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Linkage Disequilibrium Between Incompatibility Locus Region Genes in the Plant *Arabidopsis lyrata*

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ABSTRACT

We have studied diversity in *Arabidopsis lyrata* of sequences orthologous to the *ARK3* gene of *A. thaliana*. Our main goal was to test for recombination in the *S*-locus region. In *A. thaliana*, the single-copy *ARK3* gene is closely linked to the non-functional copies of the self-incompatibility loci, and the ortholog in *A. lyrata* (a self-incompatible species) is in the homologous genome region and is known as *Aly8*. It is thus of interest to test whether *Aly8* sequence diversity is elevated due to close linkage to the highly polymorphic incompatibility locus, as is theoretically predicted. However, *Aly8* is not a single-copy gene, and the presence of paralogs could also lead to the appearance of elevated diversity. We established a typing approach based on different lengths of *Aly8* PCR products and show that most *A. lyrata* haplotypes have a single copy, but some have two gene copies, both closely linked to the incompatibility locus, one being a pseudogene. We determined the phase of multiple haplotypes in families of plants from Icelandic and other populations. Different *Aly8* sequence types are associated with different *SRK* alleles, while haplotypes with the same *SRK* sequences tend to have the same *Aly8* sequence. There is evidence of some exchange of sequences between different *Aly8* sequences, making it difficult to determine which ones are allelic or to estimate the diversity. However, the homogeneity of the *Aly8* sequences of each *S*-haplotype suggests that recombination between the loci has been very infrequent over the evolutionary history of these populations. Overall, the results suggest that recombination rarely occurs in the interval between the *S*-loci and *Aly8* and that linkage to the *S*-loci can probably account for the observed high *Aly8* diversity.

PLANT self-incompatibility loci are subject to frequency-dependent selection with a fertility advantage to rare alleles, a form of balancing selection that acts to keep allele frequencies intermediate and prevents loss of alleles from populations (Wright 1939; Vekemans and Slatkin 1994; Schierup *et al.* 1998; Uyenoyama 2000). This long-term maintenance of alleles is predicted to lead to high diversity at sites genetically closely linked to the sites where selection acts (Nordborg *et al.* 1996; Charlesworth *et al.* 1997; Innan and Nordborg 2003; Schierup *et al.* 2000a; Wiuf *et al.* 2004). In *SRK*, which encodes the pistil receptor kinase, high diversity has indeed been found in the intracellular kinase domain, which is unlikely to be subject to balancing selection (Charlesworth *et al.* 2003a).

In Brassica, there are three closely linked *S*-locus genes, only two of which are involved in the incompatibility recognition response. *SRK* encodes the stigma receptor kinase, and *SCR* encodes the pollen ligand recognized by the *SRK*'s extracellular *S*-domain to give the self-incompatibility (SI) phenotype (Nasrallah 2000). To maintain functional incompatibility, the *SCR* and *SRK* genes must be in linkage disequilibrium, with each haplotype carrying alleles at the two loci that are recognized as incompatible. Recombination between these *S*-genes will lead to nonfunctional (self-compatible) haplotypes. Low recombination may thus have evolved in the *S*-locus region (Casselman *et al.* 2000) to maintain incompatible combinations of *SRK* and *SCR* alleles during the long time periods during which incompatibility alleles exist.

If recombination is reduced in the whole region, diversity should be high in loci across the nearby genome region. Several nonincompatibility loci are present in the *S*-locus region, physically close to the genes involved in the SI response (Kusaba *et al.* 2001), and molecular diversity studies of such genes are thus of interest in testing these ideas and gaining an understanding of the combined effects of both balancing selection and recombination across this region. Here we study one such locus, *Aly8*, in the self-incompatible plant, *A. lyrata*, a distantly related species in the same plant family as Brassica, which is closely related to *A. thaliana*. *Aly8* is the ortholog of the *A. thaliana* *ARK3* gene, which is not involved in incompatibility

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(Kusaba *et al.* 2001). In two haplotypes physically mapped by Kusaba *et al.* (2001), *Aly8* is located at differing distances from the *S*-genes (in haplotype *a*, it is ~5 kb from *SCR* and 10 kb from *SRK*, and in haplotype *b* the distances are 50 and 30 kb, respectively). Other haplotypes may have gene arrangements and physical distances different from both of these, as large size differences are seen in Brassica (Suzuki *et al.* 2000).

