

BACK-AND-FORTH HERMAPHRODITISM: PHYLOGENETIC CONTEXT OF REPRODUCTIVE SYSTEM EVOLUTION IN SUBDIOECIOUS *DAPHNE LAUREOLA*

Conchita Alonso^{1,2} and Carlos M. Herrera¹

¹Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC). Apdo 1056, 41080, Sevilla, Spain

²E-mail: conalo@ebd.csic.es

Received October 29, 2009

Accepted January 19, 2011

Recent phylogenetic analyses of sexual reproductive systems supported the evolutionary pathway from hermaphroditism to dioecy via gynodioecy in different groups of angiosperms. In this study, we explore the evolution of sexual reproductive systems in *Daphne laureola* L. (Thymelaeaceae), a species with variation in reproductive system among population. Sequences from the ITS region of the nuclear ribosomal cistron and two plastid markers (*psbA-trnH* and *ndhF*) were analyzed and used to map the population reproductive system along the molecular phylogeny. Our results support *D. laureola* as a monophyletic lineage with three different clades within the Iberian Peninsula. The hermaphroditic populations belong to two different clades, whereas gynodioecy is ubiquitous but characteristic of the third clade, which grouped together all the North-Western Iberian populations sampled, including the apparently oldest haplotype sampled. Gynodioecy appears as the most likely basal condition of the 13 analyzed populations, but different evolutionary transitions in reproductive sexual system were traced within each *D. laureola* clade. Both ecological conditions and (meta)population dynamics may help explain plant reproductive system evolution at the microevolutionary scale. Phylogenetic studies in which the historical relationships between populations differing in reproductive system can be ascertained will help to clarify the process.

KEY WORDS: Geographic variation, gynodioecy, Iberian Peninsula, ITS, microevolution, molecular markers, phylogeography.

Hermaphroditism is considered the ancestral condition from which all other sexual reproductive systems have evolved in the angiosperms (see Barrett 2002 for a review). Gynodioecy, where hermaphrodite and female individuals coexist within populations, has been traditionally viewed as one intermediate evolutionary stage between the two most common reproductive systems, hermaphroditism and dioecy. The evolutionary pathway from hermaphroditism to dioecy via gynodioecy has been satisfactorily modeled theoretically, and requires the spreading of male-sterile mutants into cosexual populations followed by selection against female function in the remaining hermaphrodites (Schultz 1994; Charlesworth 1999). At the first stage, to spread

and persist within populations, the male-sterile mutants must have some consistent fecundity advantage that compensates for their gametic disadvantage. In theory, the female frequency within single populations of infinite size would be regulated by the relative seed production of each sexual morph, and the degree of selfing and inbreeding depression (Charlesworth 1999). Metapopulation models have further indicated that whenever a population is subdivided into local groups, individual fitness will be also a function of local morph frequency and pollen limitation of seed production, which would determine if evolutionary processes allow the maintenance of polymorphism (McCauley and Taylor 1997). The stability of gynodioecy can be also favored by nucleo-cytoplasmic

sex determination (Bailey et al. 2003; Bailey and Delph 2007; Dufay et al. 2007; but see Schultz 1994).

In the last two decades important efforts have been made to verify these models, explain some seeming exceptions to the rule, and understand the ecological conditions favoring such microevolutionary transitions in sexual reproductive system (Delph and Carroll 2001; Medrano et al. 2005; Ashman 2006; McCauley and Bailey 2009). In addition, some phylogenetic analyses of reproductive system evolution based on molecular markers (see Weller and Sakai 1999 for a review; Weiblen et al. 2000; Vamوسي et al. 2003; Navajas-Pérez et al. 2005) have also supported the theoretical pathway for a few plant groups in a macroevolutionary context, and reversals from dimorphism to hermaphroditism have been also inferred at the interspecific level (e.g., Weller et al. 1995; Desfeux et al. 1996; Weiblen et al. 2000).

Links between the micro and macroevolutionary approaches are being provided by studies of geographical variation in species that exhibit among-population variation in reproductive system (hereafter referred to as subdioecious). These investigations have shown that environmental stress, founder effects, and interactions with pollinators, are commonly correlated with the frequency of females in subdioecious species (e.g., Case and Barrett 2004; Nilsson and Ågren 2006; Ramsey et al. 2006; Alonso et al. 2007; Caruso and Case 2007). Finally, intraspecific phylogeographic studies in which the historical relationships between populations differing in reproductive system can be ascertained using molecular markers may also provide crucial information to identify the most common transitions in reproductive system evolution at the intraspecific level. By mapping selected characters on a phylogeny, such studies can suggest whether other morphological, functional, and ecological characters have played a role in this microevolutionary process. However, such studies are scarce (see e.g., Fénart et al. 2006), most likely because of the difficulties in finding well-supported intraspecific phylogenetic trees and intraspecific variation in reproductive system simultaneously for the same species.

Our study uses a phylogenetic approach to provide a historical frame for the evolutionary transitions between hermaphroditism and gynodioecy at an intraspecific level. *Daphne laureola* L. (Thymelaeaceae) is a shrub widely distributed in the understory of European forests. Monomorphic (i.e., hermaphroditic) and gynodioecious populations are patchily distributed over the Iberian Peninsula (Alonso et al. 2007). Gynodioecious populations prevail in the northwest and southeast of this large geographic area and, most likely, also at other disjunct areas of the species range (gynodioecious populations occur also in Slovenia and Morocco; C. Alonso, unpubl. data). In addition, there are at least two disjoint, distant areas in the northeast and southwest of the Iberian Peninsula in which pure hermaphroditic populations exist. We attempt to understand such a geographic

pattern using molecular phylogenetic tools. Combining different marker systems is usually advantageous to unravel intraspecific evolutionary histories; thus we analyze the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron, and two plastid markers (*psbA-trnH* and *ndhF*). Nuclear and plastid markers show contrasting modes of inheritance. It is thus particularly interesting to combine both to track plant reproductive system evolution because, as mentioned above, gynodioecy is frequently determined by the interaction between maternally inherited cytoplasmic male sterility (CMS) factors and biparentally inherited nuclear genes that restore male fertility (Kaul 1988; but see McCauley and Olson 2008).

The following questions are addressed in this article. What are the phylogenetic relationships between *D. laureola* populations from different Iberian regions and how does the sexual reproductive system change along the molecular phylogeny? Are *D. laureola* populations from the hermaphroditic disjunct areas genetically more related to each other rather than to gynodioecious populations? If so, is hermaphroditism the ancestral trait among *D. laureola* populations? Or alternatively, in which direction and how many times has the transition between gynodioecy and hermaphroditism occurred?

Materials and Methods

PLANT MATERIAL AND STUDY SITES

Daphne laureola L. (Thymelaeaceae) is an early-season flowering, insect-pollinated and bird-dispersed, evergreen shrub widely distributed in Europe (Brickell and Mathew 1976). Typically it grows in the undergrowth of shady mountain forests. In the Iberian Peninsula, the species is frequent in the northern Cantabrian and Pyrenean Mountains, characterized by Atlantic climate and deciduous and mixed forest, and also in the southern Mediterranean Betic Ranges, where it inhabits evergreen sclerophyllous and subsclerophyllous woodlands (Alonso et al. 2007). At this large geographic scale, *D. laureola* exhibits among-population variation in reproductive system. Gynodioecy is the most common sexual system and prevails in the northwestern and southeastern Iberian populations but there are at least two disjunct, distant areas in the northeast and southwest of the Iberian Peninsula in which purely hermaphroditic populations exist (Alonso et al. 2007).

