Y chromosome specific markers and the evolution of dioecy in the genus *Silene*

Y. Hi. Zhang, Veronica S. Di Stilio, Farah Rehman, Amy Avery, David Mulcahy, and Rick Kesseli

Abstract: Sex determination in plants has been most thoroughly investigated in *Silene latifolia*, a dioecious species possessing heteromorphic sex chromosomes. We have identified several new Y chromosome linked RAPD markers and converted these to more reliable sequence characterized amplified region (SCAR) markers by cloning the RAPD fragments and developing longer primers. Of the primer pairs for seven SCARs, five amplify a single, unique fragment from the DNA of male *S. latifolia*. Two sets of primers also amplify additional fragments common to males and females. Homology between the X and Y chromosomes is sufficient to allow the amplification of fragments from females under less stringent PCR conditions. Five of the SCARs also distinguish between the sexes of closely related dioecious taxa of the section *Elisanthe*, but not between the sexes of distantly related dioecious species. These markers will be useful for continued investigations into the evolution of sex, phylogenetic relationships among taxa, and population dynamics of sex ratios in the genus *Silene*.

Key words: *Melandrium*, RAPDs, sex chromosomes, SCARs.

Résumé : La détermination du sexe chez les plantes a été étudiée le plus en détail chez le *Silene latifolia*, une espèce dioïque qui présente des chromosomes sexuels hétéromorphes. Plusieurs nouveaux marqueurs RAPD liés au chromosome Y ont été identifiés et convertis en marqueurs SCAR (région amplifiée de séquence connue) suite au clonage des fragments RAPD et au développement d'amorces plus longues. Parmi sept paires d'amorces SCAR, cinq génèrent un seul fragment unique à partir de l'ADN d'individus mâles du *S. latifolia*. Deux paires d'amorces amplifient également des fragments additionnels communs aux mâles et femelles. L'homologie entre les chromosomes X et Y est suffisante pour qu'il y ait amplification de fragments chez les femelles sous des conditions d'amplification moins sélectives. Cinq des marqueurs SCAR permettent également de distinguer les deux sexes chez des espèces dioïques très apparentées de la section *Elisanthe*, mais non pas chez des espèces dioïques distantes. Ces marqueurs seront utiles pour approfondir les connaissances en matière de l'évolution du sexe, les relations phylogénétiques parmi les taxons et la dynamique des populations au niveau du ratio des sexes chez le genre *Silene*.

Mots clés : *Melandrium*, RAPD, chromosome sexuels, SCAR.

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Introduction

A striking dichotomy exists between higher plants and animals with regard to the system of sexual reproduction. Dioecy (separate male and female individuals) is well established in animals, but occurs sporadically in the plant kingdom. Yampolsky and Yampolsky (1922) noted that while only 5% of plant genera are wholly dioecious, 75% of flowering plant families have some dioecious species. Charlesworth (1985) extended the compilation of Yampolsky and Yampolsky (1922) and identified 1303 genera and 170 families in which dioecy is well established. The sporadic distribution of dioecy has led many authors to surmise that dioecism has arisen repeatedly in the angiosperms (Westergaard 1958; Charlesworth 1991). These phylogenetically independent events are particularly appealing subjects for study of the evolution of dioecy (Charlesworth 1991; Read and Nee 1991).

The genus *Silene* and related genera of the Caryophyllaceae are particularly suitable for studying the evolution of breeding systems, in particular, dioecy. This largely temperate northern-hemisphere group possesses several hundred species (Chater and Walters 1964; Oxelman and Liden 1995). Breeding systems range from cleistogamous self-pollinators to obligately outcrossed dioecy (Desfeux et al. 1996). Most species of *Silene* are self-compatible hermaphrodites, but at least two sections of the genus are predominantly dioecious. In the most studied section, *Elisanthe*, five of the six species are dioecious. The sixth, *Silene noctiflora*, a self-compatible hermaphroditic annual, has been described as a recent derivative of *Silene latifolia* (Prentice 1978) but, on the basis of chloroplast DNA variation, appears to be more distantly related to the other members of the section and therefore is likely not derived (Sandbrink et al. 1989). Three of the six species, *S. latifolia, Silene dioica,* and *S. noctiflora*, are widely distributed in both Europe and North America (Chater and Walters 1964; McNeill 1978; Prentice 1978). The remaining three species, *Silene diclinis*, *Silene heuffelii*, and *Silene marizii*, are restricted endemics in southern Europe.

