, sexual system	Locality of counted material	Meiotic/ mitotic	Reference
B. alba L.,	Poland	–/20	Pogan et al., 1990
monoecious	Poland	–/20	Turaetla-Szybowska, 1990
	Armenia	–/20	Nazarova, 1997
	Germany	–/20	This study. Voucher Volz 6 (MSB), DNA SV 95
	Germany	10/20	(Brabec & Pohlmann, 1969)
	Germany	10/20	Albers & Pröbsting, 1998
	Germany	10/20	Tischler, 1950
	Macedonia	–/20	Sopova et al., 1983
	Ukraine	–/20	Magulaev & Vu, 1984
	Russia	–/20	l.c.
	Bulgaria	–/20	l.c.
	Georgia	–/20	l.c.
B. aspera Stev. ex Ledeb.,	Iran	20/–	Aryavand, 1980
monoecious	Iran	–/40	This study. (Voucher Zarre 35812 (MSB), DNA SV 161)
	Turkey	10/–	Bilge, 1955 (syn. *B. macrostylis*)
B. cretica L.,	Greece	30/60	Montmollin, 1986
dioecious	Greece	–/60	This study. Voucher Kocyan 115 (M), DNA SV 42
B. dioica Jacq.,	Austria	–/20	Hasitschka-Jenschke, 1961
dioecious	Austria	–/20	Dobes et al., 1997
	Czech Republic	–/20	Javurkova, 1981
	Great Britain	–/20	Dempsey et al., 1994
	Germany	10/–	This study. Voucher Volz 1b (MSB), DNA SV 46
	Germany	10/20	Brabec & Pohlmann, 1969
	Germany	10/20	Albers & Pröbsting, 1998
	Germany	10/–	Strasburger, 1910
	Germany	10/20	Tischler, 1950
	Germany	10/–	Lindsay, 1930
	Denmark	10/–	Meurman, 1925
	Russia	–/20	Magulaev & Vu, 1984
	Netherlands	–/20	Van Den Brand et al., 1979
B. marmorata Petit, dioecious	Corsica	20/40	Contandriopoulos et al., 1987
B. monoica Aitch. & Hemsl., monoecious	Uzbekistan	–/20	This study. (Voucher Volz 21 (MSB), DNA SV 208)
B. multiflora Boiss. & Heldr., dioecious	Turkey	See note	Heilbronn & Basarman, 1942; fertile crosses with *B. dioica* imply a number of $N = 10$
B. syriaca Boiss., dioecious	Israel	–/20	This study. Voucher Volz 26 (M)
B. verrucosa Ait., dioecious	Tenerife	10/20	Brabec & Pohlmann, 1969
	Canary Islands	–/20	This study. Voucher Erben 20 Mar. 2004 (MSB), DNA SV 11
E. elaterium subsp. *dioicum* (Batt.) Costich,	Cyprus	–/18	Slavick et al., 1993
dioecious	Spain	9/18, 24	Castroviejo, 1993
	Botanical Garden	12/–	Whitaker, 1933
E. elaterium subsp. *elaterium* (L.) A. Rich., monoecious	Spain	9/18	Castroviejo, 1993

Discussion

This study set out to infer sexual system evolution in *Bryonia* and to look for possible associations between hybridization, polyploidy, and sexual system. Of particular interest were the relationships among *B. alba*, *B. dioica*, and *B. aspera*, the monoecious and dioecious species of *Bryonia* used in the classic experiments that led to the inference of monofactorial dominant inheritance of dioecy in *Bryonia* and of the presence of XY sex determination in *B. dioica* (Correns, 1903, 1907; Bateson, 1909; Heilbronn and Basarman, 1942; Heilbronn, 1953; Westergaard, 1958). We discuss these aspects in turn, bringing in the geographic and temporal context as relevant.

Evolutionary transitions between monoecy and dioecy in *Bryonia*

Given the unresolved backbones of the nuclear and plastid gene phylogenies (Figs. 1 and 3) and considering that the closest relative of *Bryonia* is a single species with a monoecious and a dioecious subspecies, the ancestral sexual system of *Bryonia* cannot be inferred with confidence. If the common ancestor of *Bryonia* was dioecious, this would imply two transitions to monoecy, one in the common ancestor of *B. alba* and *B. aspera*, and one in the ancestor of *B. monoica*. The alternative scenario of ancestral monoecy may require four character transitions, viz. evolution of dioecy in *B. verrucosa*, in *B. syriaca*, in *B. multiflora*, and in the common ancestor of the *B. dioica* polytomy (*B. acuta*, *B. cretica*, *B. dioica*, and *B. marmorata*). The picture is complicated by *B. cretica* sequences appearing in three places in the nuclear tree (Fig. 3) and in two places in the chloroplast tree (Fig. 1). A further complication is that the monoecious species *B. alba* and *B. monoica* may occasionally be dioecious, although this has only been inferred from herbarium specimens (Jeffrey, 1969; see Materials and Methods). The family Cucurbitaceae in general is well known for changes between dioecy and monoecy at the genus, species, and population level (Roy and Saran, 1990; Zhang et al., 2006; Renner et al., 2007).

Although the ancestral sexual system of *Bryonia* is not securely known, there is unequivocal evidence for minimally two transitions in sexual systems, implying that the evolution of XY sex determination, i.e., the suppression of recombination between a pair of chromosomes carrying sex-determining genes, probably was dynamic. This fits with the phylogeny of a male-linked locus obtained from a broad sample of *B. dioica* (from Sweden to North Africa; R. Oyama, S. Volz, and S. Renner, unpublished manuscript). All northern sequences are clearly male or female, while in some southern populations the Y chromosome

appears to undergo occasional recombination.

The species involved in the early genetic experiments, *B. alba* and *B. dioica* (Correns; 1903, 1907; Bateson, 1909), and *B. aspera* and *B. dioica* (Heilbronn, 1953, Heilbronn and Basarman, 1942; Bilge, 1955), are not particularly close to each other. Judged from the molecular clock estimates and the distinctly different *rbcL* sequences of *B. alba* and *B. dioica* (compared to almost 200 other Cucurbitaceae sequences), they are separated by several million years of evolution. Based on a strict clock, the deepest split in *Bryonia* dates back 8.4 ± 1.8 Myr. Such a long history of *Bryonia* in Eurasia fits with the existence of an oligolectic *Bryonia*-specialist, the halictid bee *Andrena florea* whose range overlaps that of *Bryonia* (Westrich, 1989; Schröder and Lunau, 2001) except on the Canary Islands, and which is roughly as old as *Bryonia* based on a molecular clock (L. Larkin, University of New Mexico, Albuquerque, personal communication).

Hybridization and polyploidy in the evolution of *Bryonia*

The only contradictions between the nuclear and chloroplast topologies involve *B. multiflora* and *B. cretica*. Our sampling of *B. cretica* is confined to the western part of its range, namely, the islands Kythera and Crete, and the Peloponnesian Peninsula (Appendix S1). On Kythera, *B. cretica* shows clear evidence of a hybrid origin, with individuals from the same location having the M or the C cp haplotype. The M haplotype points to *B. dioica* as one of the progenitors of *B. cretica* because this haplotype is common in *B. dioica* populations from France, Spain, Algeria, and Morocco (Fig. 2; the M haplotype otherwise only occurs in *B. marmorata*, a species endemic to Corsica and Sardinia). This hypothesis receives support from the nuclear tree (Fig. 3) in which some *B. cretica* and *B. dioica* alleles group together. The ranges of *B. cretica* and *B. dioica* come closest in southern Italy (Fig. 2), but our sampling there is insufficient to resolve the fine-scale distribution of the M haplotype. It may be relevant in this context that Jeffrey (1969, p. 447) found "distinct" specimens of *B. dioica* in southern Italy and Sicily that resemble *B. cretica* in their coarsely punctate leaves, but differ from that species in having glandular-pubescent inflorescences. Other parents of *B. cretica* may have been *B. multiflora* and/or *B. syriaca*, either of which could have contributed the C haplotype to *B. cretica*. The ranges of both partly overlap that of *B. cretica* (Fig. 2). Where *Bryonia* individuals are in flower at the same time, they automatically share at least one pollinator, namely, the aforementioned oligolectic sand bee *Andrena florea*, which has a foraging range of up to 1 km (Edwards and Williams, 2004).

Fitting with its inferred hybrid origin, *B. cretica* has several morphological variants, a typical one from southern Greece, Crete, and the Aegean Islands (which is the one sampled; the plant described by Linnaeus as *B. cretica* came from Crete) and another that occurs in Cyprus, Syria, Turkey, and Palestine (Jeffrey, 1969, p. 448). The latter variant is similar to *B. multiflora*, one of the suspected progenitors (discussed previously). A further indication of past hybridizations among some species of *Bryonia* may be the substitutions found in the 5.8S gene (Appendix S3), which indicate coexisting pseudogenic copies, perhaps due to inactived 35S rDNA loci (H. Weiss-Schneeweiss, University of Vienna, personal communication).

The other polyploid species of *Bryonia*, the tetraploid *B. marmorata*, is endemic in Corsica and Sardinia. Cloning and direct sequencing yielded a single chloroplast haplotype and quite homogeneous *LFY* alleles (Table 1). This suggests that *B. marmorata* may have originated via autopolyploidy, perhaps from an individual of *B. dioica*, a species with which *B. marmorata* shares the M haplotype and to which its *LFY* alleles are related (Figs. 2 and 3). However, only two individuals of *B. marmorata* were sequenced (and only one counted for chromosomes), and more data are needed to firmly infer autopolyploidy.

Associations between polyploidy and sexual systems

Polyploidization can lead to a breakdown of some forms of nuclear-determined dioecy (Westergaard, 1958; Smith, 1955, 1969). In *Bryonia*, however, this does not seem to be the case; the hexaploid *B. cretica* and the tetraploid *B. marmorata* are both dioecious. In *Mercurialis*, with an unknown sex determination mechanism, a loose negative association between polyploidy and dioecy may result from a combination of homoploid speciation, genome duplication, and hybridization, with the precise sequence of events unclear (Durand and Durand, 1992; Obbard et al., 2006). As is likely the case in *Bryonia*, monoecy in *Mercurialis* sometimes evolved from dioecy (Obbard et al., 2006). However, the life histories of *Bryonia* and *Mercurialis* are so different that fruitful comparison is difficult; all *Bryonia* are climbers that annually sprout from large long-lived underground tubers; species of *Mercurialis* are annuals or woody perennials. The hypothesis that polyploidy may favor dioecy as an outcrossing mechanism because polyploid species may experience a breakdown of their self-incompatibility system (Miller and Venable, 2000; Yeung et al., 2005) is unlikely to apply to *Bryonia*, which is capable of selfing (S. Volz, personal observation).

Different from what we expected, hybridization appears to play a minor role in

Bryonia, which turned out to be a relatively old clade of genetically (mostly) distinct entities. The genetic distinctness of most species was unexpected from the morphological situation, where species can be difficult to distinguish (Jeffrey, 1969). It was also surprising to find (minimally) two shifts in sexual system in a genus of just 10 species. Together, these results provide a basis for developing *Bryonia* into a model system for the study of plant sex chromosome evolution, which so far has focused exclusively on species with heteromorphic sex chromosomes.

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Supplementary Material

APPENDIX 1-1. *Bryonia*, *Ecballium*, and *Austrobryonia* species and their authors, voucher information, collecting locality, DNA accession number, chloroplast haplotypes (Cp HT), and GenBank accession numbers for all sequences produced as part of this study. Herbarium acronyms follow Holmgren et al. (1990). BG stands for botanical garden. Specimens or total DNAs marked with an asterisk were used for chromosome counts or in bulk analyses for AFLP and SCAR work (Oyama,R. K., S. M. Volz, and S. S. Renner , unpublished manuscript).