We studied the phylogenetic relationship between individuals from four hermaphroditic and seven gynodioecious Iberian populations of *D. laureola* (see Fig. 1 for locations). Those populations were a subsample of a broader survey conducted to determine the geographic variation in the proportion of female and hermaphrodite plants per population (Alonso et al. 2007). All of them have more than 100 individuals. Gynodioecious populations were located in NW, NE, and S Iberian regions and their frequency of females (11%–54%; Table 1) encompassed the range

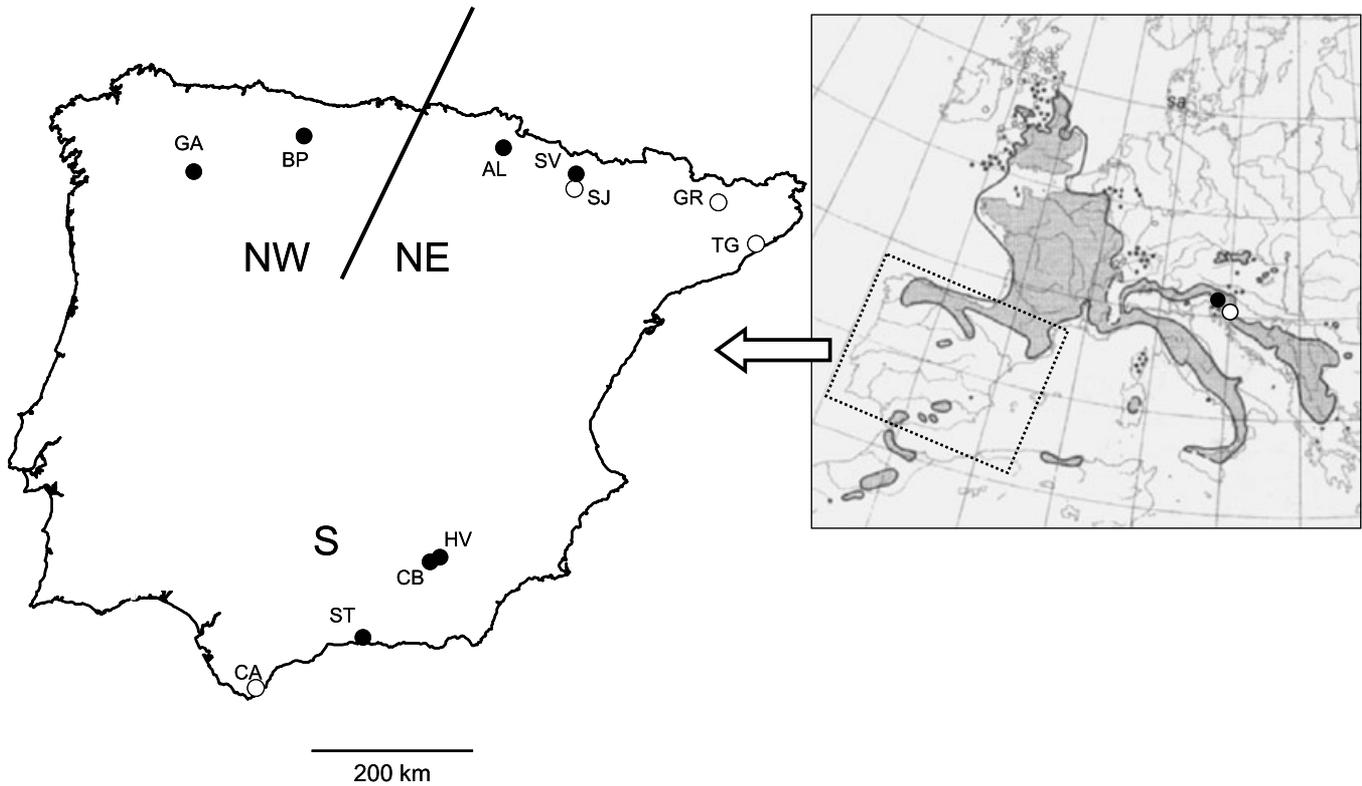


Figure 1. *Daphne laureola* L. distribution map according to Meusel et al. (1978). Locations of the two Slovenian populations are marked in the distribution map and the 11 Iberian populations sampled are shown in more detail, with the three a priori geographical regions we distinguished indicated. Black and white dots denote gynodioecious and hermaphroditic populations, respectively, population codes as in Table 1.

of variation recorded at the broad geographic scale (see Alonso et al. 2007 for details). Three hermaphroditic populations were selected from the NE region where they are frequent, and one from the S region where they are not so common. In addition, we included two European populations outside the Iberian Peninsula, one hermaphroditic and one gynodioecious, to better char-

acterizing the phylogeographic relationships by broadening the geographic distances (Fig. 1). The two populations were located in two different Slovenian mountain ranges, distant about 70 km from each other, and the hermaphroditic population was smaller (22 individuals) than the gynodioecious population (>100 individuals).

Table 1. Location, reproductive system, female percentage, ribotypes (ITS) and haplotypes (*psbA-trnH* and *ndhF* sequences combined) of the *Daphne laureola* populations studied. Regions and population codes as in Figure 1.

Region	Population (code)	Latitude N/Longitude W	Reproductive system	% females	Ribotype	Haplotype
S Iberia:	Huerta Vieja (HV)	38.12/−2.81	Gynodioecy	35.5	A	3/4
	Cuevas Bermejas (CB)	37.96/−2.85	Gynodioecy	32.2	A	3
	La Maroma (ST)	36.91/−4.02	Gynodioecy	10.9	C	3
	Hoyos de D. Pedro (CA)	36.10/−5.51	Hermaphroditism	0	C	5
NW Iberia:	Las Cruces (GA)	42.59/−7.04	Gynodioecy	39.4	D	1
	Bosque Peloño (BP)	43.17/−5.13	Gynodioecy	36.0	D	1
NE Iberia:	Aldatz (AL)	43.02/−1.86	Gynodioecy	54.0	D	1
	Selva Villanúa (SV)	42.68/−0.50	Gynodioecy	54.0	D	1/2
	S. Juan de la Peña (SJ)	42.51/−0.67	Hermaphroditism	0	A	6
	Gresolet (GR)	42.26/1.72	Hermaphroditism	1.0	A	6
	Tagamanent (TG)	41.75/2.30	Hermaphroditism	0	A	6
Slovenia:	ca. Vrhnika (SL1)	45.96/14.19	Hermaphroditism	0	B	6
	ca. Kocevje (SL3)	45.67/14.99	Gynodioecy	15.0	A/B	6

Each population was represented in the phylogenetic analyses by two individuals of each sex ($N = 42$ individuals) to allow detecting possible polymorphisms linked to sex (e.g., McCauley and Olson 2003). The small sample size per population was chosen on the assumption of reduced levels of polymorphism in DNA sequences at the within-population level, as frequently found for plastid markers (e.g., Cornman and Arnold 2007).

Finally, leaf samples from two to six individuals of other six European *Daphne* species (*D. blagayana*, *D. cneorum*, *D. gnidium*, *D. mezereum*, *D. oleoides*, and *D. rodriguezii*) were also collected and similarly analyzed to allow the rooting of the phylogenetic trees because no molecular phylogeny of *Daphne* is currently available.

DNA ISOLATION AND MOLECULAR ANALYSIS

Total genomic DNA was isolated from freshly frozen bud tissue maintained at -80°C , using a DNeasy 96 Plant Kit (QIAGEN, Inc., Valencia, CA). We selected one nuclear and two cpDNA regions for sequencing: the ITS region of the 18S-5.8S-26S nuclear ribosomal cistron and the *psbA-trnH* intergenic spacer and the *ndhF* gene, respectively.

