Hybridization, polyploidy, and evolutionary transitions between monoecy and dioecy in *Bryonia* (Cucurbitaceae)[1]

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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.

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 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, in press). 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. However, distinguishing dioecy from monoecy based on herbarium specimens alone is problematic because incomplete collections of these climbing species may easily suggest unisexuality.

Because experimental interspecific crosses result in fertile progeny (as shown by the Mendelian studies described above), 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, C. Heibl, and S. S. Renner, 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′ AAATTTC-TATATCATGTCAAGAGG 3′) and *ccSSr4* R2 (5′ GAACGTTTT CTACT-TCAAGACC 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′ TCTTCCACCTSTATGARCAGT-GTCGTGAAT 3′ and *LFY* CucR 5′ CGAAATCACAAA AGYTATTGSG-YAKTYCA 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$_2$O 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 100000 trees, with a burn-in of 10000 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 crown group of Cucurbitaceae, which is about 60 Myr old, based on *Trichosanthes*-like 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 3 : 1 ethanol : acetic 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
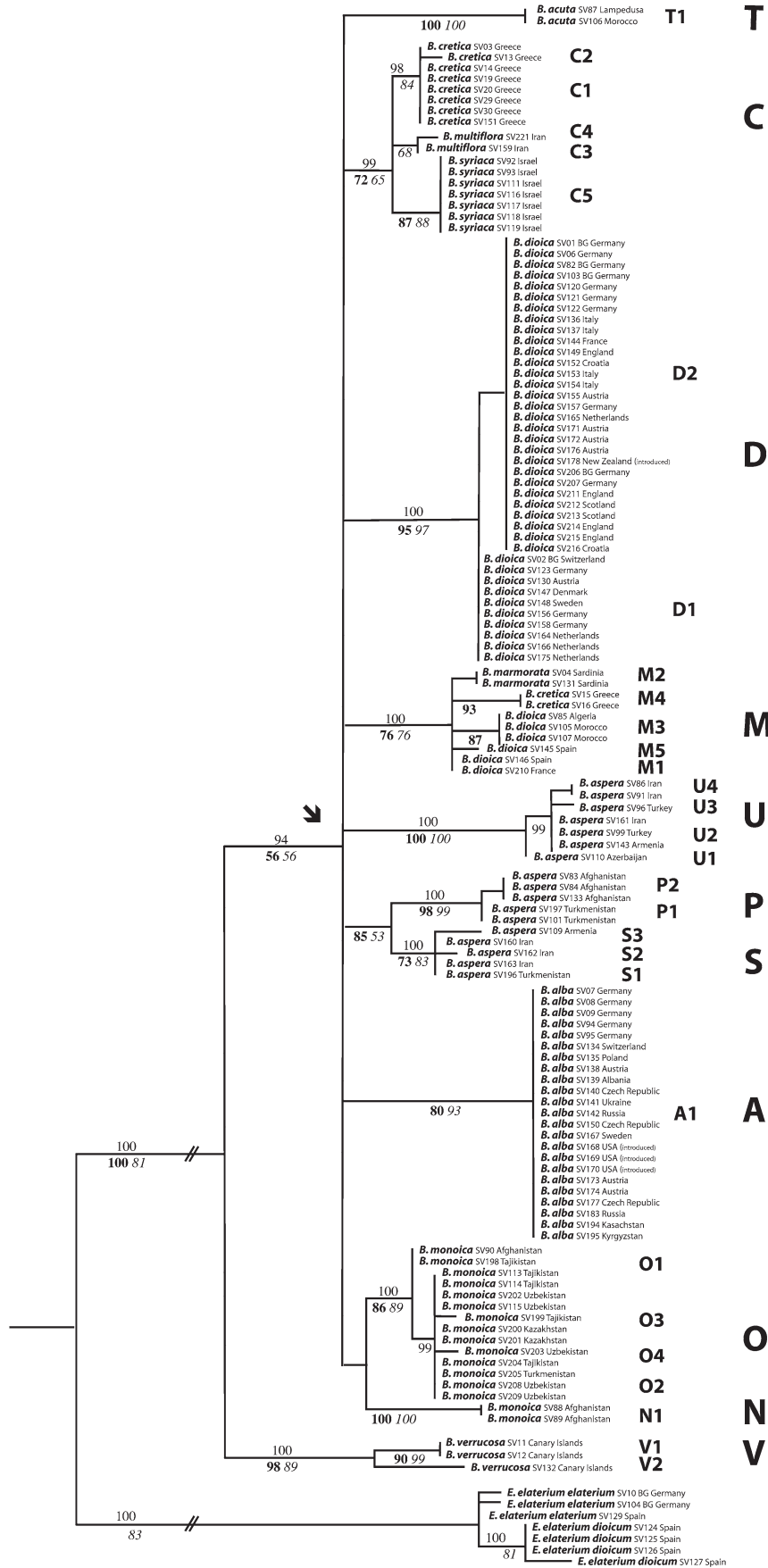
GenBank accession numbers are listed in Appendix S1 (see Supplemental Data with online version of this article). Alignments and trees have been submitted to TreeBASE (http://www.treebase.org, submission name Stefanie M. Volz, PIN 25754). Because these chloroplast loci represent a single linkage group, 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, see online Supplemental Data). 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.