Like *SRK*, *Aly8* has an *S*-domain sequence and a kinase region, and it has been shown to have high diversity (Charlesworth *et al.* 2003b), but it is not involved in SI. Expression studies of *ARK3* in *A. thaliana* and of the probable ortholog, *SFR2*, in *Brassica oleracea* (Kai *et al.* 2001) show responses to bacterial infection and wounding (Pastuglia *et al.* 2002) and expression in vegetative, not reproductive, tissues (Dwyer *et al.* 1994).

ARK3 is a single-copy gene in *A. thaliana*, at least in the *Col*-0 strain, and in both physically mapped *A. lyrata* haplotypes (Kusaba *et al.* 2001). However, when PCR amplification products from this locus were cloned and sequenced from individual *A. lyrata* plants, more than two sequences were sometimes found, suggesting the presence of multiple, paralogous loci (Charlesworth *et al.* 2003b), which may partly explain *Aly8*'s high sequence diversity (Charlesworth *et al.* 2003b). To study diversity at this locus, and to test whether it is elevated because of linkage to the *S*-locus region, it is therefore necessary to establish how many loci are present, to assign sequences to individual loci and determine which are linked to the *S*-loci. Here, we show, using family data, that some, but not all, *S*-haplotypes have two copies, and that, in such haplotypes, both *Aly8* copies are linked to the *S*-locus.

To test for close linkage over evolutionary time, which is expected if the *S*-locus region recombines unusually rarely, we established the phase of *SRK-Aly8* haplotypes, using families made by crossing plants with known *SRK* alleles. Finding consistent associations between *SRK* and *Aly8* alleles across multiple populations (as we observe) suggests linkage disequilibrium maintained for considerable times. This constitutes much stronger evidence for low recombination in the *S*-locus region than family data, where even a few genotyping errors can give incorrect estimates (*e.g.*, Isidore *et al.* 2003). Population genetic data can stringently test for the occurrence of recombination and potentially restrict estimates of the recombination frequency to lower values than can be detected in family data, because even very infrequent recombination eliminates associations over multiple generations.

MATERIALS AND METHODS

Plant materials and notation: To investigate the genetics of the *Aly8* sequences, and determine whether there are only two loci or more than two, and their linkage relations, we initially analyzed five full-sib families of *Arabidopsis lyrata*, originating from crosses between plants from a single population at Mt. Esja near Reykjavik, Iceland, with known *SRK* sequences; the parents of these crosses are designated as 00B or 99R, followed by individual identification numbers (see also Bechsgaard *et al.* 2004; Mable *et al.* 2004). The numbering system for the families and sequences that is used in the tables, based on the sequence lengths, is described below in *Fragment length analysis*. To test whether the associations between *SRK* and *Aly8* are maintained in independent plants with the same *S*-alleles, we also studied segregation in five additional families—42, 43, 44, 47, and 49—whose parents originated from several different Icelandic populations (Bechsgaard 2002). The locations of the populations are shown in Figure 1.

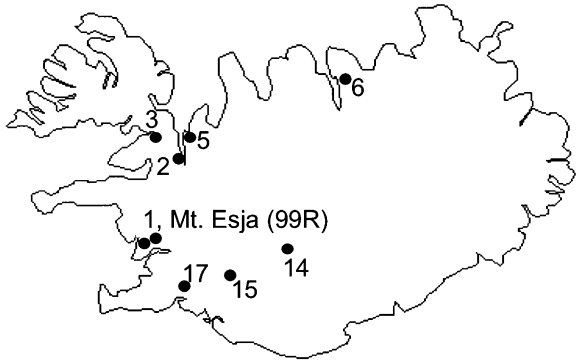


Figure 1.—Locations of the Icelandic populations from which the plants were sampled.

Among the *A. lyrata* haplotypes tested, we included genotypes carrying *SRK* corresponding to two of the three *A. thaliana SRK* haplogroups (Shimizu *et al.* 2004). These were haplotypes carrying the *A. lyrata* S_{16} allele (Schierup *et al.* 2001) and *A. lyrata* S_{37} (similar to Hap A); amplification from the S_{36} haplotype (Hap C) showed that the *Aly8* sequence was probably of the long type, but no useful sequence was obtained. In addition, BAC clones from the two physically mapped haplotypes, carrying the S_{13} and S_{20} alleles, originating from Indiana (Kusaba *et al.* 2001), were kindly provided by June Nasrallah (Cornell University). To assess *Aly8* sequence variability within haplotypes with a given *SRK*, we included as many non-Icelandic individuals as possible with known *SRK* alleles. In what follows, we refer to the *SRK* sequences as *S*-alleles and denote them by subscript numbers, for brevity. S_1 and S_9 haplotypes were studied in plants from an *A. lyrata* mapping family derived from crossing plants from two populations, Mjallom from Sweden, and Karhumaki from Russia (Kuittinen *et al.* 2004), and an S_1 haplotype was studied from an S_1/S_{34} heterozygote individual from Plech, Germany, provided by M. H. Schierup (the *Aly8* sequence from the S_{34} haplotype was not determined).