PCR-amplification was performed by using PCR-Beads kits (PuReTaq Ready-To-GoTM, Amersham Biosciences, UK) with approx. 25 ng of DNA template, and 0.2 mM of each primer in a final volume of 25 μL . The following primers were used for each region: P1A/P4 for the complete ITS region (ITS1, 5.8S gene, ITS2; White et al. 1990; Downie and Katz-Downie 1996), *psbA/trnH* for their intergenic spacer (Sang et al. 1997), and primers 1318 and 2110R for the 3' end of the *ndhF* plastid gene (Olmstead and Sweere 1994). The PCR mix underwent the following conditions on a 9700 thermal cycler (Perkin-Elmer, Norwalk, CT): 10 min denaturing at 95°C , 37 cycles of 30 s denaturing at 95°C , 30 s annealing at 52°C , and 1 min extension at 72°C and a final extension step at 72°C for 7 min. The PCR products were then purified using spin filter columns (UltraCleanTM PCR Clean-upTM Kit, MoBio Laboratories, Inc., Carlsbad, CA) following the protocols provided by the manufacturer and directly two-way sequenced with an ABI Prism BigDye Terminator Cycle kit on a ABI Prism 3730 (Applied Biosystems, Norwalk, CT). The resulting electropherographs were manually proofread and the complementary strands combined into one sequence to identify ambiguous positions assisted by Sequencher 4.8 (Gene Codes Corporation 2007).

DATA ANALYSES

Sequences and alignment

Forty-two ITS sequences of *D. laureola* and 18 of the other *Daphne* species analyzed were generated from forward and reverse sequences (GenBank accession numbers GQ167491–GQ167548, HQ268822–HQ268823). Forty-two sequences of the

psbA-trnH marker were obtained for *D. laureola*, and 16 of the other *Daphne* species (GenBank accession numbers GQ167433–GQ167490). Thirty-seven sequences of the *ndhF* marker were obtained for *D. laureola*, and 10 of the other *Daphne* species (GenBank accession numbers GQ167388–GQ167432, HQ268820–HQ268821). For sequence analyses, multiple alignments were performed using ClustalX, with default parameters for gap opening and extension (Thompson et al. 1997). The ITS and *ndhF* regions analyzed did not show any indel variation within the *D. laureola* sequences that constitute the focal purpose of this study (see Appendices S1 and S2). Consequently, gaps were treated as missing data. The alignment of the *psbA-trnH* intergenic spacer was not so straightforward. First, at the interspecific level a 5 bp inversion occurred at position 36, that was coded as a single character change, with all ingroup taxa exhibiting the same character state for the inversion. Second, the initial alignment of our study sequences (see Appendix S3) suggested that two insertion/deletion events occurred within the *D. laureola* sequences analyzed that would be ignored if treated as missing data. The first one involved an A rich region with 9, 10, or 11 repeats, and the second one involved a TA motif with 7, 10, or 13 repeats. The complex architecture of this plastid marker that can affect automatic alignment (Storchova and Olson 2007; Morrison 2009), and the absence of sequences of other Thymelaeaceae phylogenetically close to *Daphne* that could improve the phylogenetic interpretation of these events precluded us from coding indels and thus gaps were conservatively treated as missing data.

Haplotype detection

Statistical parsimony network implemented in the TCS program (Clement et al. 2000) was used to detect haplotypes, estimate the mutational differences among them justified by the parsimony criterion, and establish the resulting network that would allow incorporating the frequently nonbifurcating genealogical information associated with population-level divergences. Confidence level was set at 95%, and gaps were treated as missing data.

Phylogenetic analysis

Five ITS sequences of the sister genus *Thymelaea* (Galicia-Herbada 2006) retrieved from GenBank (AJ549483 *T. villosa*, AJ549489 *T. argentata*, AJ549468 *T. dioica*, AJ549470 *T. granatensis*, and AJ549442 *T. passerina*), were included into the analyses to explore the phylogenetic relationships of *Daphne* with respect to another Thymelaeaceae and improve the rooting of our focal sequences. Phylogenetic relationships among taxa were estimated using maximum parsimony analysis (unordered characters, equal weights; referred as MP hereafter) as implemented in PAUP 4.0b10 (Swofford 2002). Heuristic searches with random trees used as a starting point and 10 random addition sequences of taxa were performed with the TBR branch-swapping algorithm

and MulTrees options in effect. Bootstrap support (referred as BS hereafter) values were calculated on 1000 replicates.

Bayesian inference phylogenetic analyses were also conducted using MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001). Analyses were run for 100,000 generations with four MCMC chains and two independent runs with trees sampled every 100th generation, and the first 80–90 sampled trees discarded by burn-in. Modeltest version 3.7 (Posada and Crandall 1998) was used to determine the DNA substitution model that best fitted the data among those available in MrBayes. The model chosen for our data using hierarchical likelihood ratio tests was HKY + G, which assumes a time-reversible process, a nonuniform distribution of nucleotides and different rates for transitions and transversions, and accounts for among-site rate variation using the gamma distribution. Thus, we set Nst = 6 and rates = gamma options in MrBayes analyses.

We performed maximum likelihood ancestral state reconstructions of the population reproductive system on the MP tree based on the three sequenced regions analyzed simultaneously, that was topologically similar but with a better resolution than those obtained from cpDNA and rDNA markers analyzed independently. As reproductive system is a population-level feature, we reduced our sample to just a single individual per population. This procedure should not affect phylogenetic signal in any important way, because in most cases all the samples from the same population were invariant in sequence and exhibited identical ribotypes and haplotypes (Table 1). In the only three populations where polymorphism occurred at some marker, variability involved a single individual out of the four sampled, and we represented each population using the commonest variant. We used Mesquite 2 (Maddison and Maddison 2007) and coded population sexual system as a binary character (hermaphroditism vs. gynodioecy). The rate of a character's evolution was estimated under the simple Markov k-state one-parameter stochastic model, assuming that transition between hermaphroditism and gynodioecy had the same probability in both directions.

Results

DAPHNE LAUREOLA IN THE CONTEXT OF OTHER EUROPEAN DAPHNE SP.

ITS-based phylogenetic analyses

The percentage of potentially parsimony-informative sites in the complete ITS region analyzed was 19.2% from 604 characters. The score of the best trees found was 246, and the strict consensus tree had consistency index (C.I.) = 0.825 (Fig. 2). Both parsimony and Bayesian analyses yield a similar topology in their resulting trees where all *D. laureola* sequences strongly group together in a single lineage (BS and posterior probability 100%). The lineage splits into two separate lineages both with a high BS. One

encompasses the representatives from NW Iberian populations including the two Pyrenean gynodioecious populations (AL and SV), and the second groups the remaining sequences. This second group remains largely unresolved due to lack of variation, except for two small groups of sequences. The first group singles out (BS 86%) those sequences coming from the two westernmost Southern Iberian populations (ST and CA), and the second groups with a low BS (68%) several Slovenian sequences.

The results of TCS analysis identify four different ribotypes A, B, C, and D in *D. laureola* (Table 1), A being the most frequent within the sequences analyzed and the one identified as the oldest in the sample. Different *Daphne* species yielded different unconnected networks (results not shown). One important feature is that all the samples from the same population invariably share the same ribotype, except for the gynodioecious Slovenian population (Table 1). In this population three individuals have ribotype A and the other one has ribotype B, an infrequent ribotype present only in the second Slovenian population studied and differing by only a single change with regard to ribotype A. Hermaphroditic populations in NE Iberian Peninsula all have ribotype A, which was also shared with the two easternmost Southern Iberian populations (CB and HV) and three of four individuals of the gynodioecious Slovenian site mentioned above. Ribotype C differed from A in two changes and was only present in the two westernmost Southern populations (ST and CA). Ribotype D differed in nine changes with regard to ribotype A and was present in the four gynodioecious populations studied in Northern Iberian Peninsula.