Species	Vouchers	Collecting locality	DNA number	Cp HT	tmL-F	atpA-trnR	psbA-trnH	LFY	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.	E. Dörr 28 Feb. 1987 (M)	Germany, Bavaria, Erlangen	SV 07	A1	EU102289	EU102422	EU096317		
	G. Cocora-Tiez & 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); chromosomes counted: 2n = 20	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		
	Karwowskij 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)	USA, Washington, Whitman Co.	SV 168	A1	EU102302	EU102436	EU096331	EU102562	
	J. Brokaw 139 (M)	USA, Washington, Whitman Co.	SV 169	A1	EU102303	EU102437	EU096332		
	J. Brokaw 140 (MSB)	USA, 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 Baschkortostan, Sterlitamak	SV 183	A1	EU102308	EU102442	EU096337	
	N. Sipcinsnogo 210 (MHA)	Kazakhstan, Alatauy Zhotasy	SV 194	A1	EU102309	EU102443	EU096338	
	Gorbunova 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	EU102576
	K. Rechinger 18076 (M)	Afghanistan, Kabul	SV 83	P2	EU102395	EU102530	EU096425	EU102577
	K. Rechinger 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
	T. Heideman 5323-3000 (MO)	Azerbaijan, Naxcivan	SV 110	U1	EU102405	EU102540	EU096435	
	G. Fayvush et al. 1201 (M)	Armenia, Prov. Kotayk	SV 143	U2	EU102408	EU102543	EU096438	EU102566
	S. Zarre 35812 (MSB); chromosomes counted: 2n = 40	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. Rechinger 49111 (M)	Azerbaijan, Sardasht	SV 86	U4	EU102411	EU102546	EU096441	
	K. Rechinger 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 115 (M); chromosomes counted: 2n = 60	Greece, Kythera	SV 42*					
	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, Fanö	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 et 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 1b (MSB), chromosomes counted: 2n = 20	Germany, Munich	SV 46*						
	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, Regensburg	SV 122	D2	EU102344	EU102479	EU096374	EU102624	
	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, Vrbnik	SV 215	D2	EU102365	EU102500	EU096395	
	M. Kropf, no voucher	Croatia, Vrbnik	SV 216	D2	EU102366	EU102501	EU096396	EU102635
	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-Villaraco 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 et 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), chromosomes counted: 2n = 20	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		
	S. Volz 26 (M); chromosomes counted: 2n = 20	Israel, Ramat Yishay	*						
B. verrucosa Ait.	M. Erben 20 March 2004 (MSB), chromosomes counted: 2n = 20	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 ssp. *elaterium* (L.) A. Rich.	S. Renner et 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 ssp. *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

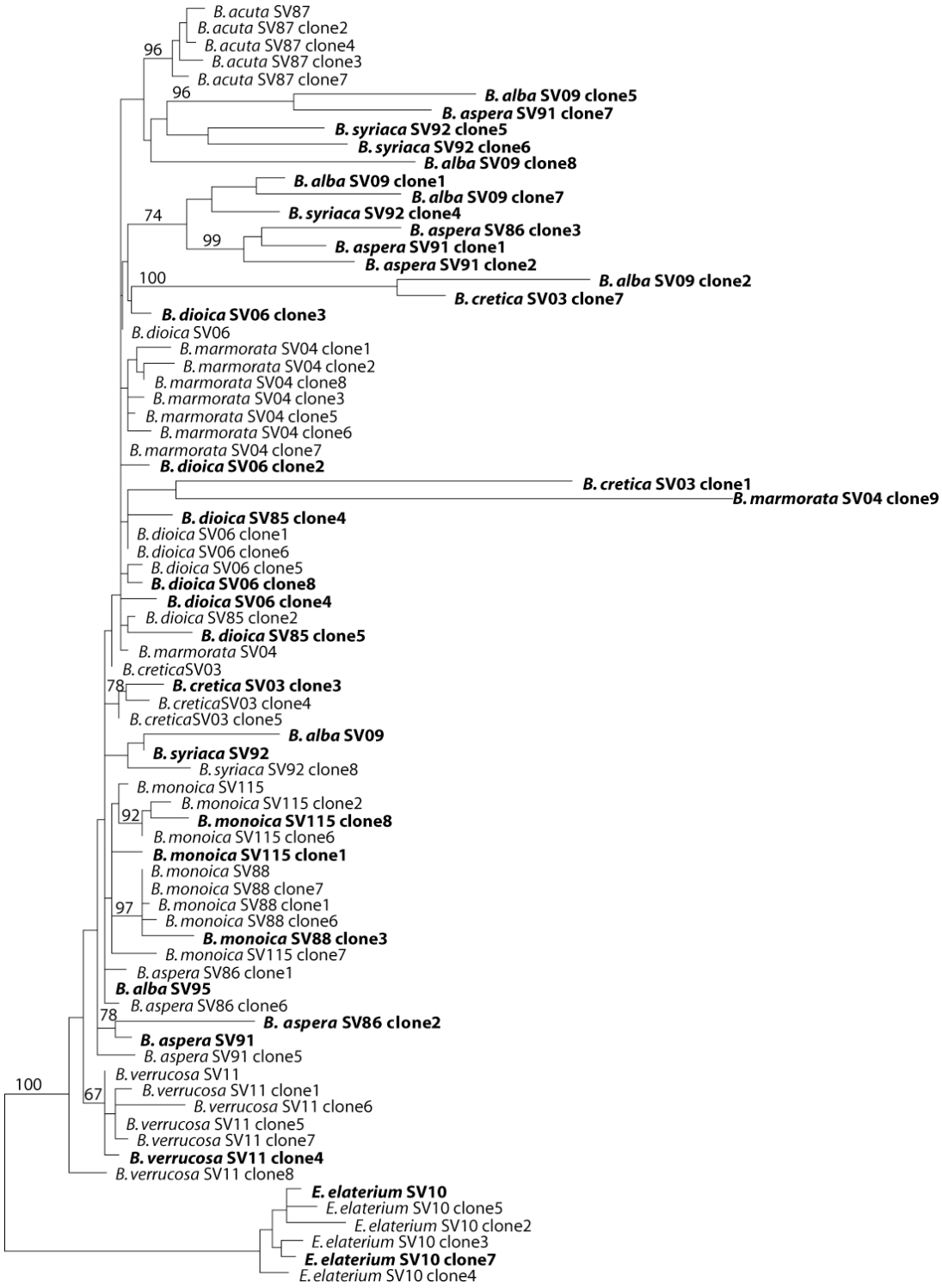
APPENDIX 1-2: Individuals of *Bryonia* and *Ecballium* sequenced for nuclear ribosomal DNA; see Appendix 1 for the herbarium voucher for each plant. GenBank accession numbers are followed by the C/G content (in percent) and the length (in nucleotides) of the 3' end of 18S, ITS1, 5.8S, ITS2, and the 5' end of 25S. For the rRNA-coding areas the numbers of single nucleotide polymorphisms (SNP) and indels are given. Synapomorphies are marked by an asterisk; n.a. = not applicable. Mutations >1 and length variation in the rDNA, indicating potential pseudogenic copies, are shown in bold.

	GenBank	18S			ITS1		5.8S			ITS2		25S		
Species	Accession #	C/G%	length	SNP/indels	C/G%	length	C/G%	length	SNP/indels	C/G%	length	C/G%	length	SNP/indels
B. acuta														
SV87	EU102672	n. a.	n. a.	n. a.	62,44	213	58,23	158	0	68,24	256	50,55	91	0
SV87 clone 2	EU102673	55,47	128	0	62,44	213	58,23	158	0	67,97	256	50,55	91	0
SV87 clone 3	EU102675	55,47	128	0	62,91	213	58,23	158	1	68,75	256	50,55	91	0
SV87 clone 4	EU102674	55,47	128	0	62,44	213	58,86	158	0	68,09	257	n. a.	n. a.	n. a.
SV87 clone 7	EU102676	56,25	128	1	62,44	213	58,23	158	0	68,36	256	50,55	91	0
B. alba														
SV09	EU102677	55,52	128	**1**	60,25	227	n. a.	n. a.	n. a.	65,27	264	48,81	91	**2**
SV09 clone 1	EU102678	54,33	128	**1***	58,14	215	54,87	**113**	**0/1**	67,70	257	49,45	91	**2**
SV09 clone 2	EU102679	50,00	128	**6***	58,43	216	54,43	158	**6**	60,39	255	43,96	91	**6**
SV09 clone 5	EU102680	52,76	128	**3***	59,45	217	53,80	158	**10**	64,12	262	48,35	91	**4**
SV09 clone 7	EU102681	54,33	128	1*	58,14	215	58,23	158	**2**	66,02	256	49,45	91	1
SV09 clone 8	EU102682	51,18	128	**8***	59,54	215	55,70	158	**6**	64,73	258	49,45	91	1
SV95	EU102683	55,91	**127**	**0/1**	62,50	216	58,23	158	0	68,75	256	50,55	91	1
B. aspera														
SV86 clone 1	EU102684	55,47	128	0	63,07	226	58,23	158	0	68,09	257	50,55	91	0
SV86 clone 2	EU102685	55,47	128	1	60,00	215	56,96	158	**2**	67,97	256	49,45	91	**2**
SV86 clone 3	EU102686	45,75	**94**	**1/1**	60,00	215	51,46	**103**	**6/1**	66,67	216	47,25	91	**3**
SV86 clone 6	EU102687	55,47	128	0	63,59	225	58,23	158	0	68,36	256	50,55	91	1
SV91	EU102688	55,47	128	0	58,16	225	57,06	158	**2**	68,61	256	50,55	91	0
SV91 clone 1	EU102689	46,81	**94**	**0/1**	59,54	215	52,21	**113**	**4/1**	66,93	257	47,25	91	**5**
SV91 clone 2	EU102690	55,47	128	0	58,92	227	53,98	**113**	**5/1**	68,12	229	47,25	91	**4**
SV91 clone 5	EU102691	55,47	128	0	61,86	212	58,23	158	0	67,97	256	51,65	91	1
SV91 clone 7	EU102692	51,56	128	**5**	58,61	215	53,80	158	**6**	64,34	258	n. a.	n. a.	n. a.

B. cretica														
SV03	EU102693	55,47	128	0	62,46	216	58,23	158	0	68,53	256	50,55	91	0
SV03 clone 1	EU102694	52,34	128	**8**	57,48	214	55,06	158	**5**	61,32	243	48,35	91	**8**
SV03 clone 3	EU102695	55,47	128	0	62,86	210	59,87	**157**	**2/1**	67,97	256	50,55	91	**2**
SV03 clone 4	EU102696	55,47	128	0	63,33	210	58,86	158	1	68,87	257	50,55	91	0
SV03 clone 5	EU102697	55,47	128	0	62,86	210	58,23	158	0	68,24	255	50,55	91	0
SV03 clone 7	EU102698	55,47	128	0	62,86	210	53,80	158	**8**	60,39	255	43,96	91	**6**
B. dioica														
SV06	EU102703	55,47	128	0	62,44	216	58,23	158	0	69,26	256	50,55	91	0
SV06 clone 1	EU102699	55,47	128	0	61,86	215	58,23	158	0	69,14	256	50,55	91	0
SV06 clone 2	EU102700	55,47	128	0	62,96	216	57,96	**157**	**0/1**	68,36	256	50,55	91	0
SV06 clone 3	EU102701	55,47	128	1	62,33	215	57,60	158	1	68,87	257	50,55	91	0
SV06 clone 4	EU102702	55,47	128	**2**	62,04	216	58,86	158	1	69,14	256	50,55	91	0
SV06 clone 5	EU102704	56,25	128	1	62,50	216	58,23	158	0	68,75	256	49,45	91	1
SV06 clone 6	EU102705	55,47	128	0	62,33	215	58,23	158	0	69,14	256	50,55	91	0
SV06 clone 8	EU102706	55,04	**129**	**0/1**	62,50	216	58,23	158	1	68,75	256	50,55	91	0
SV85 clone 2	EU102707	55,47	128	0	62,50	216	58,23	158	1	69,53	256	50,55	91	0
SV85 clone 4	EU102708	55,12	**127**	**0/1**	61,68	214	58,23	158	0	69,65	257	50,55	91	**2**
SV85 clone 5	EU102709	56,25	128	1	61,57	216	58,23	158	**2**	69,26	257	50,55	91	0
B. marmorata														
SV04	EU102710	55,47	128	0	62,50	216	58,23	158	0	69,26	257	50,55	91	0
SV04 clone 1	EU102711	55,47	128	0	62,04	216	57,60	158	1	68,99	258	50,55	91	0
SV04 clone 2	EU102712	54,69	128	1	62,04	216	58,23	158	0	69,02	255	50,55	91	0
SV04 clone 3	EU102713	55,47	128	1	62,33	215	58,23	158	0	69,14	256	50,55	91	0
SV04 clone 5	EU102714	55,47	128	0	62,04	216	58,23	158	0	69,14	256	50,55	91	0
SV04 clone 6	EU102715	55,47	128	0	61,11	216	58,23	158	0	68,75	256	50,55	91	0
SV04 clone 7	EU102716	55,47	128	0	62,50	216	58,23	158	0	69,14	256	50,55	91	0
SV04 clone 8	EU102717	55,47	128	0	62,50	216	58,23	158	0	69,14	256	50,55	91	0
SV04 clone 9	EU102718	51,56	128	0	58,02	212	50,63	158	**14**	61,03	213	46,15	91	**6**
B.monoica (*B. melanocarpa*)														

SV115	EU102719	n. a.	n. a.	n. a.	63,74	216	58,23	158	0	68,36	256	51,11	91	0
SV115 clone 1	EU102720	54,69	128	1	64,06	217	58,23	158	1	68,75	256	50,00	**90**	**0/1**
SV115 clone 2	EU102721	55,47	128	0	62,96	216	58,86	158	1	67,84	255	50,55	91	0
SV115 clone 6	EU102722	55,47	128	0	62,70	223	58,23	158	0	67,58	256	50,55	91	0
SV115 clone 7	EU102723	55,47	128	0	62,50	216	58,23	158	1	69,14	256	51,65	91	1
SV115 clone 8	EU102724	55,47	128	0	63,55	214	57,96	**157**	**2/1**	68,36	256	50,55	91	0
B. monoica														
SV88	EU102725	55,47	128	0	63,43	216	58,23	158	0	67,58	256	50,55	91	0
SV88 clone 1	EU102726	55,47	128	0	63,43	216	58,23	158	0	67,97	256	50,55	91	0
SV88 clone 3	EU102727	54,69	128	1	63,43	216	57,60	158	**5**	67,06	255	50,55	91	0
SV88 clone 6	EU102728	55,47	128	0	63,89	216	58,23	158	0	67,97	256	50,55	91	0
SV88 clone 7	EU102729	55,47	128	0	63,43	216	58,23	158	0	67,58	256	50,55	91	0
B. syriaca														
SV92	EU102730	55,47	128	0	62,67	217	57,50	**160**	**0/2**	68,75	256	50,55	91	0
SV92 clone 4	EU102731	53,13	128	**5**	60,19	216	54,87	**113**	**0/1**	67,32	257	49,45	91	1
SV92 clone 5	EU102732	55,47	128	0	61,93	220	57,86	**159**	**4/1**	65,89	258	50,55	91	0
SV92 clone 6	EU102733	54,69	128	1	58,60	215	56,33	158	**5**	66,54	257	n. a.	n. a.	n. a.
SV92 clone 8	EU102734	n. a.	n. a.	n. a.	61,14	226	58,23	158	0	68,85	260	50,55	91	0
B. verrucosa														
SV11	EU102735	55,47	128	0	62,39	218	58,23	158	0	69,14	256	50,55	91	0
SV11 clone 1	EU102736	n. a.	n. a.	n. a.	61,50	226	57,60	158	1	68,75	256	50,55	91	0
SV11 clone 4	EU102737	n. a.	n. a.	n. a.	62,02	211	58,13	**160**	**0/2**	69,14	256	50,55	91	0
SV11 clone 5	EU102738	n. a.	n. a.	n. a.	62,67	218	58,23	158	0	69,14	256	50,55	91	0
SV11 clone 6	EU102739	n. a.	n. a.	n. a.	60,49	226	58,23	158	0	68,87	257	n. a.	n. a.	n. a.
SV11 clone 7	EU102740	n. a.	n. a.	n. a.	61,69	211	58,23	158	0	69,14	256	50,55	91	0
SV11 clone 8	EU102741	56,25	128	1	62,79	215	58,23	158	0	69,14	256	50,55	91	0
E. elaterium														
SV10	EU102742	55,81	**129**	**1/1**	55,85	232	58,23	158	0	60,86	256	50,55	91	0
SV10 clone 2	EU102743	55,47	128	0	55,17	232	57,60	158	1	61,57	255	50,55	91	0
SV10 clone 3	EU102744	54,69	128	1	55,60	232	58,23	158	1	60,16	256	50,55	91	0