PCR and sequencing: Previously published *Aly8 S*-domain sequences were compared with sequences from the paralogous loci *Aly13* and *Aly10* (Charlesworth *et al.* 2003b) and primers specific for different portions of *Aly8* were designed; the regions studied include parts of the *S*-domain and parts of intron 1 (Figure 2). To find genetic markers for studying segregation, we first sequenced *Aly8* from plants in families from one population, Mt. Esja. PCR reactions were run using genomic DNA with QIAGEN (Valencia, CA) Taq polymerase under standard conditions, using the primers J4F 5′-GGTCAGATGGGTGTGTGCGA-3′ and J4R 5′-CCGAACGCGTCAACATCTCAATCA-3′ or J4F and J1R 5′-GGCATTTGGGTGTACCTTAAACCA-3′. Other primers used are listed in supplemental Table 1 at http://www.genetics.org/supplemental/. The *Aly8* allele linked to allele S_6 (see

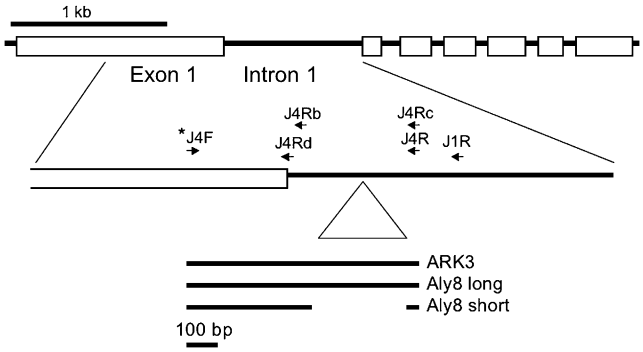


FIGURE 2.—Diagram of the *Aly8* locus, showing the whole gene, the region sequenced, and the locations of the primers used for amplification and detection of alleles, including the primer labeled with the fluorescent dye (indicated by *). The forward primer is located in the first exon and all reverse primers are in the first intron. The triangle indicates the large indel distinguishing the "L" alleles. The part of *Aly8* previously sequenced (SCHIERUP *et al.* 2001) is located just upstream of the region studied here.

below) did not amplify with the original primer pair (see RESULTS), but could be amplified and sequenced using the J1R reverse primer, allowing a primer specific for this allele to be designed (J4Rc 5′-CACATACGAACGCGTCAAGAAGT-3′). When possible, parental plant alleles were sequenced, but offspring were used if the parental DNA samples failed to amplify. GenBank accession numbers are given in the Figure 3 legend.

We tested offspring selected to maximize the chances of finding and identifying all *Aly8* alleles, on the assumption (based on preliminary results of M. H. Schierup) that at least one of the *Aly8* loci should be linked to *SRK* (this is confirmed by our results, see below). Amplified fragments from several plants were cloned using the Invitrogen (San Diego) TOPO TA cloning kit for sequencing. Bacterial colonies were picked and used for an additional round of PCR with the same primers, followed by automated sequencing on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA). Fragments were sequenced in both forward and reverse directions, and the resulting sequences were examined using the Sequencher software (version 4.2.2; GeneCodes, Ann Arbor, MI) with alignment and base-calling adjustments after manual comparison of the chromatograms. Multiple colonies were sequenced from each individual to check for PCR errors and PCR recombination. The sequences from a set of 18 Mt. Esja plants (see Table 2 and Figure 3 for details) have many single-nucleotide differences and a strikingly high indel-to-polymorphism ratio.

Fragment length analysis: Length differences were used to genotype individuals in the families (see below). To score presence of sequence variants in the progeny plants from our families, PCR was run using the forward primer J4F labeled with a fluorescent dye (6-Fam, Applied Biosystems), together with the reverse primer J4R. The PCR products were separated and the alleles detected in an ABI 3730 capillary sequencer (Applied Biosystems) and compared with a size standard ladder (ABI GeneScan LIZ500). The resulting output was analyzed with GeneMapper 3.0 (Applied Biosystems). *Aly8* sequences were denoted by their lengths (*e.g.*, 477 denotes an estimated size of 477 bp). However, the amplified products are rather large for this kind of analysis, and sequencing showed that the true lengths of alleles 560–575 are typically ~10 bp shorter than the values these names suggest.