Plastid DNA-based phylogenetic analyses

The percentage of potentially parsimony-informative sites was 4.1% from 758 characters in the *ndhF* region and 8.8% from 318 characters in the *psbA-trnH* intergenic spacer (after coding one inversion of five characters as a single change). The two markers suggested a similar topology and thus we combined their analyses, discarding the individuals that lacked one of the two markers. The score of the best trees found was 75 and the strict consensus tree had C.I. = 0.947.

Plastid DNA based phylogenies also revealed *D. laureola* as a monophyletic lineage where no haplotypes are shared between different species (Fig. 3). The monophyly of the lineage is supported by a BS value and posterior probability of 100% (Fig. 3). The study sequences appear included in a large polytomy where all the NW Iberian individuals, singled out according to the ITS analyses, are nested. Within this polytomy three lineages arise, all supported by strong Bayesian posterior probabilities (Fig. 3): the first one gathers NE Iberian and Slovenian populations (BS 99%), the second one includes most sequences from the Southern Iberian populations, and the last one joins two single sequences from two Southern Iberian populations (BS 85%).

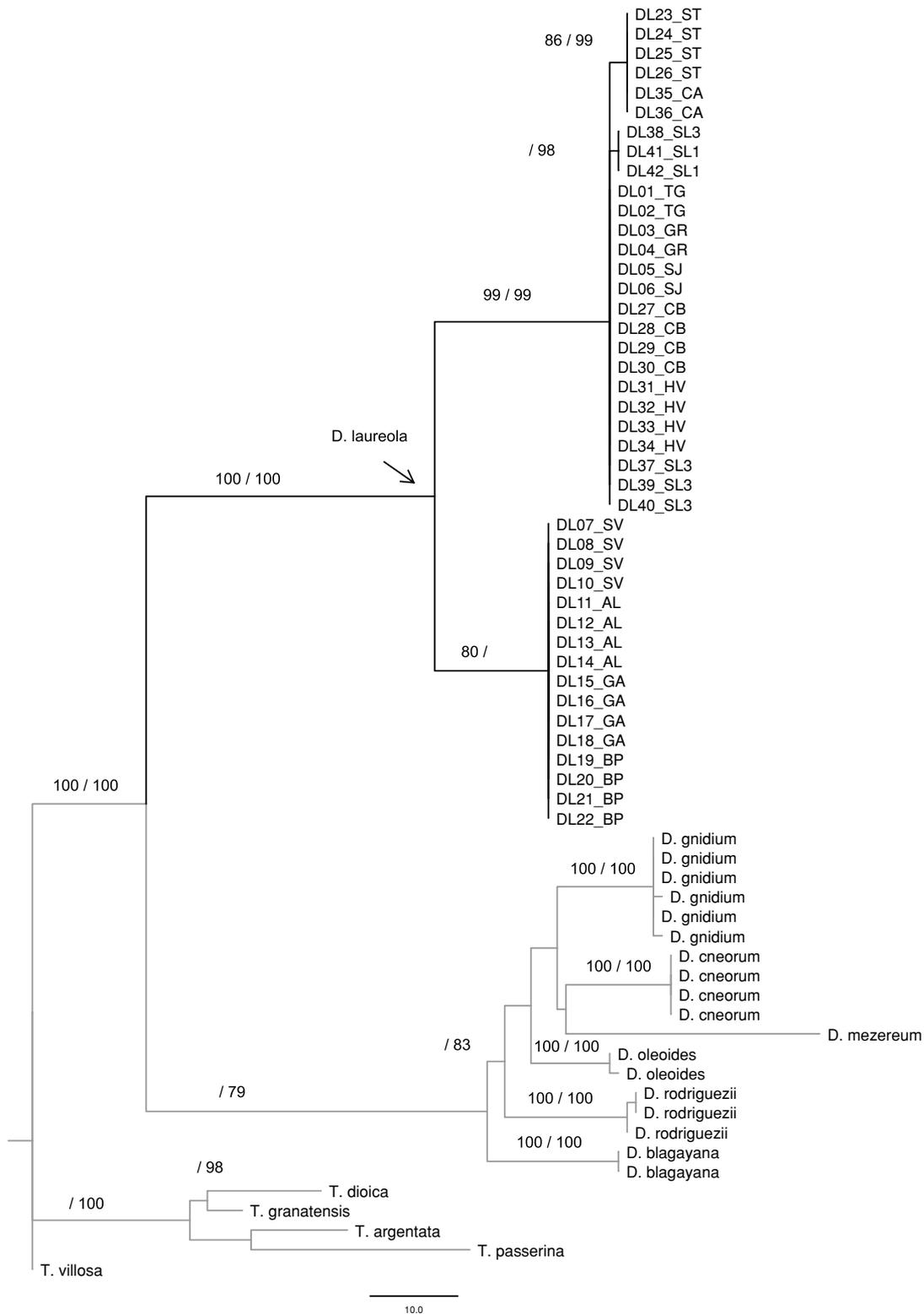


Figure 2. Phylogenetic relationships based on ITS sequence of the 42 *D. laureola* individuals from 13 populations (in black, population codes as in Table 1) and the other six *Daphne* species analyzed in this study, together with five *Thymelaea* species retrieved from GenBank. Maximum Parsimony strict consensus tree (submitted to TreeBase <<http://purl.org/phylo/treebase/phyloids/study/TB2:S10851>>) obtained with branch lengths proportional to inferred DNA changes. Bootstrap values (>70) and Bayes posterior probabilities (>80) noted near branches left and right of a slash, respectively. The scale of substitution per site is indicated.

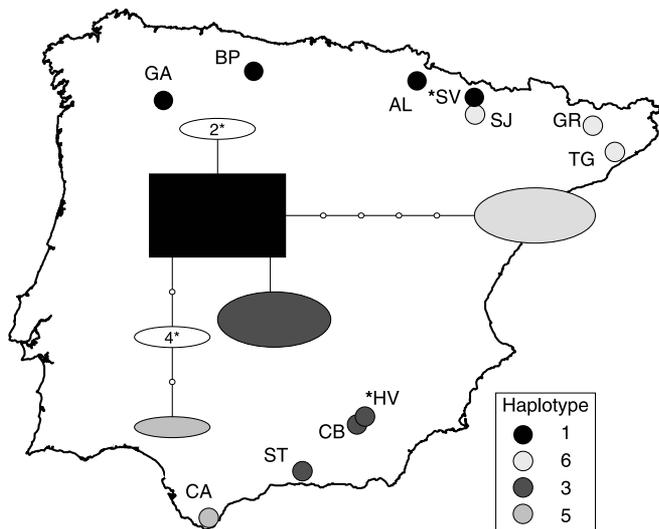


Figure 4. Haplotype network of the study sequences based on the two plastid regions studied (*psbA-trnH* intergenic spacer and *ndhF* gen). The maximum number of steps connecting parsimoniously two haplotypes is indicated. The haplotype with the highest outgroup probability, that correlates with haplotype age, is displayed as a square, whereas other haplotypes are displayed as ovals whose size corresponds to the haplotype frequency in the sample. A map is provided with the geographic distribution of main *D. laureola* haplotypes within the Iberian Peninsula, the Slovenian populations not shown in the map have haplotype 6. Haplotypes only represented by a single individual each and the populations bearing those are marked with an asterisk.