SV10 clone 4	EU102745	55,47	128	0	55,60	232	57,60	158	1	59,77	256	50,55	91	0
SV10 clone 5	EU102746	55,47	128	0	56,47	232	57,60	158	1	61,33	256	50,55	91	0
SV10 clone 7	EU102747	54,69	128	**2**	55,60	232	58,23	158	0	60,55	256	50,55	91	0
Mean		54,06	127,21		61,20	217,55	57,40	155		67,22	254,79	50,64	90,99	



APPENDIX 1-3 Maximum likelihood tree obtained from 76 nuclear rDNA internal transcribed spacer sequences obtained from 14 individuals representing the ten species of *Bryonia* and *Ecballium elaterium* (835 nucleotide characters plus 48 coded indels). The name *B. melanocarpa* is a synonym of *B. monoica*. Suspected pseudogenes (based on the SNPs and indels marked in Appendix 2) are shown in bold. Maximum likelihood bootstrap support values >75 are given above branches.

Chapter 2

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

STEFANIE M. VOLZ AND SUSANNE S. RENNER

Abstract

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 (cp) and nuclear (nr) 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 cp and nr 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.

Key words: 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 12 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, in press).

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; Reynold, 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* subspecies *dioica* are now invasive in the Rangitikei River area (Webb & al., 1995; T. Gilbertson, Department of Conservation, Mangaweka, New Zealand, personal communication, 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 sampled for DNA (L. Bagmet, curator of the herbarium of the N. I. Vavilov Institute of Plant Industry in Saint Petersburg, personal communication, 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 US 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; Jakob & Blattner 2006; Bänfer & al., 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 12 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 (in press); Appendix 2-1 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 *trn*L intron and *trn*L-*trn*F intergenic spacer (IGS) were amplified using the Taberlet & al. (1991) primers c, d, e, and f. The *psb*A-*trn*H spacer was amplified with the forward primer of Sang & al. (1997) and a *Bryonia*-adapted reverse primer 5' CGCGCATGGTGGATTCACAATCC 3'. The *trn*R-*atp*A 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' TCTTCCACCTSTATGARCAGTGTCGTGAAT 3' and *LFY* CucR 5' CGAAATCACAAA 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). 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 data set 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. ML 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 *psb*A-*trn*H spacer (last 7 nucleotides excluded), a poly-A run of up to 14 nucleotides in the *trn*L intron (5 excluded), and a poly-T run of up to 31 nucleotides (23 excluded) again in the *trn*L intron.

Bayesian analyses relied on the GTR + G models in MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003); maximum likelihood analyses relied on the RAxML Blackbox with the default GTR + I + G model of substitution (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 ML-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 1 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 4.201 (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, und 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 cpDNA haplotypes were sorted into regional groups, based on geography.

Results

Trees and networks from the chloroplast data

The *trn*L region in *Bryonia* comprises 928-939 bases, the *psb*A-*trn*H spacer 237-245, and the *trn*R-*atp*A spacer 517-537. A combined matrix of these loci, sequenced for 139 plants (including the outgroups *Ecballium* and *Austrobryonia*), had a length of 1913 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 cp haplotypes that could be assigned to 11 major groups (labeled in large letters in Fig. 1).

A permutational contingency test (10000 resamples) on 81 accessions representing the 11 main haplotype groups rejected the null hypothesis of no clustering of haplotypes with geographic location (c2= 75.3, P = 0.034), and a minimum-spanning network of the cp haplotypes is shown in Figure 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 and 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 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 data set of 55 accessions (alignment length 523 characters, including 50 coded indels). A ML 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 nr tree and the cp tree that is statistically supported is that *B. multiflora* groups with *B. cretica* and *B. syriaca* in the cp tree, but with *B. alba* and *B. aspera* in the nr tree.

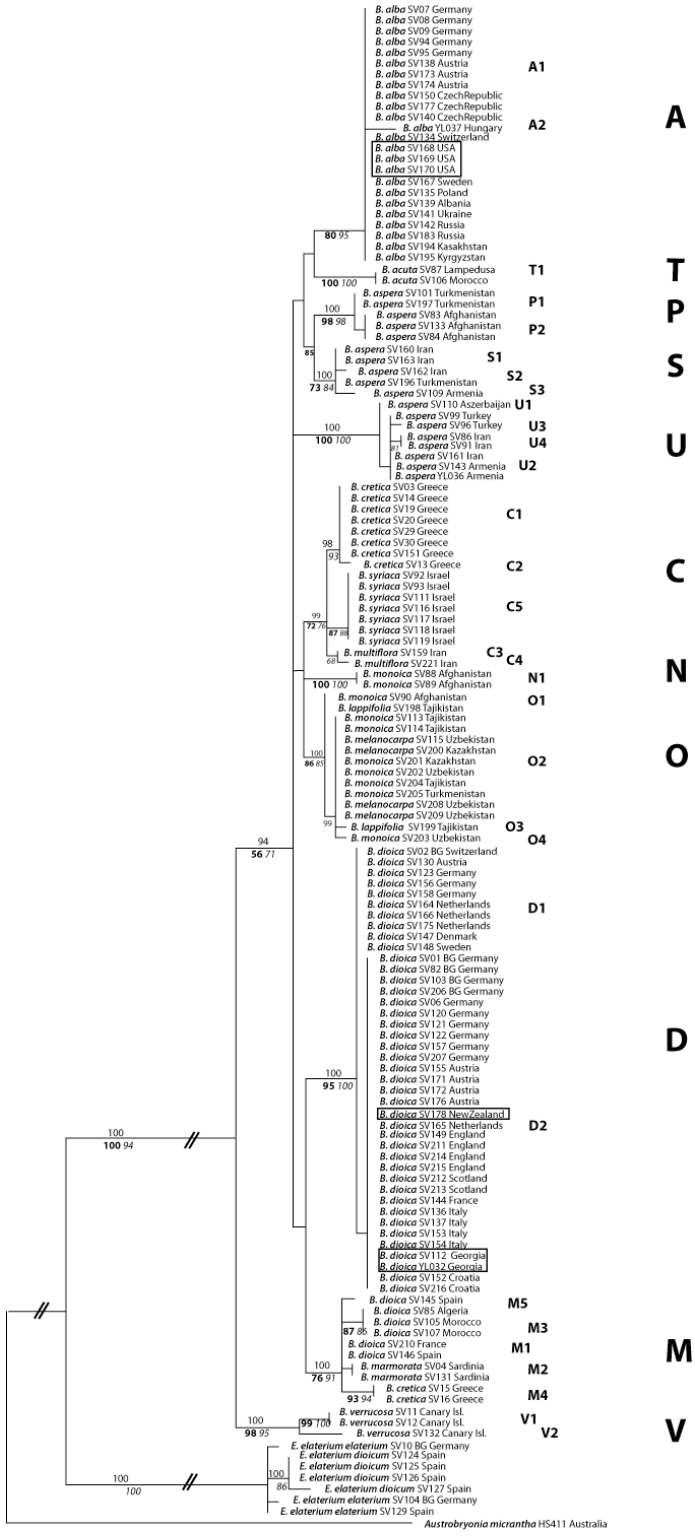


FIGURE 2-1 Maximum likelihood tree for 139 *Bryonia* and outgroup chloroplast sequences (1913 characters including 49 coded indels). Following each species name are DNA number, geographic origin, and the acronym of the respective haplotype (A to V, with the subtypes indicated in smaller letters and numbers, C1, C2, C5, etc.). Numbers above branches indicate Bayesian posterior probabilities >95, those below, bootstrap support >75 under parsimony (bold) and >70 under ML (italics). Sequences from naturalizing invasive populations are boxed.

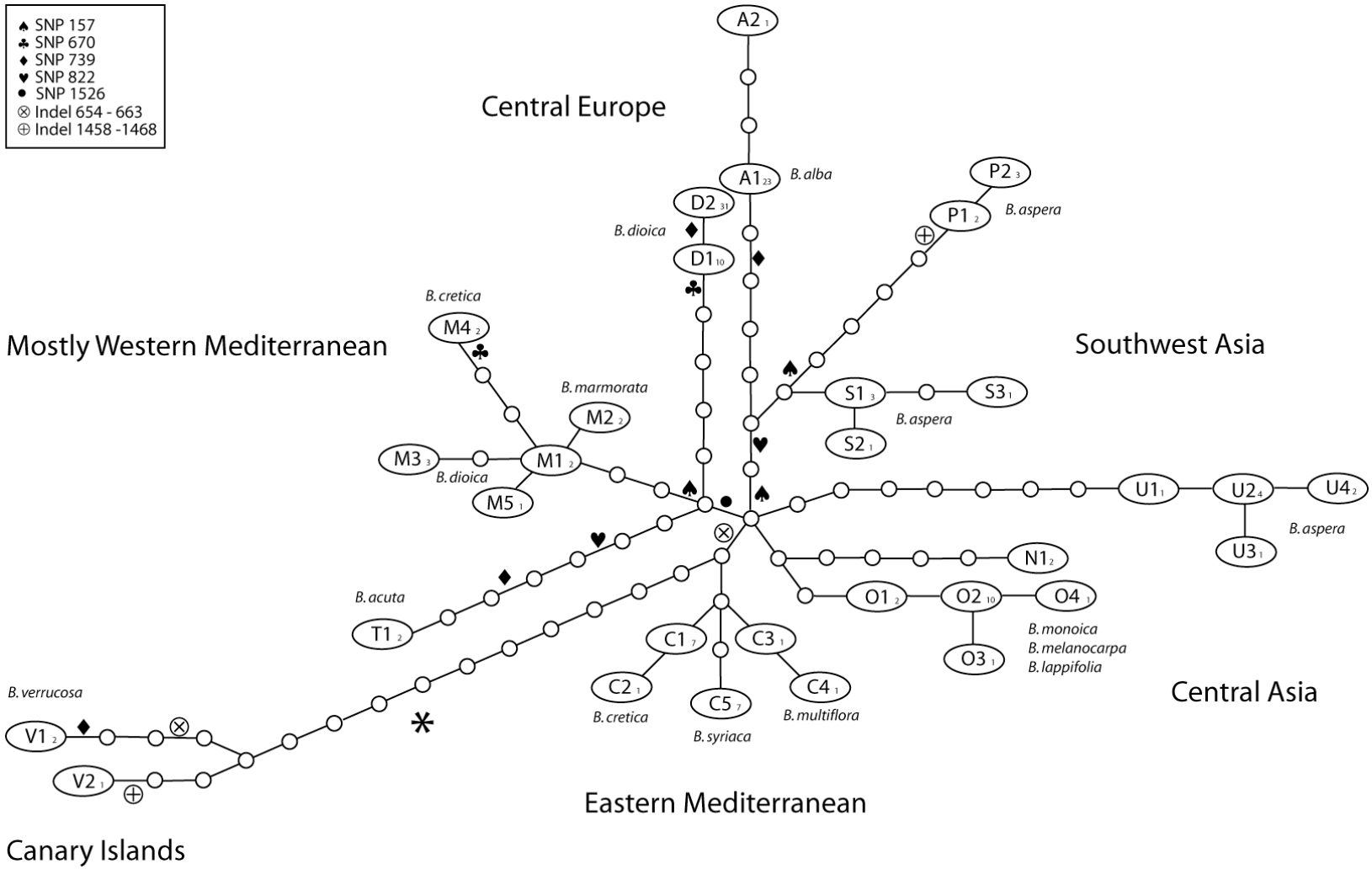


FIGURE 2-2 Minimum-spanning network of the 31 chloroplast haplotypes found among 131 individuals of *Bryonia* from throughout the range of the genus. Haplotypes are labeled as in Figure 1; numbers in subscript refer to the number of individuals in which each haplotype was found. Small open circles indicate 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).