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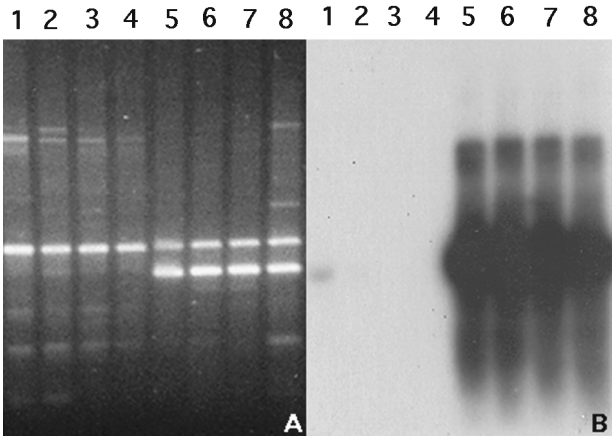
Fig. 1. Identification and verification of the cloning of Y chromosome specific fragments. (A) Ethidium bromide stained agarose gel of RAPD fragments amplified with primer OPD12. The DNA from four female (lanes 1–4) and four male (lanes 5–8) *S. latifolia* was amplified. The size of the male-specific fragment is approximately 800 bp. (B) Autoradiograph of the Southern blot of the RAPD profile shown in A hybridized to the radiolabeled clone of the 800 bp male specific fragment.



Sex determination in plants has been most thoroughly investigated in *S. latifolia*. Early work demonstrated that males are heterogametic and possess morphologically distinct sex chromosomes (Correns 1928; Winge 1931; Warmke 1942; Westergaard 1958). The Y chromosome is approximately 50% larger than the X chromosome and easily identified by standard cytological techniques (Ciupercescu et al. 1990). More recent studies have focused on the molecular bases of sex determination and its evolution. Genes involved in male and female function and markers linked to sex chromosomes have been identified. Hardenack et al. (1994) and Matsunaga et al. (1996) have identified several genes differentially expressed in male and female structures of *S. latifolia* that are likely involved in sex differentiation. Mulcahy et al. (1992) have previously identified RAPD (random amplified poymorphic DNA) markers linked to the Y chromosome of *S. latifolia*, and these markers have now been used by other investigators to monitor sex ratios in natural populations (Taylor 1996; Lyons et al. 1995). Neither the genes that affect sex expression, because they are not Y- linked, nor the random markers, because they are not coding sequences, are the analog of *Sry*, the sex determination gene of mammals.

In this paper, we have continued the work of identifying Y chromosome linked RAPD markers and have converted these to more reliable sequence characterized amplified region (SCAR) markers by cloning the RAPD fragments and developing longer primers. These markers have advantages over RAPDs, since they often show codominance (for autosomal sequences) and can be used to identify heterologous sequences in related taxa (Paran and Michelmore 1993; Kesseli et al. 1993, 1994; Witsenboer et al. 1995). We describe seven SCARs designed from RAPDs for the Y chromosome of *S. latifolia.* We also show that five of these SCARs distinguish between the sexes of closely related dioecious taxa of section *Elisanthe*, but not between the sexes of distantly related dioecious species.

Materials and methods

Plant material

Silene latifolia Poirt (= *Melandrium album* (Miller) Garcke = *Lychnis alba* Miller) from western Massachusetts and *S. dioica* from England were used for developing SCAR markers. Seeds of *S. latifolia*, *S. dioica*, and the other taxa, *S. diclinis, Silene otites*, *Silene vulgaris*, *Silene roemeri*, and *Lychnis flos-cuculi*, were obtained from Kew Botanical Gardens (Kew, England) and maintained in the greenhouse at the University of Massachusetts, Boston.