The initial results showed that some sequences contain a large insertion relative to others. However, fragments approaching an expected size of 800 bp could not be detected in the fragment length analysis or, if detected, could not be sized accurately. A second, internal, primer, J4Rb 5′-GGTGG GAAGACGACAAATCATTC-3′ (Figure 2), was therefore designed to amplify a reliably scorable portion of the longer alleles. In what follows, an "L" is added to the lengths of sequences that were amplified with this reverse primer.

In three families (43, 47, and 49), the region used in the typing included no length polymorphism between the alleles of one parent. The *Aly8* sequences in the first two families were resolved by sequencing, while in family 49 the S_{12}- and S_1-linked *Aly8* sequences are distinguishable after digestion with *Mfe*I (which cut the S_{12}-linked *Aly8* sequence, but not that linked to S_1). In addition, two haplotypes, carrying *S*-alleles S_{38} and S_1 (see below), both amplified a sequence 570 bp long. These were distinguished by an additional PCR amplification with labeled J4F primer together with a new primer, J4Rd 5′-GCCCATTATCTATCAACCGTTA-3′, which gave a band of length 399 bp for haplotype S_{38} and 395 bp for the S_1 haplotype; these alleles were thus labeled 570^{399} and 570^{395}, respectively. This primer combination also amplified some of the other alleles, helping to confirm presence or absence of these sequences.

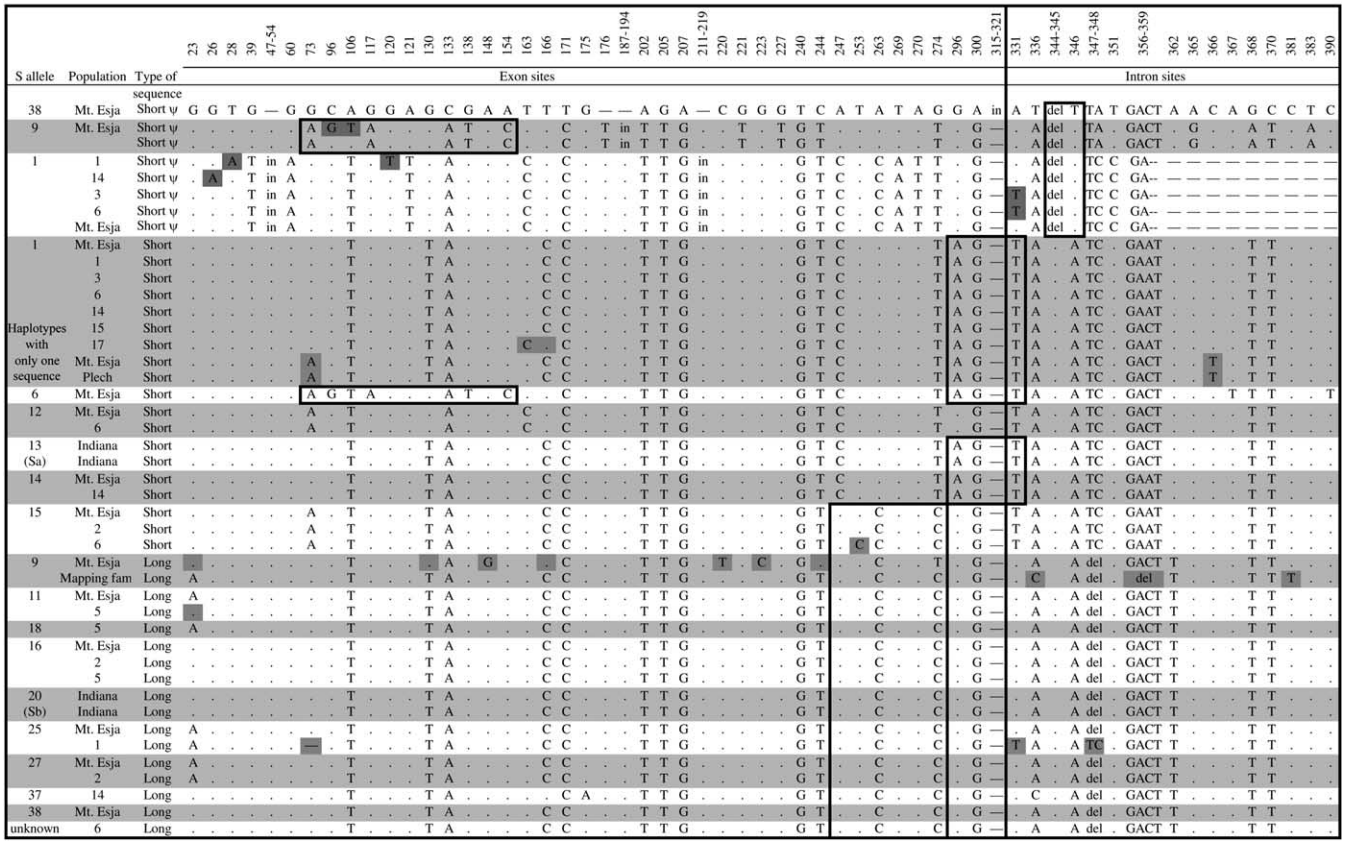
To determine the number of loci, it was important to detect all *Aly8* sequences present. We therefore made repeated PCR amplifications, using multiple primers, decreased annealing temperatures, and a range of template concentrations. All discrepancies were rechecked with new PCR reactions. In some cases (see below), allele-specific primers were designed to detect *Aly8* sequences.