Six haplotypes can be inferred from the TCS analysis of *D. laureola* combined plastid sequences *psbA-trnH* and *ndhF* (Table 1). None of them is present in any of the other sampled species, for which the haplotype networks remain unconnected to the main ingroup network (results not shown). Three haplotypes (2, 4, and 5) are represented by only a single individual each and thus should be interpreted with caution. Haplotype 1, the most frequent one and plausibly so the oldest in our sample (Clement et al. 2000), is characteristic of NW Iberian populations (Fig. 4), except for a single individual in SV population with haplotype 2, differing in a single change in the *ndhF* sequence. The Haplotype 3 characteristic of most of the Southern Iberian populations differs from the previous one in one change in the *psbA-trnH* region. Haplotype 6 is characteristic of hermaphroditic NE Iberian and the two Slovenian populations and differs from the previous ones in five and six changes, respectively. Finally, Haplotype 5, present in the CA Southern Iberian population is the most dissimilar one (Fig. 4).

MAPPING REPRODUCTIVE SYSTEM IN THE PHYLOGENY

The MP tree based on a single individual per population and the three sequenced regions analyzed simultaneously showed a topology similar to those obtained using all the study samples,

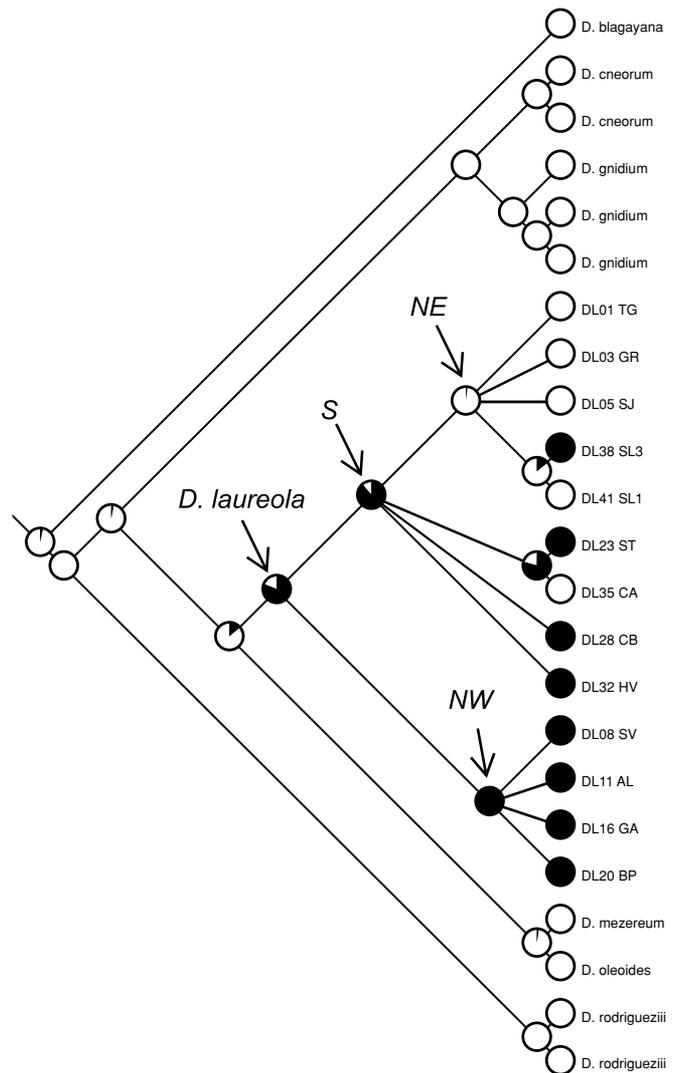


Figure 5. Maximum likelihood ancestral-state reconstruction of reproductive system evolution on the strict MP consensus tree obtained using the three sequenced regions simultaneously and a single individual per population. White and black circles in the tips denote hermaphroditic and gynodioecious populations, respectively. In the reconstructed internal nodes white and black display the proportional likelihood support for hermaphroditic and gynodioecious states, respectively. *Daphne laureola* lineage and the three different clades NW, NE (including the two Slovenian populations), and S obtained are indicated. Population codes as in Table 1.

with monophyly of *D. laureola* supported by 100% BS, and the three major lineages distinguished within this clade (NW, S, and NE; Fig. 5) being consistent with those obtained with all the available samples (Figs. 2 and 3). None of the rest of species showed evidence of gynodioecy (Brickell and Mathew 1976), and consequently the most likely ancestor between *D. laureola* and the closest species in the phylogeny was hermaphroditism with 86% support (Fig. 5).

The hypothetical ancestor common to all the *D. laureola* study populations showed an 81% likelihood of being gynodioecious (Fig. 5). Within *D. laureola*, the NW clade (91% BS) that grouped all the Northern gynodioecious populations had a 100% likelihood of having a common ancestor gynodioecious (Fig. 5). The common ancestor to all S and NE populations had 89% likelihood of being gynodioecious, hermaphroditism being thus a derived condition in the CA Iberian population. Furthermore, the NE clade (100% BS) had 100% likelihood of having a common ancestor hermaphroditic, gynodioecy being thus a derived condition in the Slovenian SL3 population (Fig. 5).

Summing up, gynodioecy appeared as the most likely ancestral state common to all the *D. laureola* populations studied, and the different evolutionary transitions in reproductive system traced within each clade account for current geographic variation at the within-species level (Fig. 5).

Discussion

PHYLOGEOGRAPHY OF *D. LAUREOLA* IN THE IBERIAN PENINSULA

The Iberian Peninsula is a relatively isolated, physiographically complex area in the western part of the Mediterranean Basin, with strong climatic gradients between the Atlantic, Mediterranean and continental territories, and contrasted soil types. The Iberian mountain ranges, that predominantly run east-west, have apparently provided multiple Pleistocene glacial refugia for many temperate European tree species, which currently retain a higher genetic diversity than in more northern areas of their distribution ranges (Gómez and Lunt 2007; de Heredia et al. 2007). Such long-term historical processes might have affected even more strongly the genetic diversity and phylogeography of an understory species like *D. laureola*. The disjunct distribution of *D. laureola* within the Iberian Peninsula (Fig. 1) coupled with the recent discovery of among-population variation in reproductive system in both the Northern and Southern areas of the Iberian distribution range (Alonso et al. 2007) prompted this phylogeographic study aimed to describe the genetic relatedness between several populations broadly distributed geographically and to unravel the evolutionary transitions between hermaphroditism and gynodioecy at the intraspecific level.