Screening of RAPD markers

DNA was extracted from the leaf tissue of progeny of known crosses of *S. latifolia* or *S. dioica,* using a modified method of Bernatzky and Tanksley (1986). To identify Y chromosome specific markers, we used bulked segregant analysis (BSA; Michelmore et al. 1991); the DNA of male and female individuals (minimum of five individuals per pool) were pooled separately and these pools screened for RAPDs as described previously (Mulcahy et al. 1992). Arbitrary decamers were obtained from Operon Technologies (kits A, B, C, D, F, G, H, I, K, L, M, Q, R, S, T, U, and X).

Development of SCAR markers

The Y chromosome specific bands amplified with random decamers were excised, and the DNA was isolated using either GeneClean II (Bio101 Inc., La Jolla, Calif.) or Qiagen gel purification kit (Qiagen, Chatsworth, Calif.). These fragments were ligated to either pT7Blue T vector (Novagen, Madison, Wis.) or pGEM 5Z (Promega, Madison, Wis.), following the company's protocols. The derived clone was shown to be correct and Y-specific by hybridizing the ^{32}P-labeled insert back to a RAPD profile (Fig. 1). After verification, each clone was sequenced and this information used for designing longer primers. Sequencing was performed either manually, using *fmol* DNA Cycle Sequencing System from Promega, or automatically, using ABI Prism 377 from Perkin Elmer (Foster City, Calif.). The sequences adjacent to the original decamers were scored, and SCAR primers (17–30 bp) were designed using the program Oligo 3.0 (National Biosciences); these primers were synthesized by Operon Technologies (Alameda, Calif.). Generally, the SCAR primers contain the 10 b of the original decamer. Occasionally, because of primer dimerization or disparities in annealing temperatures, SCAR primers that have none or only part of the original decamer were chosen. The PCRs using SCAR primers were optimized from the following basal conditions: 2.5 μL of 10× buffer (100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, plus 0.1% gelatin), 2.0 μL of dNTP (1.25 mM each of dATP, dCTP, dGTP, and dTTP), 0.5 μL each of forward and reverse primer (10 mM), 2.0 μL of $MgCl_2$ (10 mM), 0.2 μL of Taq DNA polymerase (5 U/μL), 5 μL of DNA (5 ng/μL), and 12.3 μL of H_2O. The basal cycling parameters were 1 cycle of 94°C for 1 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; and 1 cycle of 72°C for 5 min. For hot start, Taq polymerase was added after the first cycle.

Characterizing Y chromosome specific SCAR markers in related taxa

The primers for each SCAR were used to amplify the DNA of different species of *Silene* and *L. flos-cuculi*, using the optimized conditions established for *S. latifolia* and at lower annealing temperatures (50°C). The amplified fragments for the different taxa were separated in 2% agarose gel in 1× TBE (90 mM Tris-borate plus 2 mM EDTA, pH 8.0) to obtain banding profiles. The gels were blotted to a nylon membrane (Hybond N$^+$, Amersham, Arlington Heights, Ill.) via alkaline transfer (Sambrook et al. 1989). Each of the original Y-specific cloned inserts used to develop a SCAR was labeled with [^{32}P]dCTP (Multiprime DNA Labeling System, Amersham, Arlington Heights, Ill.) and subsequently hybridized to the blots. Prehybridization and hybridization were carried out in a hybridization oven (Techne,

Table 1. Sequence information for seven pairs of SCAR primers for Y-chromosome fragments from *S. dioica* and *S. latifolia*.

Species	Locus	Primer	Sequence[a]	Polymorphism
S. dioica	$ScB07_{750}$	ScB07F	GGTGACGCAGTTGTGGAGATG	Dominant
		ScB07R	GGTGACGCAGACCCAAATTAT	
S. latifolia	$ScD05_{1350}$	ScD05F	TGAGCGGACACGGGTGGGGC	Dominant
		ScD05R	TGAGCGGACATTGTGAGGTTACCTCC	
	$ScD12_{800}$	ScD12F	TTCCCTCCTCCTTTCTCTCTC	Dominant
		ScD12R	TAGAAGAAGATGGGTGATTTGG	
	$ScK02_{850.}$	ScK02F	GCAAATGGGTTTAGTGTAGTGGTT	Dominant
		ScK02R	GTCTCCGCAATTATCACACTAAGT	
	$ScQ14_{700}$	ScQ14F	GGACGCTTCATGACCCATTTACTC	Dominant
		ScQ14R	GGACGCTTCAGCGGGCGGGATT	
	$ScX11_{400}$	ScX11F	GGAGCCTCAGGGATTAGAAAGCCT	Dominant
		ScX11R	GGAGCCTCAGTACTAATAACATCA	
	$ScX18_{1000}$	ScX18F	GACTAGGTGGGATCGGCTG	Dominant
		ScX18R	GACTAGGTGGCCATACTAGGA	