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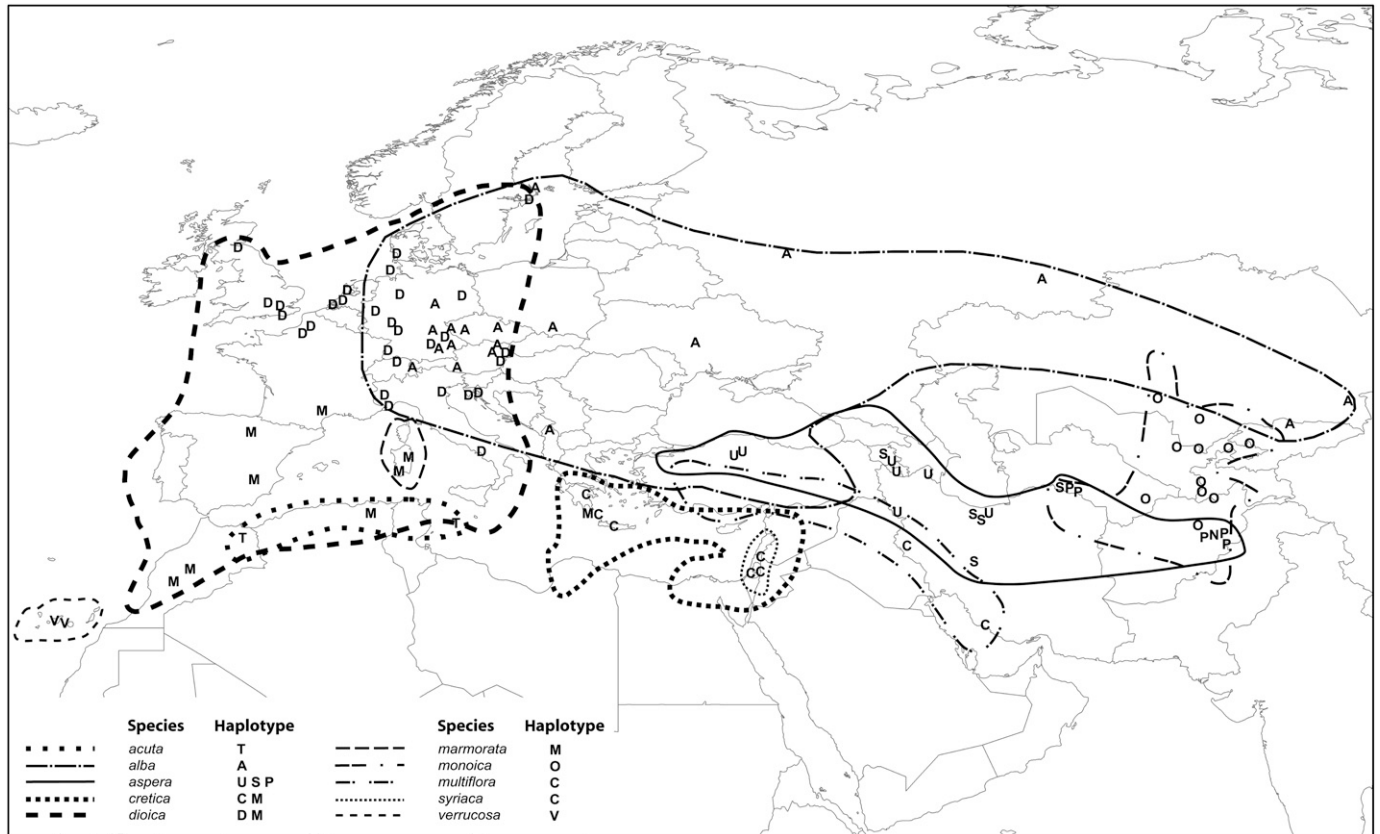
Fig. 2.   Geographic distribution of the 10 major chloroplast haplotypes found in *Bryonia*. The labeling of haplotypes corresponds to that in Figs. 1 and 3. Species ranges modified from Jeffrey (1969).