Our results supported *D. laureola* as a monophyletic lineage. No haplotypes or ribotypes were shared with different species. At intraspecific level, on one hand we found out that the a priori distinction between NE and NW regions, based on the reduced abundance of *D. laureola* in the Eastern part of the Cantabrian mountains, did not accurately reflect the phylogenetic history of the species because the two gynodioecious populations sampled in the Western Pyrenees (AL and SV) were genetically akin to a well-supported NW clade based on ITS, characterized by ri-

botype D, and they also share the distinctive NW haplotype 1, plausibly the oldest in our sample, with the only exception of a single sequence with the infrequent haplotype 2 (Fig. 4). Furthermore, the plastid markers also indicated that all samples from the hermaphroditic NE Iberian populations, including the one located at a southern pre-Pyrenean foothill (SJ), were genetically inter-related and grouped together with the Slovenian samples, characterized by the distinctive haplotype 6 (Fig. 5). Thus, genetic differentiation of *D. laureola* in the North apparently occurred at the central region of the Pyrenees, somewhere between the pre-Pyrenean (SJ) and Pyrenean (SV) populations, distant only a few kilometers (Fig. 1), but with a sharp contrast in geological history and climate (García-Castellanos et al. 2003). Interestingly, genetic differentiation occurred together with disparity in population reproductive system, with the NE Iberian populations, characterized by drier and warmer locations, being all hermaphroditic (Alonso et al. 2007) and more genetically related to Slovenian populations than to the other Iberian populations sampled. On the other hand, the Southern populations showed characteristic haplotypes 3, 4, and 5 and two different ribotypes, one of them shared with the NE clade, suggesting also certain genetic differentiation within this region, further supported by analyses of amplified fragment length polymorphisms (AFLP) variation (A. R. Castilla, C. Alonso and C. M. Herrera, unpubl. data). To ascertain whether genetic differentiation is due to different *D. laureola* refugia during the last glaciation in the Iberian Peninsula, like in several of the forest tree species such as *Fagus sylvatica* (Magri et al. 2006), deciduous oaks (Olalde et al. 2002), and evergreen oaks (de Heredia et al. 2007), or to local adaptation and diversification would require a more comprehensive phylogeographic sampling of the species. The remarkable diversity of ribotypes and haplotypes already obtained with our limited geographic sample apparently supports the hypothesis of multiple Iberian refugia. However, it could be further related to variation in reproductive system itself if gynodioecious species harbor more old haplotypes, as recently found with mitochondrial genes (Touzet and Delph 2009).

EVOLUTION OF REPRODUCTIVE SYSTEM IN *D. LAUREOLA*

A recent review of gender variation within Thymelaeaceae has shown the existence of unisexual flowers and diverse reproductive systems in a large proportion of genera (Beaumont et al. 2006). Such variation was concentrated in the largest subfamily Thymelaeoideae, to which most genera including *Daphne* belong, in contrast to the other three smaller subfamilies in which hermaphroditism is ubiquitous. In particular, the sister genus *Thymelaea* (Van der Bank et al. 2002; Galicia-Herbada 2006) is highly variable in reproductive system, and in the Iberian Peninsula only *T. passerina* of 21 species has a monomorphic

hermaphroditic reproductive system (Pedrol 1997). Although the genus *Daphne* (ca. 70 spp) apparently is not so variable in reproductive system, gynodioecy is known from *D. kamtschatica* (Kikuzawa 1989), and unisexual flowers have been occasionally reported from *D. acutiloba*, *D. jezoensis*, *D. mezereum*, *D. odora*, and *D. oleoides* (Brickell and Mathew 1976; Nieto Feliner 1997), which suggests that “further study of the genus is required to determine the significance of these characters” (Brickell and Mathew 1976, p. 6).

Recent studies on the ecological context of reproductive system variation in *D. laureola* showed that hermaphroditic Iberian populations were associated with higher ambient temperatures and lower annual precipitation than gynodioecious ones, and suggested that the abiotic features conditioning gender polymorphism would be similar at both Northern and Southern regions (Alonso et al. 2007). Different selective and stochastic processes might prevail at different geographic locations (e.g., Nilsson and Ågren 2006) and phylogenetic analysis can be essential to unravel, for instance, if similarity in population reproductive system or average plant size could also reflect genetic similarity between populations or not. Phylogenetic analysis would be also useful to identify cryptic species, as in the *Wurmbea* genus, where the study of the evolutionary transitions of reproductive system with a phylogenetic perspective contradicted the monophyly of two sexually polymorphic taxa, *Wurmbea dioica* and *W. biglandulosa* (Case et al. 2008).

Our analysis of intraspecific genetic relationships between populations of *D. laureola* differing in reproductive system aimed to unravel whether hermaphroditic populations from NE and S Iberia were closely genetically related to each other and were placed as a basal group in the phylogeny that could be interpreted as the relict representatives of a formerly wider distribution range. That hypothesis would be consistent with hermaphroditism being the ancestral condition shared by all the analyzed *Daphne* species and the macroevolutionary pathway suggested for the whole Angiosperms (Barrett 2002). Results did however indicate that NE and S hermaphroditic populations were located in different clades within the phylogenies obtained from both nuclear and plastid markers and thus do not support a single origin of the populations in which female-sterility mutants are not currently present. Gynodioecy emerged anyway as the most likely ancestral state common to all the *D. laureola* populations sampled, being ubiquitous in the NW clade and also present in the other two sequence groups (Fig. 5).

Different evolutionary transitions in reproductive system were traced within each *D. laureola* clade, with two events of change from gynodioecy to hermaphroditism and one further reversion from gynodioecy to hermaphroditism (Fig. 5). Gynodioecy emerged as the most likely condition of the ancestor common to all the populations sampled at NW Iberian Peninsula,

genetically characterized by ribotype D and haplotypes 1 and 2. Such an ancestor would have likely survived within this interglacial refugium (see above) from which it would have eventually recolonized all the Northwestern Iberian mountain ranges including the Western Pyrenees. In contrast, hermaphroditism emerged as the most likely condition of the ancestor common to Slovenian and NE Iberian populations sampled (Fig. 5), genetically characterized by the haplotype 6, suggesting another interglacial refugia of the species located in NE Iberian mountains, that would have likely followed different recolonizing routes to Central Europe and the Southern Iberian ranges, where gynodioecy would have succeeded as the commonest reproductive system in the South (Alonso et al. 2007). Population dynamics and selective particularities of a complex trait such as reproductive system (Pannell and Dorken 2006; Case et al. 2008) can likely explain such evolutionary liability although it has been only rarely documented at intraspecific level (Fénart et al. 2006).

In fragmented landscapes, hermaphroditism and selfing would be positively selected in recently colonized populations due to reproductive assurance advantages when the number of immigrants is low (Pannell 1997; Pannell and Dorken 2006). A recent metapopulation model further suggests that gene migration among demes can be essential for the dynamics of the system under nucleo-cytoplasmic inheritance (Dufay and Pannell 2010). At the geographic scale studied here, fragmentation of the landscape with repeated colonization and extinction of local populations has likely been the commonest scenario for *D. laureola*, an understory species of shady mountainous forest, since the last glaciation. Long-distance dispersal events involving very few seeds dispersed by birds would allow the colonization of relatively small and isolated mountains and would be characterized by a reduced genetic diversity within population (Bialozyt et al. 2006). Interestingly, the Southwestern hermaphroditic population CA is characterized by absence of variability in the 73 adult individuals analyzed (C. Alonso, unpubl. data) for the three allozyme systems that were known to be polymorphic in several Southeastern populations (Medrano et al. 2005), thus suggesting that a long-distance colonizing event could be behind the only hypothesized reversion from gynodioecy to hermaphroditism observed. Ongoing studies at the Southern Iberian Ranges support a role of among-population gene flow in the dynamics of gynodioecy (Dufay and Pannell 2010), because the frequency of females and prevalence of gynodioecy were higher in the central region, with highly connected populations encompassing a broad altitudinal range, than in the marginal and highly isolated Western Betic Ranges where the hermaphroditic CA population is located (A. R. Castilla, C. Alonso and C. M. Herrera, unpubl. data). In addition, Dufay and Pannell (2010) simulations suggest that, when migration among local populations is absent for a few hundred generations, the combination of genetic drift and selection will

promote allele fixation, frequently leading to hermaphroditism within populations, and even at the metapopulation scale if female reproductive advantage is not strong. Such extreme isolation of populations is expected to happen in highly heterogeneous landscapes such as the small and isolated foothills characteristic of the NE Catalanian Coastal Ranges (García-Castellanos et al. 2003), where all *D. laureola* populations surveyed were found to be hermaphroditic and individual plants smaller, presumably reducing geitonogamous selfing and the potential for a female advantage by inbreeding avoidance (Alonso et al. 2007).