[a]Underlining indicates the sequence from the original decamer; SCAR primers were designed to optimize T_m and to limit dimerization, therefore they may or may not contain the original 10 b of the RAPD primer.

Table 2. Optimized reaction conditions for using SCAR primers.

Locus	Hot start	Annealing *T* (°C)	Cycles
ScB07	Yes	65	40
ScD05	Yes	66	30
ScD12	Yes	60	30
ScK02	No	60	30
ScQ14	Yes	67	30
ScX11	No	60	30
ScX18	Yes	67	30

Note: See Materials and methods for PCR conditions and basal cycling parameters.

Princeton, N.J.) maintained at 65°C, following the conditions described in Sambrook et al. (1989). The blots were washed at final concentrations of 0.2× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS at 65–67°C for 30 min and exposed to x-ray film at room temperature for 10 min or 1, 4, or 16 h. Ethidium bromide stained gels and autoradiographs were digitized with a Hewlett Packard Scanjet 2CX scanner. Figures were generated with Adobe Photoshop 2.5 at 600 dpi and 256 shades of gray; contrast and brightness were adjusted to accurately depict the appearance of the originals.

Results

Characterization of Y chromosome linked sequences

We have screened 340 arbitrary decamers for Y chromosome specific bands using BSA of male and female *S. latifolia* DNA. To estimate the frequency of male-specific markers, we examined a subset of 44 primers that produced 220 easily visualized bands. Twelve fragments (5%) were specific to the male pool. Since the Y chromosome represents approximately 10% of the male diploid genome (Ciupercescu et al. 1990; Matsunaga et al. 1996), it appears that we are identifying markers at one-half the expected frequency.

Seven Y chromosome specific RAPDs were cloned and subsequently converted to SCARs (Table 1). The clones were radiolabeled, and all were shown to hybridize intensely to the original male-specific RAPD fragment (Fig. 1). Hybridizations to other fragments in males were also detected, indicating either additional homologies to other male-specific sequences or, alternatively, artifacts resulting from PCR or the separation of fragments by gel electrophoresis. For *S. latifolia*, under optimized generally stringent PCR conditions, the SCAR primers amplify fragments from the DNA of male individuals only (Table 2; Fig. 2). Fragments of similar size, but of smaller quantity (faintly stained with ethidium bromide), can be amplified from the DNA of female individuals when using lower

Fig. 2. Male-specific SCARs. Ethidium bromide stained agarose gel showing the fragments amplified, under optimized PCR conditions, from the DNA of male, but not female, *S. latifolia*. Lane 1 is a 123-bp ladder. In the even-numbered lanes, female *S. latifolia* DNA was used as the template for the PCRs, and in the odd-numbered lanes, male *S. latifolia* DNA was used as the template for the PCRs. The following sets of primers were used: lanes: 2 and 3, ScB07; 4 and 5, ScD05; 6 and 7, ScD12; 8 and 9, ScK02; 10 and 11, ScQ14; 12 and 13, ScX11; and 14 and 15, ScX18. In addition to the male-specific fragment, the primer sets ScQ14 and ScX18 also amplified fragments common to both male and female individuals.