Sequences from other individuals with known *S* genotypes: When length typing did not differentiate alleles, we inferred phases from the alleles' sequences whenever informative variants allowed. Since our initially established haplotypes allowed prediction of *Aly8* sequences, we assumed that a band length consistent with the expected one, given the haplotype's *SRK* sequence, represents the same *Aly8* sequence and then checked by sequencing. For instance, S_{15} haplotypes from the Mt. Esja population yield a band of length 570, but we cannot always distinguish this from the 570 band of the S_1 haplotype (in the Mt. Esja population, these haplotypes are distinguished by the absence of the 477 bp band, indicating a second *Aly8* copy, in S_{15}, but this copy is also absent from some S_1 haplotypes, see below); in a family from a different population segregating for S_{15}, we assumed that this band represents the same *Aly8* sequence as the S_{15}-associated allele, not the S_1 one.

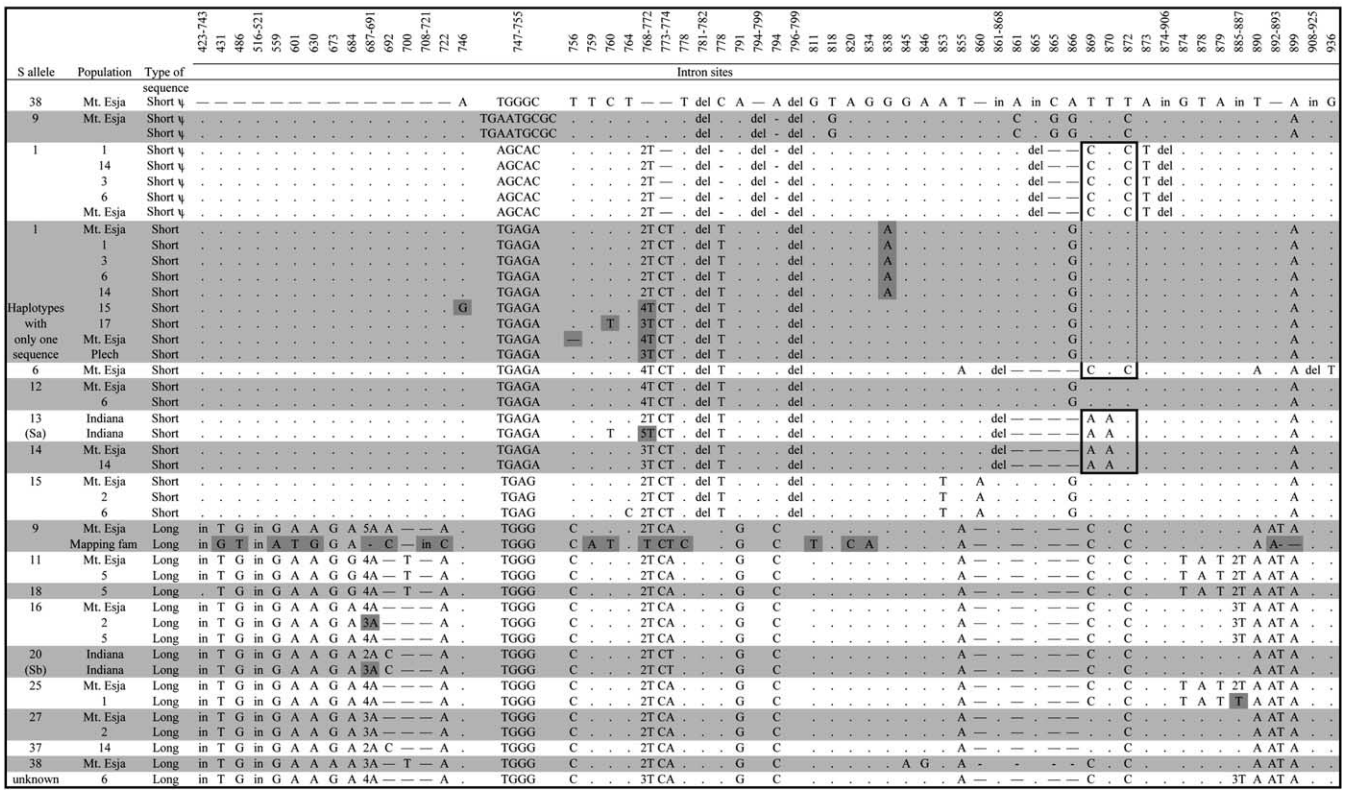
As just described, *Aly8* bands of the same size were sometimes obtained from haplotypes with different *SRK* alleles (particularly with primer J4Rb, specific to the long alleles). Multiple alleles of the same length were sequenced to test whether these alleles differ within or between different *S*-haplotypes, and sequence differences were often found between *S*-haplotypes (see RESULTS). These different *Aly8* sequences were given a superscript, as above, or an additional letter (a, b, c, etc.) to identify them uniquely.