Altogether, our study highlights that plant reproductive system is not necessarily a species-specific trait, but can be a variable, labile feature subject to eco–evo coevolutionary feedbacks (Kokko and López-Sepulcre 2007) with hermaphroditism and selfing being positively selected at the low individual frequencies characteristic of new colonized populations but with male-sterile mutants being able to spread in more dense (older) populations if they reach some reproductive advantage through quantitative seed production or inbreeding avoidance (Medrano et al. 2005, and references therein) and are able to maintain some gene flow among them (Dufay and Pannell 2010). Under this scenario, species can evolve gynodioecy from hermaphroditism as traditional models predict (Barrett 2002), but gynodioecy can also revert to hermaphroditism, favored by genetic drift and isolation. A combination of selective and stochastic processes will determine the course and polarity of evolutionary transitions, the role of stochasticity being expected to increase with population isolation and limitations to gene flow, as commonly found at the borders of species distribution ranges (Herrera and Bazaga 2008). Broad phylogeographic analysis of species that exhibit among-population variation in reproductive system are bound to contribute in important ways to understand how the macroevolutionary history of colonization/extinction and diversification inferred from neutral genetic markers, and the microevolutionary population dynamics of reproductive system, converge to explain the evolution of plant polymorphic reproductive systems.

ACKNOWLEDGMENTS

We are grateful to G. N. Feliner and J. F. Aguilar for enthusiastically supporting the onset of this project and introducing us to phylogenetic techniques; P. Bazaga and B. Guzmán, for assistance with technical issues; V. Fernández, D. Gómez, and R. Brus for their help in locating populations at the Baetic Ranges, Pyrenees, and Slovenia, respectively; and M. Burd and two anonymous referees for their constructive reviews of the work. The study was funded by the Spanish Ministerio de Educación y Ciencia through the research project CGL2006-01355/BOS, Consejo Superior de Investigaciones Científicas (PIE200730/001), and the Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía, through Excellence Research Project RNM156-2005.

LITERATURE CITED

- Alonso, C., P. Mutikainen, and C. M. Herrera. 2007. Ecological context of breeding system variation: sex, size and pollination in a (predominantly) gynodioecious shrub. *Ann. Bot.* 100:1547–1556.
- Ashman, T.-L. 2006. The evolution of separate sexes: a focus on the ecological context. Pp. 204–222 in L. D. Harder and S. C. H. Barrett, eds. *Ecology and evolution of flowers*. Oxford Univ. Press, Oxford, UK.
- Bailey, M. F., and L. F. Delph. 2007. A field guide to models of sex-ratio evolution in gynodioecious species. *Oikos* 116:1609–1617.
- Bailey, M. F., L. F. Delph, and C. M. Lively. 2003. Modeling gynodioecy: novel scenarios for maintaining polymorphism. *Am. Nat.* 161:762–776.
- Barrett, S. C. H. 2002. The evolution of plant sexual diversity. *Nat. Rev. Genet.* 3:274–284.
- Beaumont, A. J., T. J. Edwards, and F. R. Smith. 2006. The first record of gynodioecy in a species of *Gnidia* (Thymelaeaceae) from South Africa. *Bot. J. Linn. Soc.* 152:219–233.
- Bialozyt, R., B. Ziegenhagen, and R. J. Petit. 2006. Contrasting effects of long distance seed dispersal on genetic diversity during range expansion. *J. Evol. Biol.* 19:12–20.
- Brickell, C. D., and B. Mathew. 1976. *Daphne*. The genus in the wild and in cultivation. The Alpine Garden Society, Surrey.
- Caruso, C. M., and A. Case. 2007. Sex ratio variation in gynodioecious *Lobelia siphilitica*: effects of population size and geographic location. *J. Evol. Biol.* 20:1396–1405.
- Case, A., and S. C. H. Barrett. 2004. Floral biology of gender monomorphism and dimorphism in *Wurmbea dioica* (Colchicaceae) in Western Australia. *Int. J. Plant Sci.* 165:289–301.
- Case, A., S. W. Graham, T. D. Macfarlane, and S. C. H. Barrett. 2008. A phylogenetic study of evolutionary transitions in sexual systems in Australasian *Wurmbea* (Colchicaceae). *Int. J. Plant Sci.* 169:141–156.
- Charlesworth, D. 1999. Theories of the evolution of dioecy. Pp. 33–60 in M. A. Geber, T. E. Dawson, and L. F. Delph, eds. *Gender and sexual dimorphism in flowering plants*. Springer-Verlag, Berlin.
- Clement, M., D. Posada, and K. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9:1657–1660.
- Comman, R. S., and M. L. Arnold. 2007. Phylogeography of *Iris missouriensis* (Iridaceae) based on nuclear and chloroplast markers. *Mol. Ecol.* 16:4585–4598.
- Delph, L. F., and S. B. Carroll. 2001. Factors affecting relative seed fitness and female frequency in a gynodioecious species, *Silene acaulis*. *Evol. Ecol. Res.* 3:487–505.
- Desfeux, C., S. Maurice, J.-P. Henry, B. Lejeune, and P.-H. Gouyon. 1996. Evolution of reproductive systems in the genus *Silene*. *Proc. R. Soc. Lond. B* 263:409–414.
- Downie, S. R., and D. S. Katz-Downie. 1996. A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *Am. J. Bot.* 83:234–251.
- Dufay, M., and J. R. Pannell. 2010. The effect of pollen versus seed flow on the maintenance of nuclear-cytoplasmic gynodioecy. *Evolution* 64:772–784.
- Dufay, M., P. Touzet, S. Maurice, and J. Cuguen. 2007. Modelling the maintenance of male-fertile cytoplasm in a gynodioecious population. *Heredity* 99:349–356.
- Fénart, S., P. Touzet, J.-F. Arnaud, and J. Cuguen. 2006. Emergence of gynodioecy in wild beet (*Beta vulgaris* ssp. *maritima* L.): a genealogical approach using chloroplastic nucleotide sequences. *Proc. R. Soc. Lond. B* 273:1391–1398.
- García-Herbada, D. 2006. Origin and diversification of *Thymelaea* (Thymelaeaceae): inferences from a phylogenetic study based on ITS (rDNA) sequences. *Plant Syst. Evol.* 257:159–187.