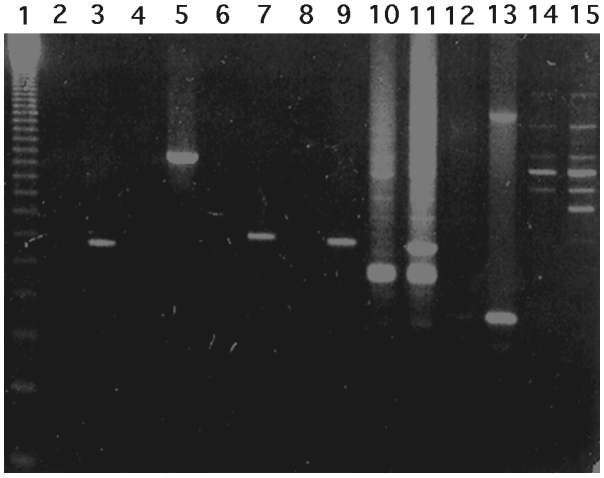
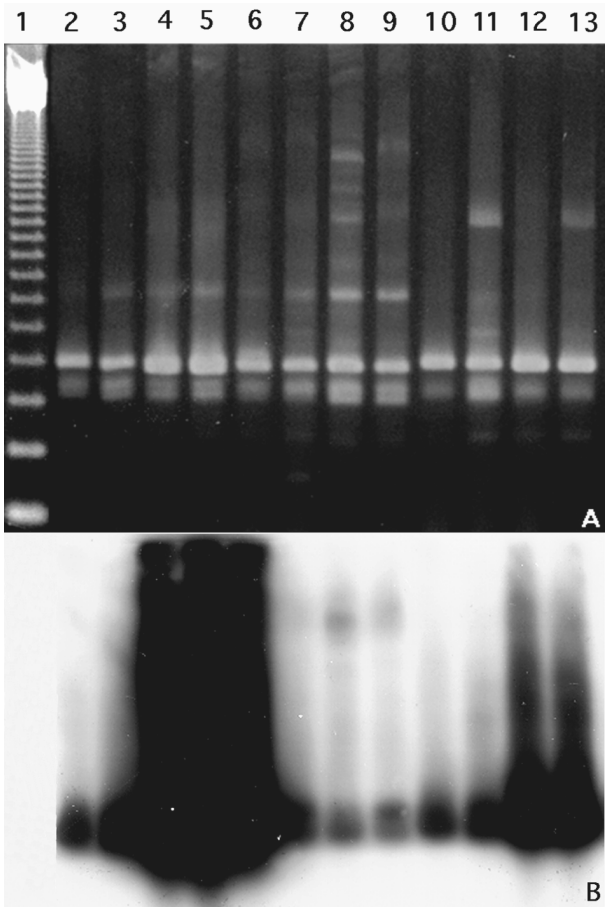


Fig. 3. Amplification, under less stringent conditions, of fragments from female *S. latifolia* that are homologous to the male-specific sequences. (A) Ethidium bromide stained agarose gel of PCR products amplified with primer set ScX11 at annealing temperatures of 50°C. Lane 1 is a 123-bp ladder. The DNA templates for the PCRs are the following: lanes: 2 and 3, female *S. latifolia*; 4 and 5, male *S. latifolia*; 6 and 7, female *S. diclinis*; 8 and 9, male *S. diclinis*; 10 and 11, female *S. dioica*; and 12 and 13, male *S. dioica*. (B) Autoradiograph of a Southern blot of the agarose gel shown in A hybridized with the radiolabeled clone of the male-specific fragment ScX11.



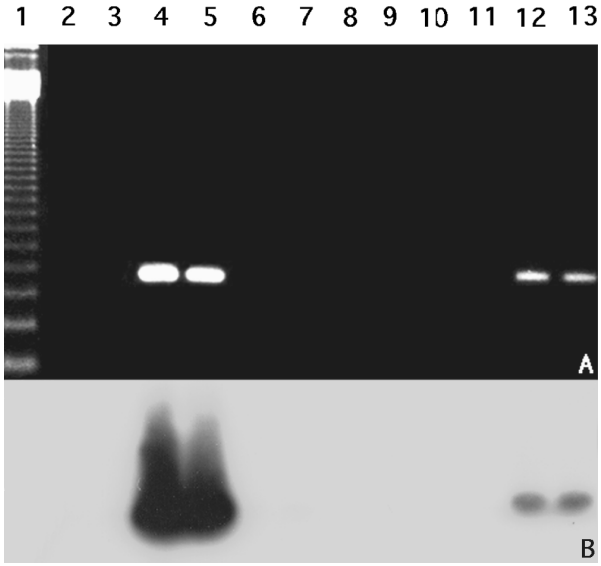
annealing temperatures (50°C). The male-derived clones hybridize, although weakly, to these fragments (Fig. 3).