Sequence analysis: Sequence differences between clones from the same *S*-haplotype of a single individual were treated as errors during amplification and cloning, and a consensus sequence for the *Aly8* from the haplotype was deduced on the basis of all clones of the haplotype from the plant. For some individuals, however, only one clone of a given allele was sequenced, so that some of our variants may not be true differences. Since our aim is to test for associations between particular *SRK* and *Aly8* sequences, any such errors will be conservative for our conclusions (*i.e.*, will overestimate variability within a given *S*-haplotype and obscure associations).

A



B



Diversity estimates were calculated using the software DNAsp (Rozas and Rozas 1999), which was also used to test for selection (see below) and to detect gene conversion tracts. We also tested for gene conversion using GeneConv (http://www.math.wustl.edu/~sawyer/geneconv/) (Sawyer 1989). The alignment was also used to construct neighbor-joining (NJ) trees using MEGA software (version 3, Kumar *et al.* 2004). When the sequences of the *A. thaliana* paralog of *ARK3*, or the Brassica ortholog, *SFR2* (accession no. X98520), were included, only the exon sequences aligned with the *Aly8* sequences.

RESULTS

Sequences and polymorphisms of *Aly8*: Table 1 lists the Mt. Esja families initially studied and their *Aly8* sequences, using the notation described in materials and methods for *Aly8* allele lengths and superscripts or additional letters to distinguish between alleles with the same length but different sequences. The number of *Aly8* sequences detected in individual plants ranged from one, in some individuals homozygous for the S_6 allele, to at most four sequences in some individuals in family 12 (see below). Thus, no more than two copies of the locus appear to exist. All attempts to detect more alleles, including additional PCR and cloning using the alternative reverse primers, were unsuccessful.

In three of the haplotypes with two *Aly8* copies, one sequence is a pseudogene. S_9 and S_{38} haplotypes have long plus short (pseudogene) sequences, while in the S_1 haplotypes, both sequences were short. Each of the three pseudogene sequences has a unique mutation making it nonfunctional, although all three share a deletion of 2 bp near the start of intron 1 (see Figure 3 below). The 477 (S_1) and 570^{399} (S_{38}) sequences have indels causing frameshift mutations, while the 575 (S_9) sequence has an in-frame insertion that introduces a stop codon. Indels in exons were present only in these pseudogene sequences.