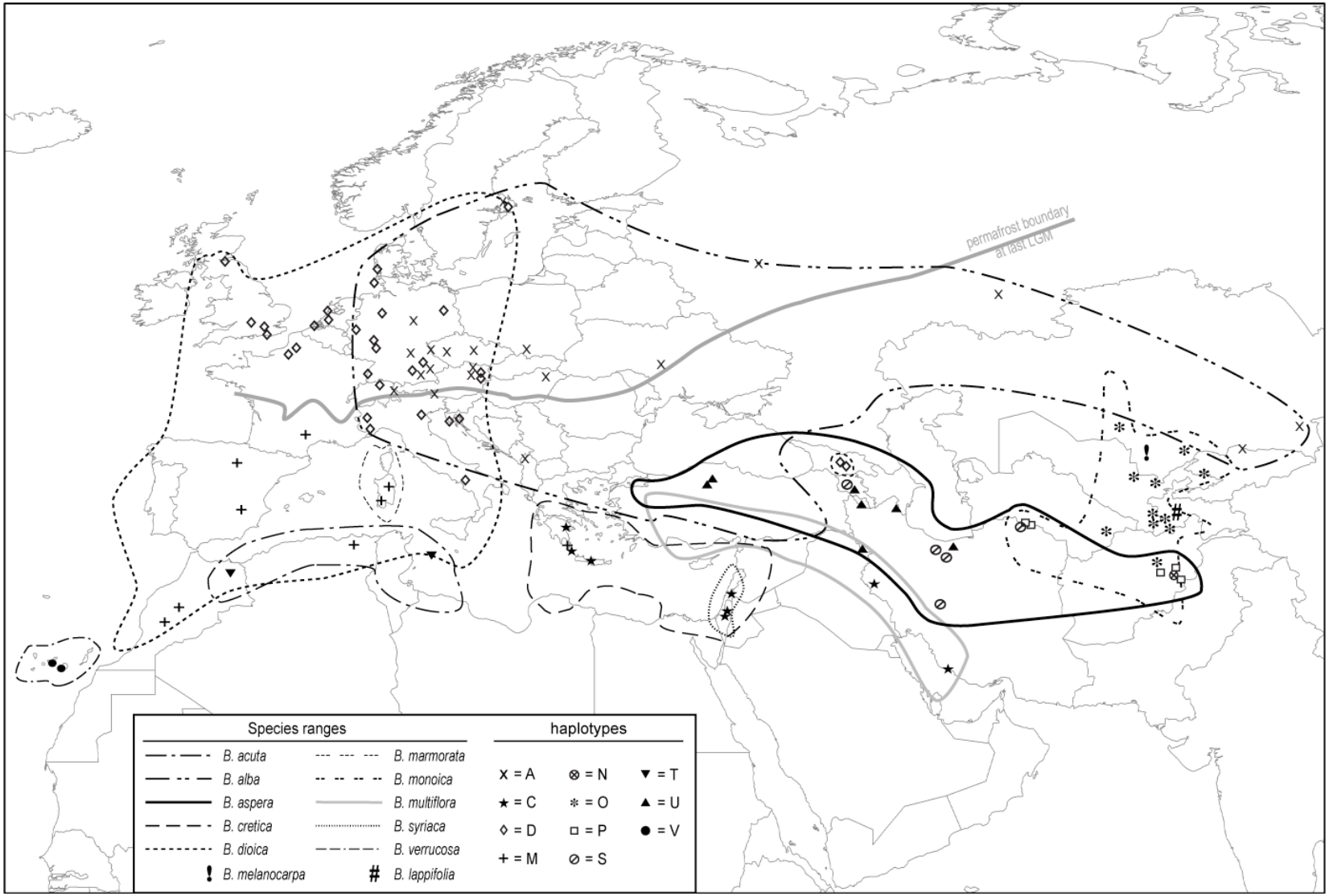


FIGURE 2-3 Geographic distribution of the major chloroplast haplotypes found in *Bryonia*. Species boundaries after Jeffrey (1969), but including newly discovered occurrences of *B. acuta* in Morocco and *B. dioica* in Georgia. The labeling of haplotypes corresponds to that in Figs. 1 and 2.

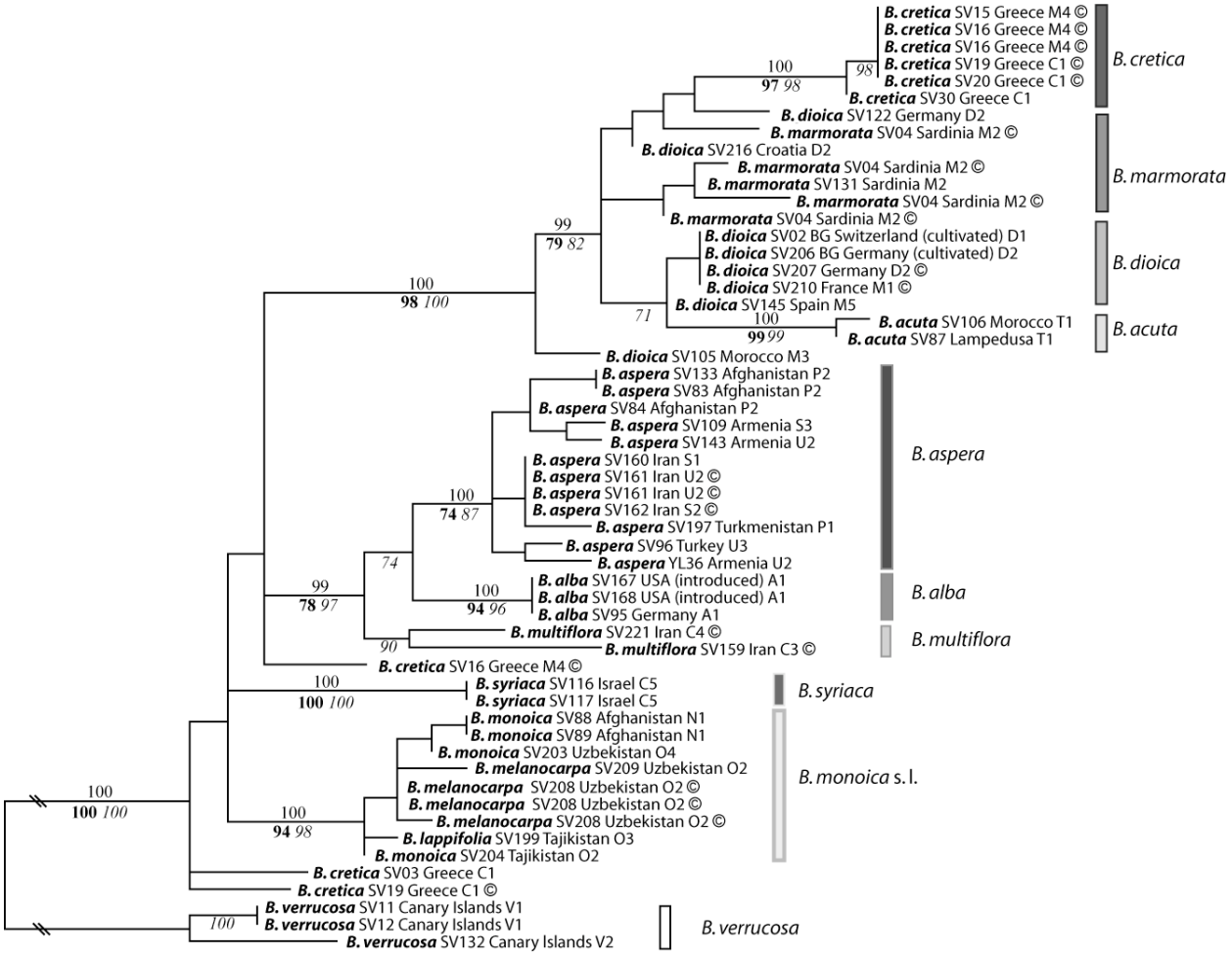


FIGURE 2-4 Maximum likelihood tree from 55 nuclear LFY sequences of *Bryonia*, rooted on *B. verrucosa*. Following the species names are DNA number, geographic origin, and chloroplast haplotype labeled as in Figures 1 and 2. Cloned sequences are marked by a ©. Numbers above branches indicate Bayesian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

Discussion

How many biological species of *Bryonia*?

A comparison of the morphology-based species circumscriptions of Jeffrey (1969) who distinguished 12 species of *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 and 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 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 and 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, in press). 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 Central Asia, the tubers can reach a length of 75 cm and a weight of 27 kilogram (Nabiev, 1961; 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).

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 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 et al., 2004) and *Hypochaeris* (Cerbah et 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

FIGURE 2-5 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.

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 et 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). 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, and Iraq), and thus probably extended into northern Europe from the 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; Reynold, 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, personal communication, 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 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 c. 2300 km between its occurrence in Tbilisi (in Georgia at E 44°47') 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 deg. 38 min. E, and one from Trencin, 18 deg. 02 min. 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, personal communication, 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. diocia* 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* subspecies *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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Supplementary Material

APPENDIX 2-1 *Bryonia*, *Ecballium*, and *Austrobryonia* species and their authors, voucher information, collecting locality, DNA number, chloroplast haplotype (Cp HT), and GenBank accession numbers for all sequences used in this study. Herbarium acronyms follow Holmgren et al. (1990). BG stands for botanical garden.

Species	Vouchers	Collecting locality	DNA	Cp HT	*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	EU683744	EU683741	EU683738		
	E. Dörr 28 Feb. 1987 (M)	Germany, Bavaria, Erlangen	SV 07	A1	EU102289	EU102422	EU096317		
	G. Cocora-Tiez & 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		
	Karwowskij 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)	USA, Washington, Whitman Co.	SV 168	A1	EU102302	EU102436	EU096331	EU102562	
	J. Brokaw 139 (M)	USA, Washington, Whitman Co.	SV 169	A1	EU102303	EU102437	EU096332		
	J. Brokaw 140 (MSB)	USA, 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 Baschkortostan, Sterlitamak	SV 183	A1	EU102308	EU102442	EU096337	
	N. Sipcinsnogo 210 (MHA)	Kazakhstan, Alatauy Zhotasy	SV 194	A1	EU102309	EU102443	EU096338	
	Gorbunova 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	EU102576
	K. Rechinger 18076 (M)	Afghanistan, Kabul	SV 83	P2	EU102395	EU102530	EU096425	EU102577
	K. Rechinger 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	EU683746	EU683743	EU683740	EU683747
	G. Fayvush et 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. Rechinger 49111 (M)	Azerbaijan, Sardasht	SV 86	U4	EU102411	EU102546	EU096441	
	K. Rechinger 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, Fanö	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 et 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, Saxon-Anhalt	SV 74	D2	FJ174449	FJ009140	FJ009154		
	S. Volz 4e (MSB)	Germany, Saxon-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, Vrbnik	SV 215	D2	EU102365	EU102500	EU096395	
	M. Kropf, no voucher	Croatia, Vrbnik	SV 216	D2	EU102366	EU102501	EU096396	EU102635
	R. Gagnidze et al. 269 (MO)	Georgia, Kartli, Tbilisi, Narikala, 20 June 2002	YL 32	D2	EU683745	EU683742	EU683739	
	M. Merello et al. 2220 (MO)	Georgia, Kartli, 8 June 1999	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-Villaraco 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 et 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 ssp. *elaterium* (L.) A. Rich.	S. Renner et 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 ssp. *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

Chapter 3

A sex-linked SCAR marker in *Bryonia dioica* (Cucurbitaceae), a dioecious species with XY sex-determination and homomorphic sex chromosomes

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Abstract

Genetic crosses between the dioecious *Bryonia dioica* (Cucurbitaceae) and the monoecious *B. alba* in 1903 provided the first clear evidence for Mendelian inheritance of dioecy and made *B. dioica* the first organism for which XY sex-determination was experimentally proven. Applying molecular tools to this system, we developed a sex-linked SCAR marker for *B. dioica* and sequenced it for individuals representing the full geographic range of the species from Scotland to North Africa. For comparison, we also sequenced this marker for representatives of the dioecious *B. cretica*, *B. multiflora*, and *B. syriaca*, and monoecious *B. alba*. In no case did any individual, male or female, yield more than two haplotypes. In northern Europe, we found perfect linkage between our marker and sex, with all Y-sequences being identical to each other. In southern Europe, however, the linkage between our marker and sex was weaker, with recombination detected within both the X- and the Y-homologues. Population genetic analyses suggest that the SCAR marker experienced different evolutionary pressures in northern and southern Europe. These findings fit with phylogenetic evidence that the XY system in *Bryonia* is labile and suggest that the genus may be a good system in which to study the early steps of sex chromosome evolution.

Key words: *Bryonia*, Cucurbitaceae, population structure, sex chromosome, Y-chromosome

Introduction

Sexually specialized life cycle stages that are only male or only female evolved many times among land plants. In flowering plants, however, sexual specialization of the sporophyte is rare, with only six percent of the 240,000 species being dioecious (Renner & Ricklefs, 1995). In spite of this limited overall success, dioecy has evolved thousands of times, implying the parallel evolution of different forms of sex determination. The sex determination mechanism that is most common in animals, namely heteromorphic XY or WZ sex chromosomes, in plants evolved exceedingly rarely. Only a dozen species in five genera – *Cannabis*, *Humulus*, *Rumex*, *Silene*, and *Coccinia* – are known to have morphologically distinct sex chromosomes (Ming, et al., 2007). While there is no evidence that all organisms with separate sexes are destined to acquire morphologically distinct sex chromosomes (Lynch, 2007), theory predicts the assortative accumulation of sex-determining genes and sexually antagonistic genes, accompanied by suppression of recombination on one of the gonosomes (in the case of XY sex determination, on the Y-chromosome), and culminating in the physical differentiation of the two sex chromosomes (Bull, 1983; Charlesworth B., 1991; Charlesworth D., 2002). An early-stage in sex chromosome evolution would thus involve a chromosome with a recombination-suppressed region that contains key sex-determining genes.