- García-Castellanos, D., J. Vergés, J. Gaspar-Escribano, and S. Cloetingh. 2003. Interplay between tectonics, climate, and fluvial transport during the Cenozoic evolution of the Ebro Basin (NE Iberia). *J. Geophys. Res.* 108:2347–2365.
- Gene Codes Corporation. 2007. Sequencher Program. Version 4.8. Ann Arbor, MI, USA.
- Gómez, A., and D. H. Lunt. 2007. Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. Pp. 155–188 in S. Weiss and N. Ferrand, eds. *Phylogeography of Southern European Refugia*. Springer, Dordrecht, The Netherlands.
- de Heredia, U. L., J. S. Carrion, P. Jimenez, C. Collada, and L. Gil. 2007. Molecular and palaeoecological evidence for multiple glacial refugia for evergreen oaks on the Iberian Peninsula. *J. Biogeogr.* 34:1505–1517.
- Herrera, C. M., and Bazaga, P. 2008. Adding a third dimension to the edge of a species' range: altitude and genetic structuring in mountainous landscapes. *Heredity* 100:275–285.
- Huelsenbeck, J. P., and F. Ronquist. 2001. MrBayes, Bayesian Analysis of Phylogeny. *Bioinformatics* 17:754–755.
- Kaul, M. L. H. 1988. Gene-cytoplasmic male sterility. Pp. 97–192 in M. L. H. Kaul, ed. *Male sterility in plants*. Springer-Verlag, Berlin, Germany.
- Kikuzawa, K. 1989. Floral biology and evolution of gynodioecism in *Daphne kamtchatica* var. *jezoensis*. *Oikos* 56:196–202.
- Kokko, H., and E. López-Sepulcre. 2007. The ecogenetic link between demography and evolution: can we bridge the gap between theory and data? *Ecol. Lett.* 10:773–782.
- Maddison, W. P., and D. R. Maddison. 2007. Mesquite: a modular system for evolutionary analysis. Version 2.0 <http://mesquiteproject.org>.
- Magri, D., G. G. Vendramin, B. Comps, I. Dupanloup, T. Geburek, D. Gomory, M. Latalowa, T. Litt, L. Paule, J. M. Roure, et al. 2006. A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. *New Phytol.* 171:199–221.
- McCauley, D. E., and M. F. Bailey. 2009. Recent advances in the study of gynodioecy: the interface of theory and empiricism. *Ann. Bot.* 104:611–620.
- McCauley, D. E., and D. R. Taylor. 1997. Local population structure and sex ratio: evolution in gynodioecious plants. *Am. Nat.* 150:406–419.
- McCauley, D. E., and M. S. Olson. 2003. Associations among cytoplasmic molecular markers, gender, and components of fitness in *Silene vulgaris*, a gynodioecious plant. *Mol. Ecol.* 12:777–787.
- . 2008. Do recent findings in plant mitochondrial molecular and population genetics have implications for the study of gynodioecy and cytonuclear conflict?. *Evolution* 62:1013–1025.
- Medrano, M., C. Alonso, and C. M. Herrera. 2005. Mating system, sex ratio, and persistence of females in the gynodioecious shrub *Daphne laureola* L. (Thymelaeaceae). *Heredity* 94:37–43.
- Meusel, H., E. Jäger, S. Rauschert, and E. Weinert. 1978. *Vergleichende Chorologie der zentraleuropäischen Flora*. Band III, Gustav Fischer, Jena.
- Morrison, D. A. 2009. A framework for phylogenetic sequence alignment. *Plant Syst. Evol.* 282:127–149.
- Navajas-Pérez, R., R. de la Herran, G. L. González, M. JAMILENA, R. Lozano, C. R. Rejon, M. R. Rejon, and M. A. Garrido-Ramos. 2005. The evolution of reproductive systems and sex-determining mechanisms within *Rumex* (Polygonaceae) inferred from nuclear and chloroplastidial sequence data. *Mol. Biol. Evol.* 22:1929–1939.
- Nieto Feliner, G. 1997. *Daphne*. Pp. 33–42 in S. Castroviejo, C. Aedo, C. Benedí, M. Laínz, F. Muñoz Garmendia, G. Nieto Feliner, and J. Paiva, eds. *Flora Iberica*. Vol. VIII. Real Jardín Botánico, CSIC, Madrid, Spain.
- Nilsson, E., and J. Ågren. 2006. Population size, female fecundity, and sex ratio variation in gynodioecious *Plantago maritima*. *J. Evol. Biol.* 19:825–833.
- Olalde, M., A. Herrán, S. Espinel, and P. G. Goicoechea. 2002. White oaks phylogeography in the Iberian Peninsula. *Forest Ecol. Manag.* 156:89–102.
- Olmstead, R. G., and J. A. Sweere. 1994. Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. *Syst. Biol.* 43:467–481.
- Pannell, J. R. 1997. The maintenance of gynodioecy and androdioecy in a metapopulation. *Evolution* 51:10–20.
- Pannell, J. R., and M. E. Dorken. 2006. Colonisation as a common denominator in plant metapopulations and range expansions: effects on genetic diversity and sexual systems. *Land. Ecol.* 21:837–848.
- Pedrol, J. 1997. *Thymelaea* Mill. Pp. 42–69 in S. Castroviejo, C. Aedo, C. Benedí, M. Laínz, F. Muñoz Garmendia, G. Nieto Feliner, and J. Paiva, eds. *Flora Iberica*. Vol. VIII. Real Jardín Botánico, CSIC, Madrid, Spain.
- Posada, D., and K. Crandall. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818.
- Ramsey, M., G. Vaughton, and R. Peakall. 2006. Inbreeding avoidance and the evolution of gender dimorphism in *Wurmbea biglandulosa* (Colchicaceae). *Evolution* 60:529–537.
- Sang, T., D. J. Crawford, and T. F. Stuessy. 1997. Chloroplast DNA phylogeny, reticulate evolution and biogeography of *Paeonia* (Paeoniaceae). *Am. J. Bot.* 84:1120–1136.
- Schultz, S. T. 1994. Nucleo-cytoplasmic male sterility and alternative routes to dioecy. *Evolution* 48:1933–1945.
- Storchova, H., and M. S. Olson. 2007. The architecture of the chloroplast *psbA-trnH* non-coding region in angiosperms. *Plant. Syst. Evol.* 268:235–256.
- Swofford, D. L. 2002. PAUP: phylogenetic analysis using parsimony and other methods. Version 4. Sinauer Associates, Sunderland, MA, USA.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876–4882.
- Touzet, P., and L. F. Delph. 2009. The effect of breeding system on polymorphism in mitochondrial genes of *Silene*. *Genetics* 181:631–644.
- Vamosi, J. C., S. P. Otto, and S. C. H. Barrett. 2003. Phylogenetic analysis of the ecological correlates of dioecy in angiosperms. *J. Evol. Biol.* 16:1006–1018.
- Van Der Bank, M., M. F. Fay, and M. W. Chase. 2002. Molecular phylogenetics of Thymelaeaceae with particular reference to African and Australian genera. *Taxon* 51:329–339.
- Weiblen, G. D., R. K. Oyama, and M. J. Donoghue. 2000. Phylogenetic analysis of dioecy in monocotyledons. *Am. Nat.* 155:46–58.
- Weller, S. G., W. L. Wagner, and A. K. Sakai. 1995. A phylogenetic analysis of *Schiedea* and *Alsindendron* (Caryophyllaceae: Alsinoideae): implications for the evolution of breeding systems. *Syst. Bot.* 20:315–337.
- Weller, S. G., and A. K. Sakai. 1999. Using phylogenetic approaches for the analysis of plant breeding system evolution. *Annu. Rev. Ecol. Syst.* 30:167–199.
- White, T. J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies. Pp. 315–322 in M. Innis, D. Gelfand, J. Sninsky, and T. White, eds. *PCR protocols: a guide to methods and applications*. Academic Press, San Diego, CA.

Associate Editor: M. Burd

Supporting Information

The following supporting information is available for this article:

Appendix S1. Alignment of all the ITS sequences used in this study, including their GenBank accession numbers.

Appendix S2. Alignment of all the *ndhF* sequences used in this study, including their GenBank accession numbers.

Appendix S3. Alignment of all the *psbA-trnH* intergenic spacer sequences used in this study, including their GenBank accession numbers. Note that the 5 bp inversion at position 38 was coded as a single change.

Supporting Information may be found in the online version of this article.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.