We estimated divergence values from *ARK3* separately for the short (nonpseudogene) and long *Aly8*'s, since they may be different loci (see below). For synonymous sites, these are 0.190 and 0.170, respectively (0.191 for all short sequences), somewhat higher than the average of 0.154 based on a set of 34 genes (Wright *et al.* 2003); net divergence values, after correcting for the high *Aly8* diversity (Nei 1987), are very similar to this mean. Nonsynonymous site divergence values (K_a) for short (nonpseudogene) and long *Aly8*'s are 0.047 and 0.045, and both sets have very similar K_a/K_s values, close to 20%. Thus the *Aly8* sequences that are not evident pseudogenes show evidence of selective constraint and are probably functional, or were until recently.

Testing linkage to *AlSRK*: To test *Aly8* sequences for linkage to the *SRK* gene in *A. lyrata*, and to test for the presence of multiple gene copies, we studied segregation of *Aly8* variants in five families from the Mt. Esja population (Table 1) and in further families from six other Icelandic populations. Using a combination of allele-specific PCR, fragment length analysis, and, where necessary, sequencing (mostly to distinguish between sequences of length 419), we identified, as far as was possible, the sequences present in each individual. Cosegregation with *SRK*, indicating linkage, was found, and we inferred the phase of the alleles in the haplotypes. In some families, haplotypes were not unambiguously evident, and some of our conclusions (*e.g.*, for the Mt. Esja family 23, see Table 1) are based on consistency with haplotypes in other families; *i.e.*, we assumed linkage disequilibrium between *SRK* and *Aly8*. This is supported by finding identical haplotypes in multiple families, including ones derived from different populations, only one of which is close to Mt. Esja (Figure 1, Table 1). The detailed data are not shown, for populations other than Mt. Esja, but the overall results are summarized in Table 2.

In total, 328 of 333 haplotypes with confirmed *SRK* sequences were genotyped for *Aly8* in the Mt. Esja families, yielding at most three possibly recombinant haplotypes, all in plants from which DNA was not available to check the *SRK* sequences; for all other possible recombinant plants, rechecking ruled out recombination. Given that *S*-locus genotyping is difficult, due to the presence of paralogous *S*-domain genes, and checking is always desirable, we infer linkage between the *S*-locus and the *Aly8* locus (or loci, in some haplotypes), as has also been shown in *A. halleri* (X. Vekemans and V. Castric, personal communication). Below, we briefly highlight some important points and describe the few discrepancies.

Both parents of family 12 were heterozygous for two different *SRK* alleles (Table 1), and all offspring had three or four *Aly8* sequences. Three of the four inferred parental haplotypes carried two *Aly8* copies. Of 110 progeny-inferred haplotypes in this family, there are two apparent discrepancies from the parental phases of *SRK* and *Aly8* alleles (all others proved, on retyping, to be *S*-locus typing errors). The discrepant individuals apparently inherited *Aly8* sequences of length 562 with S_9, instead of the expected S_{14}. However, not enough DNA

Figure 3.—Polymorphisms in the region of the *Aly8* gene sequenced. The positions of all SNPs and indel variants are shown (positions are numbered according to an alignment with the *A. thaliana ARK3* sequence). The different *S*-haplotypes are indicated by alternating white and gray stripes. Variants among sequences from the same *S*-haplotypes are indicated in dark gray. The top sequences are the short sequences, and those that are evidently pseudogenes are indicated, while the long sequences are given below. Regions possibly involved in gene conversion events are boxed. Note that the single-nucleotide deletion at position 73 in the coding region of one *Aly8* allele from an S_{25} haplotype was found in the only clone from this individual that yielded an S_{25} sequence, and so this variant is not certain. GenBank accession nos. are DQ394305–DQ394346.

TABLE 1

Segregation results in five full-sib families from the Mt. Esja (Iceland) population