The first experimental system for the genetics of sex determination was the Cucurbitaceae genus *Bryonia* (Correns, 1903; Correns, 1907; Rheinberger, 2000). In experiments designed to reveal the mode of inheritance of dioecy, Correns carried out numerous reciprocal pollinations between the dioecious *B. dioica* and the monoecious *B. alba*. He grew almost 1000 F1 offspring, and from the sex ratio of crosses that involved *B. dioica* as the father, inferred that half the pollen grains of *B. dioica* carried a "female tendency," the other half a "male tendency." In doing so, he provided the first genetic evidence for sex chromosomes. Because of the importance of this discovery, Bateson (1909) and Heilbronn (1948) repeated Correns's experiments, with the same results. Later studies used *B. dioica* and *B. aspera* (under the synonymous name *B. macrostylis*) as an additional dioecious/monoecious pair for reciprocal crossing (Heilbronn & Basarman, 1942; Heilbronn, 1953; Westergaard, 1958) (summarized in Westergaard). These experiments confirmed XY sex-determination, with the male the heterogametic sex. Numerous cytological investigations of *Bryonia*, however, found no evidence of a dimorphic chromosome pair (Strasburger,

1910; Meurman, 1925; Volz & Renner, *in press*).

In light of current theory on the evolution of sex chromosomes, the lack of a cytologically distinct pair of chromosomes combined with the clear evidence for an XY system of sex-determination suggests that sex chromosome evolution in *Bryonia* may be at an early stage. Support for this hypothesis comes from a phylogeny for the seven dioecious and three monoecious species of *Bryonia*, which reveals at least two switches between monoecy and dioecy within the genus (Volz & Renner, *in press*). Molecular-clock dating has yielded an age of 8-9 MY for the deepest divergence within *Bryonia* and divergence times on the order of probably a few million years for most of the nine species within core-*Bryonia*. The sister genus of *Bryonia* is *Ecballium*, which comprises a single species that also has XY sex-determination, with the male again being the heterogametic sex and the chromosomes again monomorphic, but phenotypic sex is environmentally influenced (Galán, 1946; Westergaard, 1958). The sister clade to *Bryonia* and *Ecballium* is the Australian genus *Austrobryonia*, which consists of four monoecious species (Schaefer, et al., 2008). Taken together, these observations point to an evolutionary lability of monoecy and dioecy in the *Bryonia* clade, and suggest that the sex chromosomes of *Bryonia dioica* may be relatively young.

Here we report on the relative nucleotide divergence in the X- and Y-homologues of a sex-linked SCAR (Sequence Characterized Amplified Region) marker within *Bryonia dioica*, the species that was the focus of Correns's work on the inheritance of dioecy. *Bryonia dioica* occurs from Sweden and Scotland south through Germany, France and Spain, to Algeria and Morocco in northern Africa. The thirty-three male and female individuals in this study represent most of this geographical range. For comparative analyses, we also included individuals of the geographically more restricted dioecious species *B. cretica*, *B. multiflora*, and *B. syriaca*, and an individual of the monoecious *B. alba*. The results are the first molecular data on sex-determination in this group and represent the first step in re-introducing *Bryonia dioica* as a system for studying the evolution of dioecy and sex chromosomes.

Materials and Methods

Background on study system

Of the species sampled here, *Bryonia dioica* occurs throughout Western Europe and into North Africa; *B. alba* from Central Europe to Kazakhstan; *B. cretica* in Greece, Crete,

Cyprus, Turkey, and south to Egypt and Libya; *B. multiflora* in Turkey, Iran, and Iraq; and *B. syriaca* in Lebanon, Israel, and Syria. Information about all sequenced accessions is provided in Appendix 3-1. In terms of genome ploidy, *B. alba, B. dioica, B. multiflora,* and *B. syriaca* are diploid (2n = 2x = 20), while *B. cretica* is hexaploid (2n = 6x = 60) (Volz & Renner, in press). All species of *Bryonia* are pollinated by pollen- and nectar-gathering bees, and the fruits are berries that are dispersed by birds (Kugler, 1981; Westrich, 1989; Schröder & Lunau, 2001). One of the pollinators of *Bryonia* species is the *Bryonia* pollen specialist *Andrena florea*, which has a foraging range of up to 1 km (Edwards & Williams, 2004).

DNA Extraction

Total DNA was extracted from silica gel-dried young leaves of sexed plants using NucleoSpin Plant kit (Machery-Nagel, Düren, Germany) according to the manufacturer's protocol. DNA concentration and quality was checked on 1.5% agarose gels by electrophoresis using Lambda DNA as a reference. If required, the concentration was adjusted to approximately 150 ng/µl.

Male-linked AFLP marker construction and screening

We first created DNA bulk samples for each sex by pooling the genomic DNA extractions of up to eight individuals of *Bryonia dioica* from northern and southern Europe. A restriction digest of these bulks and a ligation of the AFLP adaptor ends were performed in a single step, using the AFLP library construction kit (Invitrogen GmbH, Karlsruhe, Germany). Approximately 600 ng of DNA were combined with 1 x buffer NEB2 (New England Biolabs [NEB], Ipswich, Massachusetts), 1 x T4 DNA ligase buffer, 1 x BSA, 0.8 units of the restriction enzyme MseI, 4 units of EcoRI, 0.75 units T4 DNA ligase, 30 pm pre-annealed MseI+ and MseI- adapter, and 3 pm pre-annealed EcoRI+ and EcoRI- adapter. The adapters serve to modify the restriction enzyme recognition site to prevent repeated cutting by the enzyme. This solution was then incubated at 22º C overnight and diluted with water to a final volume of 50 µL. This was then amplified via PCR, using M02 and E01 primers (pre-selective amplification). Products were checked on 1.5% agarose gels and diluted 1:50. To reduce the number of bands to a manageable number, a selective amplification step was applied, using primers with three additional bases on the 3' end. (selective amplification) Oligonucleotide nomenclature follows Keygene guidelines (www.keygene.com), and all oligos were obtained from MWG Biotech AG, Ebersberg, Germany.

Pre-amplification was performed in a total volume of 25µl with 1 x PCR buffer

(NEB), 0.15 mM dNTP, 12.5 ng primer M02 and E01 respectively, 0.5 µL *Taq* polymerase (NEB), 4 µl of the restriction/ligation product containing approximately 48 ng DNA, and run at 72° for 2 minutes, followed by 20 cycles of 10 seconds denaturation at 94°, 30 seconds primer annealing at 56°, and 2 minutes elongation at 72°, followed by 30 minutes at 60°C. Selective amplification was performed in a total volume of 15 µl with 1 x PCR buffer (NEB), 0.15 mM dNTP, 12.5 ng selective M-primer (with three selective bases) and fluorescence 6FAM-labeled E-primer (also three selective bases), 0.25 µL *Taq* polymerase (NEB), and 3 µl of pre-amplification product, diluted 1 : 50. PCR conditions were 2 minutes at 94°, 10 cycles of 10 seconds 94°, 30 seconds 65°, 2 minutes 72°, followed by 25 cycles of 10 seconds 94°, 30 seconds 56°, 2 minutes 72°, followed by terminal 30 minutes at 60°. If necessary, additional PCR reactions with four or five selective nucleotides on the M-primer were performed to reduce the overall amount of fragments.

The AFLP products from the selective PCR were run on a 3100 Avant capillary sequencer (Applied Biosystems [ABI], Foster City, California) alongside a LIZ-labeled ladder (ABI) and evaluated with GeneMapper 3.7 (ABI) to identify AFLP markers linked to the male sex. Because the initial screening was done with pooled DNA from multiple individuals, the primary difference between the two samples was the absence or presence of a Y-chromosome. Potentially male-linked bands identified in the bulk samples were verified by repeating the procedure in several individuals of known sex.

AFLP fragment isolation and sequencing

AFLP markers that were male-linked were isolated and sequenced. Four parallel amplification reactions were pooled, concentrated, re-suspended in 10 µL of water and separated on a Spreadex Mini Gel (Elchrom Scientific, Cham, Switzerland) at 100V for 240 minutes using the SEA 2000 electrophoresis chamber (Elchrom Scientific). Gels were then stained in Ethidium Bromide and the targeted fragment was excised and incubated in 50 µL of 10 mM Tris solution at 70º for 30 minutes. The supernatant was then used as template DNA to set up a PCR reaction using the same primers. That reaction was run out on a 2.5% agarose gel, and the target band was excised and purified with the Wizard SV genomic DNA purification system (Promega, Madison, Wisconsin). The cleaned PCR product was cloned using the TEasy-System (Promega), and eight colonies were picked. The plasmid product was isolated from the cultures with the Sigma mini-prep system (Sigma-Aldrich, Munich, Germany) and sequenced, using the SP6 and T7 primers with Big Dye Terminator 3.0

chemistry (ABI). Sequence assembly and editing were carried out in Sequencher 4.6 (GeneCodes, Ann Arbor, Michigan) and checked against the GenBank database via BLAST.

SCAR marker development and testing

Sequences of AFLP markers with the appropriate length were manually aligned and used to design sequence-characterized amplified region (SCAR) primers. These primers comprised the restriction-enzyme recognition site, the three to five selective bases, and 12 to 20 additional sequence-specific bases. As the SCAR primers do not comprise the artificial adapter sequences of the AFLP primers, the expected SCAR fragment is 21 bp shorter than the AFLP fragment it amplifies.

Sex specificity of the SCAR primers was tested with genomic DNA of 20 male and seven female plants of *B. dioica*. We used touchdown PCR with 10 µM of primers, 25 µM $MgCl_2$, 1 µl Q solution (Qiagen, Hilden, Germany), 1.25 µM of each dNTP, 2.5 µl of 10x PCR-buffer, 0.5 µL of Taq DNA polymerase (Qiagen), and 10-50 ng of template DNA per 25 µl of reaction volume. The SCAR primers were also tested on other species of *Bryonia* under relaxed PCR conditions. All resulting bands were gel-cleaned and sequenced. If the sequence was not clean, the PCR product was cloned and multiple clones were sequenced in order to assess the number of alleles.

Phylogenetic and population genetic analyses

An alignment of all sequences was created in MacClade 4.08 (Maddison and Maddison, 2003) and adjusted by eye. A best Maximum-Likelihood (ML) tree and corresponding bootstrap support values were calculated using the RAxML Blackbox with the default GTR + I + Γ model of substitution (Stamatakis, et al., 2008). Trees were rooted with a sequence from *B. alba*, the only non-dioecious species in our sample. A haplotype network was also constructed of the *B. dioica* sequences using TCS 1.21 (Clement, et al., 2000), treating gaps as missing characters.

Because the distribution of *B. dioica* is along a mostly North-South axis from Sweden to North Africa, we grouped the individuals into northern European (e.g., North of the Alps) and southern European (e.g., South of the Alps and northern Africa) populations. Our northern Europe sample consisted of eleven males and six females, the southern Europe sample of thirteen males and three females. Sequences from Bavaria were assigned to the southern population (see *Results*). Calculations of Waterson's θ (Waterson, 1975), π, Tajima's D (Tajima, 1989), and F_{ST} values were made using DNAsp 4.10.9 (Rozas & Rozas,

1999).

To test whether the variation observed in our sex-linked marker was significantly different from expected variation under a neutral model, we used the program HKA from Jody Hey (http://lifesci.rutgers.edu/~heylab) (Hudson, et al., 1987). We tested the X- and Y-linked sequences from both northern and southern Europe against chloroplast sequences (see Appendix 3-2), using sequences of *B. multiflora* and *B. syriaca* as the outgroup. We adjusted the test for the different effective population sizes of the X- and Y-chromosomes, and of the chloroplast genome. Although the chloroplast genome may experience selective sweeps, thus violating the assumption of neutral evolution (Muir & Filatov, 2007), chloroplast spacer and intron sequences are a suitable yardstick against which to compare the nuclear SCAR marker sequences. A non-sex-linked nuclear marker, the *Leafy* gene, has been less densely sampled but is congruent with the chloroplast phylogeny where the accessions overlap (Volz & Renner, *in press*).

Results

SCAR marker

Six AFLP markers were unambiguously linked to the male sex. We developed one of these markers, AFLP-278, into a SCAR marker (henceforth SCAR-278), following the procedure summarized in Figure 1. The SCAR-278 primers were F-gaattcacatcgtggggtcc and R-ttaacacattatttcaagcaaataagttcc. Using these primers, we obtained PCR products from 33 individuals of *Bryonia dioica* (24 male, 9 female) as well as four individuals of *B. cretica* (2 male, 2 female), one *B. multiflora* female, two *B. syriaca* females, and one monoecious individual of *B. alba*. The resulting 61 sequences have been submitted to GenBank; GenBank accession numbers and voucher details are provided in Appendix 3-1. An alignment of all sequences was 405 characters long, including gaps. BLAST searches of GenBank with these sequences did not yield any significant hits, and stop codons exist in all three reading frames.