The cloned Y-chromosome fragments all possess high copy number sequences. The clones were radiolabeled with ^{32}P and hybridized to Southern blots of both male and female *S. latifolia* total genomic DNA that had been digested with *Eco*R1 or *Hin*dIII. All probes produced a smeared hybridization pattern for both male and female DNA digests, suggesting the presence of dispersed high copy number DNA within each clone. This was not unexpected, since RAPD fragments examined in other species often possess high copy number sequences (Kesseli et al. 1994).

Homology of SCARs among taxa

The seven sets of primers for the SCAR loci were used to amplify homologous sequences among related species (Table 3). Under high-stringency conditions that had been optimized for the amplification of male and not female fragments in *S. latifolia* (see above), all primer sets amplified fragments in related taxa, and six of the seven primer sets (not the ScQ14 primers) amplified single bands of a size comparable to the Y-specific band of *S. latifolia* (Fig. 4). For the primer sets ScQ14 and ScX18, multiple bands were amplified for some of the PCRs, and with ScQ14 primers, none of the bands appeared to be similar in size to the Y-specific product of *S. latifolia*. Each cloned fragment was radiolabeled with ^{32}P and hybridized to a Southern blot of the SCAR profiles for the species, to verify the homology of amplified bands. Only with the multibanded patterns amplified with the ScQ14 primers did there appear to be no homology in related species with the cloned fragment from *S. latifolia*. The patterns of amplification generally fit well with known species relationships (Chater and Walters 1964; Sandbrink et al. 1989; Oxelman and Liden 1995). Fragments from the species within section *Elisanthe* were amplified often; five of seven primer sets amplified a homologous fragment in each closely related dioecious species, *S. dioica* and *S. diclinis*. Homologous fragments were rarely amplified (one of seven primer sets) in the distantly related taxa, *S. vulgaris* and *L. flos-cuculi* (Table 3).

Fig. 4. Identification of sex in related species of *Silene,* using the marker ScX11. (A) Ethidium bromide stained agarose gel of PCR products amplified under conditions optimized for *S. latifolia*. Lane identification is the same as in Fig. 3A. (B) Autoradiograph of a Southern blot of the agarose gel shown in A hybridized with the radiolabeled clone of the male-specific fragment ScX11. This marker distinguishes between the sexes of *S. latifolia* and *S. dioica* but not between those of *S. diclinis.*



Not only do the SCAR loci from the Y chromosome of *S. latifolia* show homology to loci in related species, but these loci are often also Y-linked in related dioecious species. Five of the seven primer sets amplified a fragment in male, but not in female, individuals of at least one related dioecious species. Of these, the primers for ScB07 showed male-specific amplification for both *S. dioica* and *S. diclinis*; of the other four primer sets, two (those for ScD12 and ScX11) showed male-specific amplification in *S. dioica* and two (those for ScD05 and ScX18) showed male-specific amplification in *S. diclinis*.

Table 3. Homology and sex specificity for the SCARs in *Silene* spp. and *L. flos-cuculi* under optimized conditions, as determined by Southern hybridization.

		SCAR primers						
Species	Section	$ScB07_{750}$	$ScD05_{1350}$	$ScD12_{800}$	$ScK02_{850}$	$ScQ14_{700}$	$ScX11_{400}$	$ScX18_{1000}$
S. latifolia	*Elisanthe*	Y	Y	Y	Y	Y	Y	Y
S. dioica	*Elisanthe*	Y	—	Y	A	—	Y	A
S. diclinis	*Elisanthe*	Y	Y	—	A	—	A	Y
S. noctiflora	*Elisanthe*	—	A	A	A	—	A	—
S. otites	*Otites*	A	A	—	A	—	A	—
S. vulgaris	*Inflatae*	—	—	—	—	—	A	—
L. flos-cuculi	*Lichnidiformes*	A	—	—	A	—	—	—

Note: "Y" indicates that the primer amplified the Y-specific band. "A" indicates that the primer amplified the homologous band but did not distinguish sex. "—" indicates that the primer did not amplify the homologous band.