A ML tree from the nuclear ITS sequences (Appendix S2, see online Supplemental Data; 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. 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 (see online Supplemental Data) 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).
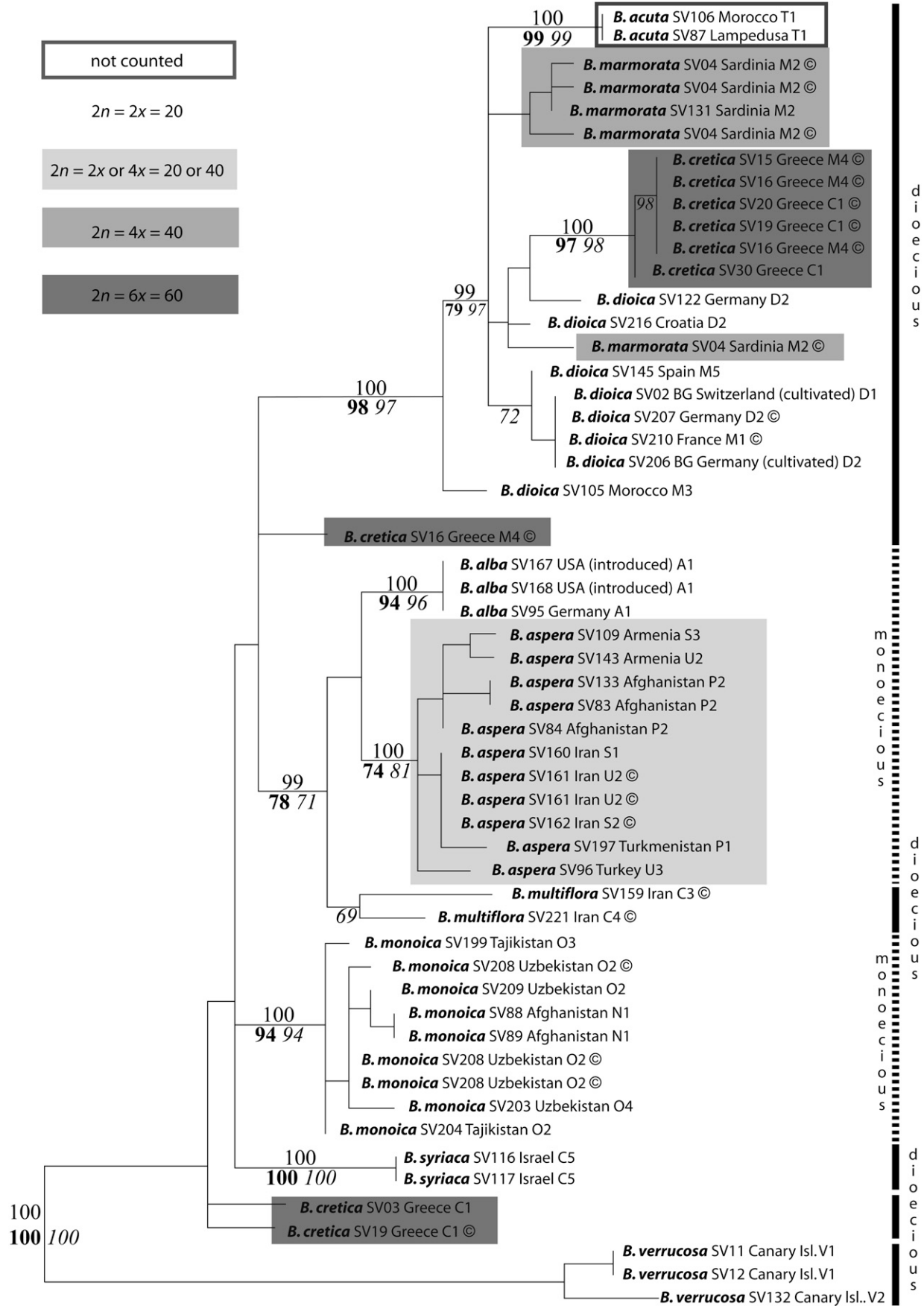
Chromosome numbers and sexual system—Table 2 lists chromosome counts for *Bryonia* and *Ecballium*. Most *Bryonia* species have $2n = 2x = 20$, but *B. cretica* is hexaploid ($2n = 6x = 60$; two individuals counted), *B. marmorata* tetraploid ($2n = 4x = 40$; one individual counted), and *B. aspera* 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

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Fig. 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 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.

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).

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, see online Supplemental Data). 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

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Fig. 3.   Maximum likelihood tree from 54 nuclear *LFY* sequences of *Bryonia*, rooted on *B. verrucosa* (see Material 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).

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

Species	Alleles	Sequences	Individuals	Interallelic HKY distances		
				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

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, see online Supplemental Data), 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 to 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

TABLE 2. Sexual system and chromosome number (meiotic/mitotic) in *Ecballium* and *Bryonia*. For complete voucher localities, see online supplemental Appendix S1. 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., monoecious	Poland	–/20	Pogan et al., 1990
	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., monoecious	Iran	20/–	Aryavand, 1980
	Iran	–/40	This study. (Voucher Zarre 35812 (MSB), DNA SV 161)
	Turkey	10/–	Bilge, 1955 (syn. *B. macrostylis*)
B. cretica L., dioecious	Greece	30/60	Montmollin, 1986
	Greece	–/60	This study. Voucher Kocyan 115 (M), DNA SV 42
B. dioica Jacq., dioecious	Austria	–/20	Hasitschka-Jenschke, 1961
	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, dioecious	Cyprus	–/18	Slavick et al., 1993
	Spain	9/18, 24	Castroviejo, 1993
	Botanical Garden	12/–	Whitaker, 1933
E. elaterium subsp. *elaterium* (L.) A. Rich., monoecious	Spain	9/18	Castroviejo, 1993

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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