Sequences of SCAR-278 from *Bryonia dioica* males that matched the original male-linked AFLP marker (AFLP-278) were classified as Y because the species is known to have XY sex-determination (Introduction). All other sequences recovered from *B. dioica* were assigned X or Y status based on the presence (Y) or absence (X) of a large indel seen between the AFLP-278 marker and the X-homologues (see Fig. 1). The Y-sequences of SCAR-278 from *B. dioica* were named BdY1 and the X-sequences BdX1 following the

system developed for Silene sex-linked regions (Filatov & Charlesworth, 2002). Sequences from the other species included were not assigned X or Y status at this point because *Bryonia alba* is monoecious and the sex-determination systems of *B. multiflora, B. syriaca*, and *B. cretica* have not been investigated. However, sequences from these other species were similar to the *B. dioica* X-sequences in that they also lacked the deletion relative to the original male-linked AFLP-278 marker.

All Y-sequences recovered from males in northern Europe (n = 10) were identical to each other and to the original male-linked AFLP maker (AFLP-278). In contrast, there was variation among the nine Y-sequences from southern European males (and one southern European female) and in the length of the deletion relative to the X-sequences. In no case did any individual, male or female, yield more than two haplotypes.

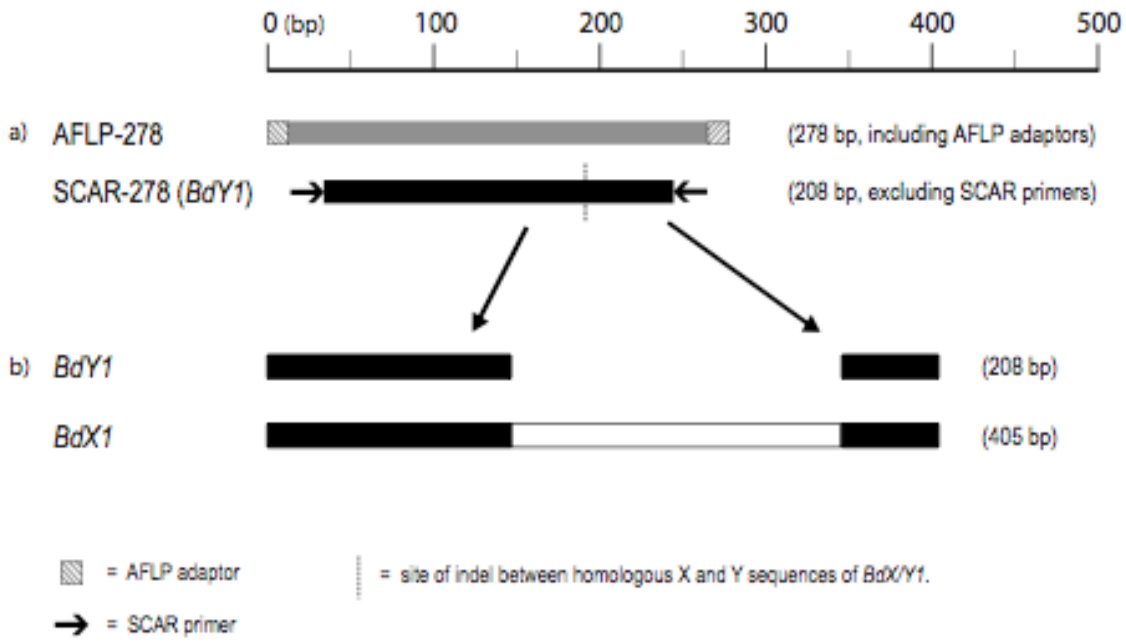

FIGURE 3-1 Schematic representation of the products at different stages of SCAR marker development. 1a) The relationship between the male-linked AFLP marker and the SCAR marker. 1b) The relationship between the male-linked region (BdY1)amplified with the SCAR primers and the homologous sequence assumed to be on the X chromosome (BdX1). The indel event is represented by the white box in the BdX1 sequence.

Phylogenetic Analysis

A Maximum-Likelihood tree of the sequences from *B. dioica* plus those from *B. alba, B. cretica, B. multiflora,* and *B. syrica* is shown as Figure 2. Sequences from two *B. syriaca* females clustered together, while two sequences (presumably alleles) from a single *B. multiflora* female did not. Instead, one sequence placed as sister to the *B. syriaca* clade, the other as sister to the *B. dioica/B. cretica* clade (Fig. 2).

To better illustrate the relationships among the *B. dioica* sequences, a pruned version of Figure 2, including only *B. dioica* sequences and rooted on one of the *B. multiflora* alleles, is shown in Figure 3. The sequences of *B. cretica* were excluded from this analysis because this taxonomic entity is hexaploid and of hybrid origin, with *B. dioica* as one of the contributing genomes (Volz & Renner, in press). The resulting phylogeny shows the Y-sequences from northern Europe forming a strongly supported clade, indicative of their distinctness (Fig. 3). The homologous X-sequences from these males, together with X-sequences from northern European females, also form a clade. Sequences from southern European accessions behaved differently. Three males from Bavaria (SV006, SV054, SV123) yielded two X-sequences and no Y-sequence. Males from Denmark (SV147), Italy (SV136, SV137), Morocco (SV107), and Spain (SV145) yielded only Y-sequences, and thus it is unknown where in the phylogeny their homologous X-sequences would place. In addition, one female from southern Europe yielded an X- and a Y-homologue for the SCAR marker (SV146).

Haplotype Network

The structure of the haplotype network recovered using TCS was sensitive to whether gaps were treated as missing data or as a fifth character (data not shown). With gaps treated as a missing character, two haplotype networks were found (Fig. 4). One consisted of the Y-sequences from Italy and Croatia. The remaining X- and Y-sequences formed a second network, and this included all of the Y-sequences identical to the original AFLP-278 as well as the Y-sequences from Spain and Morocco. The haplotype network differs from the tree in that the Y-sequences have at least two, possibly three, origins (because of the placement of two Y-sequences in Group H in Fig. 4).

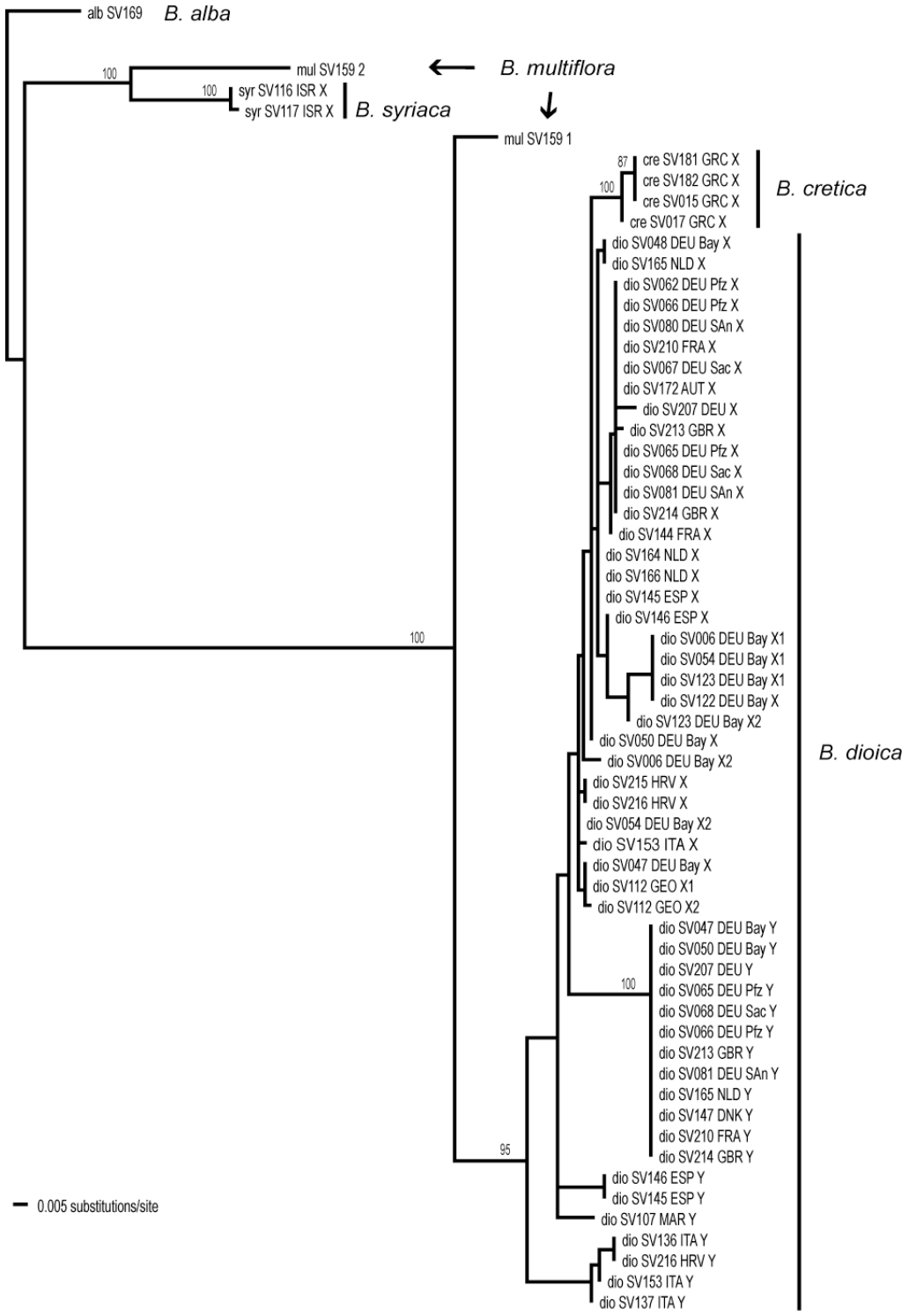

FIGURE 3-2 Neighbor-Joining phylogeny of all sequences. Numbers above branches are Neighbor-Joining bootstrap values. Support values for most clades within the *Bryonia dioica/B. cretica* group are shown in Figure 3. See Appendix 3-1 for full information on accessions. Sequences from dioecious taxa other than *B. dioica* were not assigned X or Y status because the basis for sex determination in these taxa has not been investigated

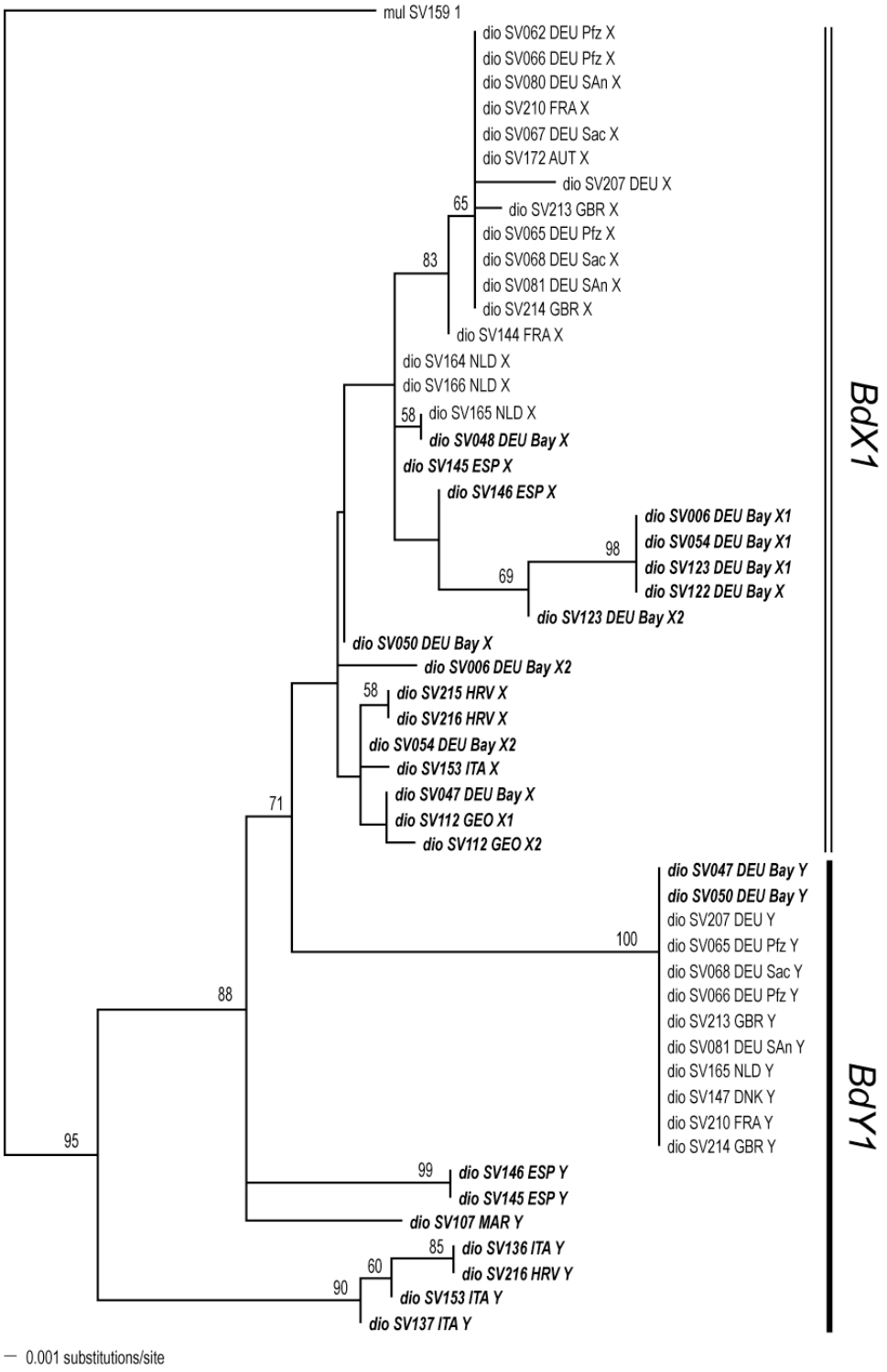

FIGURE 3-3 A Neighbor-Joining phylogeny of just the *Bryonia dioica* BdX/Y1sequences to allow the relationships to be seen more clearly. Numbers above branches are Neighbor-Joining bootstrap values.