The male-specific fragments amplified from the DNA of these dioecious taxa were generally similar in size to the corresponding fragment from *S. latifolia*. As mentioned, the primers for ScX18 amplify multiple fragments, several of which showed homology to the clone from *S. latifolia*. For this one primer set, the male-specific fragment amplified from the DNA of *S. diclinis* was larger than the corresponding fragment in *S. latifolia*.

To increase the probability of identifying homologous sequences in more distantly related species, and of characterizing sex specificity in more dioecious taxa, we relaxed the PCR conditions by lowering the annealing temperatures. In general, the amplification of homologous sequences was extended to more species for each primer set; again, homologies were confirmed by hybridizations, but sex specificity was often lost. At lower annealing temperatures, fragments identical in size to the male-specific fragment often became apparent in samples from female individuals of *S. latifolia* and the related dioecious taxa.

Discussion

Silene latifolia has a moderately large haploid genome of approximately 2.9×10^9 bp, and differences in nuclear DNA amount among male and female individuals can readily be detected by flow cytometry (Vagera et al. 1994; Meagher and Costich 1994). From cytological analyses, the Y chromosome of *S. latifolia* appears 40–50% larger than the X chromosome and comprises about 21% of the haploid genome (Westergaard 1958; Ciupercescu et al. 1990; Matsunaga et al. 1996). Assuming a random distribution of primer sites for the arbitrary decamers, roughly 10% of the fragments amplified by PCR should be from the Y chromosome. The lower frequency detected in this study could reflect a nonrandom distribution of primer sites, possibly caused by a high frequency of repetitive DNA on the Y chromosome, or by homology between the X and Y chromosomes, that decreases the number of fragments unique to males. Both possibilities are likely. Regarding repetitive DNA, Grant et al. (1994) found that the Y chromosome was rich in repetitive sequences, and all RAPD fragments that we have cloned from the Y chromosome also possess high copy number dispersed repeats. Regarding homology, dioecy is most likely a relatively recently derived state in the genus *Silene*. Despite having heteromorphic chromosomes, our data show that sequences presumably on the X chromosome are similar enough to be amplified with the primers of SCAR loci from the Y chromosome. Thus, it is also possible that many arbitrary decamers may amplify fragments from both the X and Y chromosomes and fail to provide distinguishable profiles for pooled samples of male and female DNA.

While longer primers designed from RAPDs have previously been shown to amplify alternative alleles within a taxon and homologous loci among taxa (Paran and Michelmore 1993; Kesseli et al. 1992, 1994), initially we did not expect to detect such extensive homology between the X and Y chromosomes. For autosomal sequences, SCAR primers will often amplify the alternative alleles and allow what was a dominant RAPD to be scored as a codominant marker; codominance will be observed if alternative alleles either are different sizes or possess different internal restriction sites. Since we were targeting Y-chromosome sequences in this study, we did not expect to amplify readily alternative alleles. Indeed, previous work in our laboratory studying sequences from the W chromosome of terns, the analog of the Y chromosome found in heterogametic female birds, showed that there was little homology between the sex chromosomes of these species (Sabo et al. 1994; R. Kesseli and T. Sabo, unpublished data). Studies of mammals have also shown there to be little homology between the sex chromosomes (Eicher et al. 1989). The situation in this current study was markedly different. With all SCAR primer sets except the set for ScQ14, homologous fragments from females could be amplified. In retrospect this is not surprising. Clearly the evolution of dioecy in section *Elisanthe* of the genus *Silene* is recent compared with the evolution of genetically determined dioecy in birds or mammals, which probably occurred once in a common reptilian ancestor of each lineage 150 million years ago. While there has been obvious divergence in the morphology of sex chromosomes in *S. latifolia*, sequence divergence has been more limited.