Analysis of genetic variation

Among both, the X- and the Y-sequences, the highest pairwise diversity (π) is found in southern Europe accessions. Southern and northern populations behaved inversely for Tajima's D (Table 1). Thus, the X- and Y-sequences in southern Europe had positive values, while the X in northern Europe had negative values (Tajima's D could not be calculated for the Y-sequences in northern Europe because of the absence of any variation). Although our sample lacks significant genetic structure based on FST values (Table 2), the relatively most divergent groups were the northern European X- and Y-sequences, with the Y-sequences from northern Europe being the most divergent overall. The HKA tests revealed a significant departure from neutrality for the X- and Y-sequences in northern Europe, due to the lower than expected number of segregating sites (S) observed (Table 1). Tests of recombination (Hudson & Kaplan, 1985) found evidence for at least one recombination event within the X-homologues from northern and southern Europe and the Y-homologues from southern Europe but detected no recombination events among the northern Y-homologues (see Table 1).

TABLE 3-1 Intra-population variation among the SCAR-278 sequences (*BdX1/Y1*) recovered from *Bryonia dioica*. The abbreviations stand for: n_S, number of sequences; n_H, number of haplotypes; S, variable sites; *D*, Tajima's D; R, minimum number of recombinations. The HKA test was performed using chloroplast sequences as the second marker and, as the outgroup, two haplotypes of *B. syriaca* and two of *B. multiflora*. * = significant p-value.

Population	n_S	n_H	S	π	θ_w	*D*	R	HKA	
								χ^2	p
BdX1-all	32	12	12	0.0119	0.0121	-0.0521	1	5.5024	0.064
X – North	16	6	7	0.0037	0.0058	-1.2687	0	6.5686	0.037*
X – South	17	10	10	0.0138	0.0120	0.5618	1	5.1005	0.078
BdY1-all	19	5	15	0.0380	0.0296	0.7656	2	3.3916	0.183
Y – North	10	1	0	0	0	–	0	10.9690	0.004*
Y – South	9	5	15	0.0502	0.0381	1.1453	2	3.4946	0.174

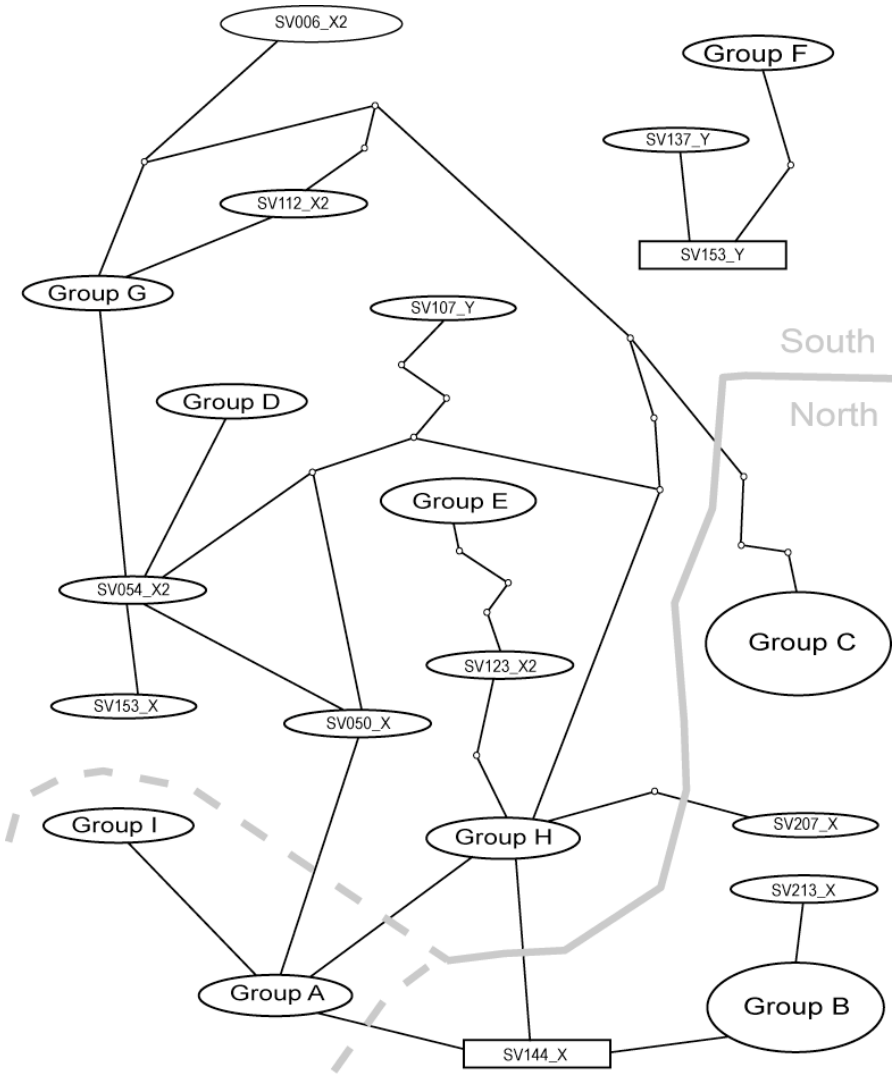

FIGURE 3-4 A haplotype network of all BdX1/Y1 sequences from *Bryonia dioica*.

TABLE 3-2 Values of π (lower left half) and F_{ST} (upper right half) from pairwise comparisons of SCAR-278 sequences (*BdX/Y1*) from each population of *Bryonia dioica*.

		F_{ST}			
		X – North	X – South	Y – North	Y – South
π	X – North		0.5340	0.9067	0.5883
	X – South	0.0116		0.4630	0.0549
	Y – North	0.0295	0.0284		0.5613
	Y – South	0.0321	0.0228	0.0380	

Discussion

Results presented here are the first molecular investigation into the evolution of sex chromosomes in *Bryonia dioica*, a classical system for studying the inheritance of dioecy. Our newly developed sex-linked SCAR marker, which we named BdX1/Y1, is perfectly linked to sex in northern Europe and supports the finding of XY sex-determination in *B. dioica* from northern Europe by Correns (1903; 1907). His experimental crosses of the dioecious *B. dioica* and the monoecious *B. alba* provided the first proof of XY sex-determination in any organism and played an important role in the early understanding of the genetics of sex (Bateson, 1909; Rheinberger, 2000). However, when sequences of BdX1/Y1 from southern Europe are included in the analyses, the picture becomes more complicated because the linkage of our SCAR marker with sex is weaker in southern than in northern Europe. Perceptions of molecular variability on the Y-chromosome of *B. dioica* are thus dependent on geographic sampling. This suggests that the sex chromosomes of *B. dioica* may be at an early stage in their evolution.

The phylogeny obtained from the sex-linked SCAR marker sequenced here is congruent with phylogenies from chloroplast and nuclear loci, to the extent that species sampling overlaps (Volz & Renner, in press). Those phylogenies, which include all species of *Bryonia*, indicate that there were at least two evolutionary transitions between dioecy and monoecy within the genus, which makes it unlikely that all dioecious *Bryonia* species inherited sex-determining chromosomes from a common ancestor (Volz & Renner, in press). This conclusion is supported by the phylogeny presented here in that the sequences of the sex-linked SCAR-278 locus group by species, rather than by their X or Y status (Fig. 2). The

primers for BdX1/Y1 also failed to work in some of the seven dioecious species of *Bryonia*, such as the Canary Island endemic *B. verrucosa*, which is sister species to the nine remaining species of *Bryonia*. These multiple lines of evidence all suggest that, at the very least, this sex-linked marker is not highly conserved across species within the genus.

The pattern of sequences from the eleven males and six females of *Bryonia dioica* sampled across northern Europe fit the expectation of XY sex-determination. No female from northern Europe yielded a Y-sequence and, with two exceptions, all males yielded both an X- and a Y-sequence. The two exceptions were accession SV172 from Austria, from which we only recovered an X-sequence, and SV147 from Denmark, from which we only obtained a Y-sequence. Although we would expect to find, respectively, a Y and an X from these males as well, these results do not contradict the findings of Correns since PCR could fail for a variety of reasons. Most importantly, all of the BdY1 sequences from northern Europe were identical to the original male-linked AFLP marker (AFLP-278) and grouped together in the phylogeny (Fig. 3). Thus, BdY1 and the male-determining locus in northern European *B. dioica* appear to be linked.

In contrast to northern Europe, sequences of BdY1 from southern Europe have weaker linkage disequilibrium to the male-determining locus. In keeping with our expectation for an XY sex-determination system, we observed both an X- and a Y-sequence from four males and only an X from one female in southern Europe. However, from the remaining accessions, the picture is mixed, with five males yielding only a Y-sequence, four males yielding two X-sequences, and one female yielding an X- and a Y-sequence. Thus, only half of the accessions from southern Europe yield SCAR-278 sequences that segregate as expected and five yield sequences that appear to contradict the finding of XY sex-determination by Correns. In addition, phylogenetic analyses place most of the southern X- and Y-sequences in a paraphyletic grade below their respective northern counterparts. Together, these results suggest geographical structure among the sequences of BdX1 and BdY1.

The suggestion of geographical structure based on phylogenetic analysis is supported by population genetic analyses. The southern European population has positive Tajima's D values for both X- and Y-homologues, while the northern European population has negative values, though none are significant. That the highest FST values were found in the pairwise comparisons with BdY1 from northern Europe suggests that this may be the most isolated group, even though the comparisons were not significant. The complete lack of variation

among the BdY1 sequences from northern Europe, meanwhile, suggests either a selective sweep or a bottleneck event for the Y-homologue in the north, while the existence of variation among BdY1 sequences in the south shows that there has not been a species-wide selective sweep. The HKA tests support an interpretation of different evolutionary histories between northern and southern populations for both BdX1 and BdY1, with the significantly lower than expected number of segregating sites among the northern BdX1/Y1 sequences suggesting either background selection or a population bottleneck in the evolutionary past. Phylogeographic analyses of chloroplast and leafy data suggest that *B. dioica* recolonized northern Europe from the south after the last glacial maximum (Volz & Renner, in press), a demographic narrative congruent with our findings.

Geographic structure and the absence of variation among BdY1 sequences from northern Europe (from a geographic sample that spans Scotland, France, Germany, Netherlands, and Denmark) are both consistent with findings in other organisms. Thus, studies across European Silene latifolia (Filatov, et al., 2000; Laporte, et al., 2005) and European domestic horses (Lindgren, et al., 2004) found no variation among the Y-linked sequences. Indeed, in ten species of animals for which data are available, levels of variation on the Y-chromosome are undetectable or substantially lower than those on either the X or the autosomes (Lynch, 2007, Table 12.2). Background selection and a local, rather than species-wide, selective sweep have also been observed in Y-linked markers of Silene (Ironside & Filatov, 2005). However, the high diversity within southern European BdX1 and BdY1 differs from the situation in Silene, where most of the polymorphic sites were fixed within geographical areas (Ironside & Filatov, 2005). In addition, the population structure of X- and Y-linked genes within the five species of Silene that have sex chromosomes may be influenced by ongoing gene flow among these species (Ironside & Filatov, 2005; Muir & Filatov, 2007; Prentice, et al., 2008). In contrast, ongoing hybridization is unlikely to be an issue in *Bryonia dioica*. Although ancient hybridization with *B. cretica* in mainland Greece is a possibility, these species do not co-occur today nor do the two other dioecious species included here (see Materials and Methods for the geographic range of each species).

The different levels of sequence variation in northern and southern Europe may be due to different levels of linkage disequilibrium between BdY1 and the male-determining locus (i.e., maleness) in the two populations. If we assume that BdY1 is perfectly linked to the male-determining region of the Y-chromosome in the north and only weakly linked in the south, then this leads to predictions that are met by our data. Thus, one would expect to find

recombination in those areas/populations where they are weakly linked and no recombination in the perfectly linked populations. And indeed, we detected recombination events in southern BdX1 and BdY1, but none in the north (Table 1). In the recombining populations, the distinction between the X- and Y-homologues of our marker should be blurred, resulting in males with two X-sequences and females with a Y-sequence. In the southern populations, we indeed found four males with two X-homologues and no Y (SV006, SV054, SV112, and SV123), and a female with an X- and Y-sequence (SV146). Where differently behaving populations come into contact, one would expect to find individuals with a mix of southern and northern X- and Y-sequences, which we did (SV047, SV050).

The evidence for recombination within BdX1 and BdY1 in southern Europe implies crossing over between X- and Y-chromosomes. According to theory for the evolution of sex-determining chromosomes, suppression of recombination should spread gradually along the Y-chromosome as male-linked genes accumulate there, with morphological differentiation of the X and Y being the last step (Charlesworth B., 2002; Charlesworth D., et al., 2005). Since the sex chromosomes of *Bryonia dioica* are not morphologically distinguishable, it is not surprising that recombination may still occur between the X- and Y-chromosomes. Thus, one explanation for the different levels of linkage disequilibrium of our marker and sex between northern and southern Europe could be that the spread of recombination suppression on the Y-chromosome in these two regions has proceeded to different degrees. That is, in northern Europe the non-recombining region encompasses BdY1, linking it to the male-determining locus, while in southern Europe BdY1 is in a pseudo-autosomal region outside the zone of recombination suppression.