The homology for SCARs within section *Elisanthe*, which possesses five dioecious and one hermaphroditic species, is evident from the data of this study. Sandbrink et al. (1989) concluded from chloroplast restriction site variability that the hermaphroditic species, *S. noctiflora*, is distantly related to the dioecious species and not a recently evolved derivative of *S. latifolia,* as proposed by Prentice (1978). There is also no indication from our data that *S. noctiflora* is a recent derivative of *S. latifolia*, since under stringent amplification conditions, homologous sequences are amplified from *S. noctiflora* no more

often than from the dioecious taxa *S. dioica* and *S. diclinis*; five of seven primer sets amplify homologous fragments from all three taxa. These data also indicate, however, that *S. noctiflora* may still belong in section *Elisanthe,* as amplification of homologous sequences is better for this taxon than for other species outside section *Elisanthe*. Recent studies by Oxelman and Liden (1995) and Desfeux et al. (1996) continue to support the close relationship of *S. noctiflora* to the dioecious species of section *Elisanthe*.

These data also provide direct evidence that there is a common origin for dioecy among the taxa of section *Elisanthe*, but that this is not shared by *S. otites*. Homologous sequences common to the Y chromosomes of three dioecious taxa of section *Elisanthe* have been detected. No such sequences have been found in *S. otites*. While these data are the first to show homology for Y chromosomes, the results are not surprising. The close relationship of the dioecious taxa used in this study, *S. latifolia*, *S. diclinis,* and *S. dioica*, as well as of the two other species, *S. heuffelii* and *S. marizii*, has been well documented by morphological, cross-hybridization, and chloroplast-DNA studies (Chater and Walters 1964; Prentice 1978; Sandbrink et al. 1989). The distinction of dioecious *S. otites* from section *Elisanthe* has also been clear. Morphological characters (Chater and Walters 1964), as well as analyses of the internal transcribed spacer (ITS) of the rRNA genes (Oxelman and Liden 1995; Desfeux et al. 1996), show section *Otites* to be distantly related to section *Elisanthe*. The fine-scale attributes of dioecy also distinguish the two sections. Subdioecy has often been reported in *S. otites* (Correns 1928; Desfeux et al. 1996; and D. Mulcahy and R. Kesseli, personal observation), but is rare or absent in section *Elisanthe*. Morphologically distinct sex chromosomes have not been reported in *S. otites*, while such chromosomes are present in species of section *Elisanthe* (Degraeve 1980 cited in Ye et al. 1991). Finally, in section *Elisanthe*, the mode of inheritance clearly involves a dominant Y chromosome and male heterogamy, while other modes, including female heterogamy, have been indicated for *S. otites* (Correns 1928). Sex determination may still prove to be male heterogametic in all dioecious species of the genus however, but the suite of other differences strongly suggests multiple origins of dioecy in the genus; a conclusion also reached by Desfeux et al. (1996) from phylogeny based on ITS sequence analyses.

The markers developed in this study will be useful for continued investigations into the evolution of sex, phylogenetic relationships among taxa, and population dynamics of *Silene*. Charlesworth (1991) proposed that at least two mutational steps are required to evolve dioecy from hermaphroditism: a male-sterility mutation converting hermaphrodites into females and a female-sterility mutation converting the remaining hermaphrodites into males. Selection for reduced recombination and gametic disequilibrium will likely follow to eliminate the production of neuters (male and female steriles). Charlesworth (1991) noted that once recombination between the male and female sterility genes, or chromosomes carrying these genes, has been eliminated, chromosomal degeneration and divergence of one of the homologs may commence. At early stages of this process, considerable homology between X and Y chromosomes will be evident, however. In the present study, for each primer set and with less stringent PCR conditions, faint bands (but homologous based on Southern hybridizations) were amplified from female individuals, indicating the recency of dioecy. We are currently characterizing these male and female bands to assay rates of divergence within and among sexes and taxa.

These primers will also be useful for the continuing study of population dynamics in *Silene*. Lyons et al. (1995) and Taylor (1996) have both used RAPDs (Taylor also converted D05 to a SCAR) that we have previously reported as Y-linked (Mulcahy et al. 1992) to score sex ratios in natural populations. Skewed sex ratios have been repeatedly reported in *Silene* for more than 70 years (Correns 1928; Mulcahy 1967). Until the advent of PCR-based markers, the examination of primary sex ratios was intractable. We now report multiple markers, all converted to more reliable SCARs, that will allow a fuller and more accurate description of sex ratio distortion and may be the tools required for characterizing the causes of this distortion.

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