In this study, we bring modern molecular tools to bear on the sex chromosomes of *Bryonia dioica*, the classical plant system for investigating the inheritance of dioecy and the first organism in which XY sex-determination was experimentally proven. Although the limited nucleotide sampling requires cautious interpretation, geographic sampling of *B. dioica* is representative, and the results can be interpreted with the help of an existing phylogenetic framework that includes all species in the genus. The main patterns found, especially the extremely low level of polymorphism of the Y-linked marker compared to the X-linked sequences in northern Europe, are consistent with the original interpretation of Correns that *Bryonia dioica* has XY sex-determination. The data from southern Europe, meanwhile, point to an as yet incompletely understood complexity of sex chromosome

evolution in *Bryonia*.

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Supplementary Material

APPENDIX 3-1 List of sequences used in this study and their accession information. Abbreviations of species names used in the figures: alb = *B. alba*, cre = *B. cretica*, dio = *B. dioica*, mul = *B. multiflora*, syr = *B. syriaca*. Country abbreviations follow the ISO 3166 (3-*alpha*) system.

Genus	species	Sex	Number	Locality	Locality (full)	Voucher	Allele	GenBank
Bryonia	*alba*	n.a.	SV169	WA	Washington - USA	J. Brokaw 139 (MSB)	-	EU514717
Bryonia	*cretica*	male	SV015	GRC	Greece, Kythera	A. Kocyan 121a (M)	-	EU514724
		male	SV017	GRC	Greece, Kythera	A. Kocyan 122a (M)	-	EU514725
		female	SV181	GRC	Greece, Kea	A. Kocyan 239 (M)	-	EU514722
		female	SV182	GRC	Greece, Kea	A. Kocyan 237 (M)	-	EU514723
Bryonia	*dioica*	male	SV006	DEU-Bay	Germany, Bavaria, Regensburg	H. Förther 5811 (M)	X1	EU514733
							X2	EU514734
		male	SV047	DEU-Bay	Germany, Bavaria, Munich	S. Volz 1c (M)	X	EU514735
							Y	EU514736
		female	SV048	DEU-Bay	Germany, Bavaria, Munich	S. Volz 1d (M)	X	*submitted*
		male	SV050	DEU-Bay	Germany, Bavaria, Munich	S. Volz 1f (M)	X	EU514737
							Y	EU514738
		male	SV054	DEU-Bay	Germany, Bavaria, Augsburg	S. Volz 2a (M)	X1	EU514739
							X2	EU514740
		female	SV062	DEU-Pfz	Germany, Rheinland-Pfalz, Germersheim	S. Volz 9b (M)	X	EU514726
		male	SV065	DEU-Pfz	Germany, Rheinland-Pfalz, Landau	S. Volz 9e (M)	X	EU514741
							Y	EU514742
		male	SV066	DEU-Pfz	Germany, Rheinland-Pfalz	S. Volz 9f (M)	X	EU514743
							Y	EU514744
		female	SV067	DEU-Sax	Germany, Saxony, Leipzig	S. Volz 3a (M)	X	EU514727
		male	SV068	DEU-Sax	Germany, Saxony, Leipzig	S. Volz 3b (M)	X	EU514745
							Y	EU514746
		female	SV080	DEU-SAn	Germany, Saxony-Anhalt, Quedlinburg	S. Volz 10a (M)	X	EU514728
		male	SV081	DEU-SAn	Germany, Saxony-Anhalt, Quedlinburg	S. Volz 10b (M)	X	EU514747
							Y	EU514748
		male	SV107	MAR	Morocco, De Beni-Mellal	D. Podlech 47692 (MSB)	Y	EU514749
		male	SV112	GEO	Georgia, Tiblisi	M. Merello et al. 2220 (MO)	X1	EU797120
							X2	EU797121
		male	SV122	DEU-Bay	Germany, Bavaria, Regensburg	F. Schuhwerk 05/222b (M)	X	*submitted*
		male	SV123	DEU-Bay	Germany, Bavaria	F. Schuhwerk 05/224 (M)	X1	EU514750
							X2	EU514751
		male	SV136	ITA	Italy, Padua	W. Lippert 04 May 1966 (M)	Y	EU797122
		male	SV137	ITA	Italy, Fontane	T. Schauer 01 June 1968 (M)	Y	EU797123
		female	SV144	FRA	France, Merval	B. de Retz 93102 (MSB)	X	EU514729
		male	SV145	ESP	Spain, Albacete	A. Garcia-Villaraco 08.1982 (M)	X	*submitted*
							Y	EU514752

Genus	species	Sex	Number	Code	Locality	Voucher	Allele	GenBank
Bryonia	*dioica* cont'd	female	SV146	ESP	Spain, Soria	Granzow & Zaballos 384 (M)	X	*submitted*
							Y	*submitted*
		male	SV147	DNK	Denmark, Fanö	L. Holm-Nielsen 374 (M)	Y	EU514753
		male	SV153	ITA	Italy, Torino	R. Camoletto 2089 (M)	X1	EU514754
							X2	EU514755
		female	SV164	NLD	Netherlands, Berheide	B. Gravendeel 2609 (M)	X	EU514730
		male	SV165	NLD	Netherlands, Berheide	B. Gravendeel 2610 (M)	X	EU514756
							Y	EU514757
		female	SV166	NLD	Netherlands, Beverwyk	W. de Wilde 22297 (MSB)	X	EU514731
		male	SV172	AUT	Austria, Vienna	M. Kropf 15 Sep. 2005b (M)	X	EU514758
		male	SV207	DEU	Germany, Helgoland	S. Volz 23 (MSB)	X	EU514759
							Y	EU514760
		male	SV210	FRA	France, Aveyron, St. Afrique	G. Gerlach, no voucher	X	EU514761
							Y	EU514762
		male	SV213	GBR-Sct	Great Britain, Scotland, Edinburgh	R. Milne 2006-BE (E)	X	EU514763
							Y	EU514764
		male	SV214	GBR-Eng	Great Britain, England, Oxford	M. Dorken, no voucher	X	EU514765
							Y	EU514766
		female	SV215	HRV	Croatia, Vrbnik	M. Kropf, no voucher	X	EU514732
		male	SV216	HRV	Croatia, Vrbnik	M. Kropf, no voucher	X	EU514767
							Y	EU514768
Bryonia	*multiflora*	female	SV159	IRN	Iran, Bushehr	S. Zarre 35811 (MSB)	a	EU514720
							b	EU514721
Bryonia	*syriaca*	female	SV116	ISR	Israel, Horshat Tal National Park	A. Dafni 1 (M)	-	EU514718
		female	SV117	ISR	Israel, Horshat Tal National Park	A. Dafni 2 (M)	-	EU514719

APPENDIX 3-2 Accession information for chloroplast sequences used in HKA analyses.

Genus	species	Number	Locality	Voucher	trnL-F	atpA-trnH	psbA-trnH
Bryonia	*alba*	SV169	Washington – USA	J. Brokaw 139 (MSB)	EU202303	EU102437	EU096332
Bryonia	*dioica*	SV006	Germany, Bavaria, Regensburg	H. Förther 5811 (M)	EU102338	EU102473	EU096368
		SV047	Germany, Bavaria, Munich	S. Volz 1c (M)	*submitted*	*submitted*	*submitted*
		SV048	Germany, Bavaria, Munich	S. Volz 1d (M)	*submitted*	*submitted*	*submitted*
		SV050	Germany, Bavaria, Munich	S. Volz 1f (M)	*submitted*	*submitted*	*submitted*
		SV054	Germany, Bavaria, Augsburg	S. Volz 2a (M)	*submitted*	*submitted*	*submitted*
		SV062	Germany, Rheinland-Pfalz, Germersheim	S. Volz 9b (M)	*submitted*	*submitted*	*submitted*
		SV065	Germany, Rheinland-Pfalz, Landau	S. Volz 9e (M)	*submitted*	*submitted*	*submitted*
		SV066	Germany, Rheinland-Pfalz	S. Volz 9f (M)	*submitted*	*submitted*	*submitted*
		SV067	Germany, Saxony, Leipzig	S. Volz 3a (M)	*submitted*	*submitted*	*submitted*
		SV068	Germany, Saxony, Leipzig	S. Volz 3b (M)	*submitted*	*submitted*	*submitted*
		SV080	Germany, Saxony-Anhalt, Quedlinburg	S. Volz 10a (M)	*submitted*	*submitted*	*submitted*
		SV081	Germany, Saxony-Anhalt, Quedlinburg	S. Volz 10b (M)	*submitted*	*submitted*	*submitted*
		SV107	Morocco, De Beni-Mellal	D. Podlech 47692 (MSB)	EU102374	EU102509	EU096404
		SV112	Georgia, Tiblisi	M. Merello et al. 2220 (MO)	EU102341	EU102476	EU096371
		SV122	Germany, Bavaria, Regensburg	F. Schuhwerk 05/222b (M)	EU102344	EU102479	EU096374
		SV123	Germany, Bavaria	F. Schuhwerk 05/224 (M)	EU102328	EU102463	EU096358
		SV136	Italy, Padua	W. Lippert 04 May 1966 (M)	EU102345	EU102480	EU096375
		SV137	Italy, Fontane	T. Schauer 01 June 1968 (M)	EU102346	EU102481	EU096376
		SV144	France, Merval	B. de Retz 93102 (MSB)	EU102347	EU102482	EU096377
		SV145	Spain, Albacete	A. Garcia-Villaraco 08.1982 (M)	EU102367	EU102502	EU096397
		SV146	Spain, Soria	Granzow & Zaballos 384 (M)	EU102368	EU102503	EU096398
		SV147	Denmark, Fanö	L. Holm-Nielsen 374 (M)	EU102330	EU102465	EU096360
		SV153	Italy, Torino	R. Camoletto 2089 (M)	EU102350	EU102485	EU096380
		SV164	Netherlands, Berheide	B. Gravendeel 2609 (M)	EU102334	EU102469	EU096364
		SV165	Netherlands, Berheide	B. Gravendeel 2610 (M)	EU102354	EU102489	EU096384
		SV166	Netherlands, Beverwyk	W. de Wilde 22297 (MSB)	EU102335	EU102470	EU096365
		SV172	Austria, Vienna	M. Kropf 15 Sep. 2005b (M)	EU102356	EU102491	EU096386
		SV207	Germany, Helgoland	S. Volz 23 (MSB)	EU102360	EU102495	EU096390
		SV210	France, Aveyron, St. Afrique	G. Gerlach, no voucher	EU102369	EU102504	EU096399
		SV213	Great Britain, Scotland, Edinburgh	R. Milne 2006-BE (E)	EU102363	EU102498	EU096393
		SV214	Great Britain, England, Oxford	M. Dorken, no voucher	EU102364	EU102499	EU096394
		SV215	Croatia, Vrbnik	M. Kropf, no voucher	EU102365	EU102500	EU096395
		SV216	Croatia, Vrbnik	M. Kropf, no voucher	EU102366	EU102501	EU096396
Bryonia	*multiflora*	SV159	Iran, Bushehr	S. Zarre 35811 (MSB)	EU102319	EU102453	EU096348
Bryonia	*syriaca*	SV116	Israel, Horshat Tal National Park	A. Dafni (M)	EU102324	EU102458	EU096353
		SV117	Israel, Horshat Tal National Park	A. Dafni (M)	EU102325	EU102459	EU096354

Curriculum vitae

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Education and Positions

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2006 – 2007	DFG-funded project Ph.D. research on "The evolution of combined versus separate sexes in *Bryonia* and *Ecballium* (Cucurbitaceae), using phylogenetic and phylogeographic approaches" (PI S. S. Renner)
June 2004	Visiting researcher Department of Taxonomy, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany (hosts: Prof. K. Bachmann and Dr. F. Blattner)
2004 – 2007	Postgraduate research, University of Munich: Systematic Botany (Prof. S. S. Renner)
2003	University degree (diploma) in biology, University of Munich, Germany
2002 – 2003	Diploma thesis: "Genomuntersuchungen bei höheren Pflanzen: *Oenothera*, *Arabidopsis* und Tabak“ University of Munich: Institut für Allgemeine und Molekulare Botanik (Prof. R. G. Herrmann)
1998 – 2002	Studies of systematic and physiological botany, ecology and biochemistry, University of Munich
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Publications

UMATE, P., SCHWENKERT, S., KARBAT, I., DAL BOSCO, C., MLCOCHOVA, L., VOLZ, S., ZER, H., HERRMANN, R. G., OHAD, I., and J. MEURER. 2007. Deletion of PsbM in tobacco alters the QB site properties and the electron flow within photosystem II. Journal of Biological Chemistry 282(13): 9758-67

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between monoecy and dioecy in *Bryonia* (Cucurbitaceae). (Submitted to American Journal of Botany)

VOLZ, S. M. and S. S. RENNER. Phylogeography of the ancient Eurasian medicinal plant genus *Bryonia* (Cucurbitaceae) inferred from nuclear and chloroplast sequences. (Submitted to Taxon)

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OYAMA, R. K., VOLZ, S. M. and S. S. RENNER. 2006. Relating clade diversification and life-history evolution: sex-linked loci in *Bryonia* (Cucurbitaceae). University of Munich, DFG-SFB site visit (poster)

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VOLZ, S., KOCYAN, A., and S. S. 2004. Assessment of the value of ITS and trnL for a study of sexual system evolution in *Bryonia* and *Ecballium* (Cucurbitaceae). Botany 2004 meeting: http://www.2004.botanyconference.org (poster)

Teaching experience

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Field experience

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