Parent 1 sequences						
477	570^{395}	570^{399}	L419b	No. of offspring	*S*-allele	Conclusion
			Family 12 (parents 00B-17/3 × 00B-17/5)			
+	+	−	−	28	S_1	Parental
−	−	+	+	27	S_{38}	Parental
Parent 2 sequences						
562		575	L415	No. of offspring	*S*-allele	Conclusion
			Family 12 (parents 00B-17/3 × 00B-17/5)			
+		−	−	19	S_{14}	Parental
−		+	+	34	S_9	Parental
+		−	+	1	S_9	Possible recombinant?
Parent 1 sequences						
477	570^{395}	570^{399}	L419b	No. of offspring	*S*-allele	Conclusion
			Family 14 (parents 00B-17/3 × 00B-22/1)			
+	+	−	−	14	S_1	Parental
−	−	+	+	18	S_{38}	Parental
+	+	+	+	1	S_{38}	Possible triploid
Parent 2 sequences						
477	570^{395}		L419a	No. of offspring	*S*-allele	Conclusion
			Family 14 (parents 00B-17/3 × 00B-22/1)			
+	+		−	14	S_1	Parental
−	−		+	18	S_{25}	Parental
+	+		+	1	S_{25}	Possible triploid
Parent 1 sequences						
539		L419c		No. of offspring	*S*-allele	Conclusion
			Family 23 (parents 00B-27/3 × 00B-29/3)			
+		−		17	S_6[a]	Parental
−		+		10	S_{27}	Parental
Parent 2 sequences						
539		575	L415	No. of offspring	*S*-allele	Conclusion
			Family 23 (parents 00B-27/3 × 00B-29/3)			
+		−	−	16	S_6	Parental
−		+	+	11	S_9	Parental
Parent 1 sequences						
572		L419d		No. of offspring	*S*-allele	Conclusion
			Family BM02-H2A, -H2B, and -H4B (parent 99R 14/1 × 99R 35/5)			
+		−		9	S_{12}	Parental
−		+		9	S_{16}	Parental
Parent 2 sequences						
L419e		575	L415	No. of offspring	*S*-allele	Conclusion
			Family BM02-H2A, -H2B, and -H4B (parent 99R 14/1 × 99R 35/5)			
+		−	−	9	S_{22}	Parental
−		+	+	9	S_9	Parental

(*continued*)

TABLE 1

(Continued)

Parent 1 sequences					
477	570	L419a	No. of offspring	*S*-allele	Conclusion
Family BM02-H5A and H5B (parents 99R 38/1 × parent 99R 19/2)					
+	+	−	17	S_1	Parental
−	−	+	16	S_{25}	Parental
+	+	+	1	S_1	Possible recombinant
Parent 2 sequences					
477	570	539	No. of offspring	*S*-allele	Conclusion
Family BM02-H5A and H5B (parents 99R 38/1 × parent 99R 19/2)					
+	+	−	11	S_1	Parental
−	−	+	23	S_6	Parental

For each family, parents 1 and 2 are named in the column heading, in that order, and each row shows the *SRK* alleles of each parent and the *Aly8* sequences we infer to be in each of the *S*-haplotypes present in the family.

[a] The primer combination J4F and J4R, used for PCR for the other plants, did not yield a product in the individuals homozygous for the S_6 allele, but amplification using the J1R reverse primer with J4F yielded a product, which was sequenced; a primer specific to this sequence was used to genotype 27 individuals (2 failed to amplify with all primers).

was available to confirm their *SRK* genotypes, which may, therefore, have been mistyped. Thus these plants do not provide strong evidence for recombination between *SRK* and *Aly8*.

In family 14, one individual yielded all three *Aly8* sequences present in the family and also apparently carries all three parental *S*-alleles. This is the one confirmed exceptional plant, but it does not suggest recombination. It could be a triploid, or the sample may be contaminated (it cannot be tested further, as the DNA is exhausted).

The fourth family group, BM02-H2A, -H2B and -H4B, confirms the phase of the S_9 haplotype already inferred and establishes three new *S*-haplotypes: 572-S_{12}, L419d-S_{16}, and L419e-S_{22}, while the fifth family, BM02-H5A (and H5B, with the same parents), includes three previously established ones (Table 1). Five progeny in this family had previously been tentatively scored as S_1 homozygotes, but with some uncertainty (B. Mable, personal communication); our *Aly8* sequences confirm this *S*-locus genotype. Two progeny (not shown in Table 1) do not fully conform to the expected patterns, but neither one strongly indicates recombination. One individual had the 477, 570^{395}, and 539 *Aly8* sequences, suggesting the *SRK* genotype S_1S_6, but only S_1 is confirmed. The other has the 477 and 570^{395} sequences (suggesting that it carries S_1) plus the L419a sequence, which, given the haplotypes inferred in this family, predicts the presence of the S_{25} allele, but this was not detected. This plant could be mistyped at the *S*-locus or possibly a recombinant.

The results, taken together, clearly support the conclusion of at most two *Aly8* loci. Most *S*-haplotypes have only one *Aly8* copy, but the three haplotypes carrying S_1,S_9, and S_{38} have two different closely linked *Aly8* sequences, one of which is nonfunctional.

Linkage disequilibrium in the Mt. Esja population and conservation of haplotypes in additional populations: The homogeneity in lengths that is seen in the Mt. Esja families just described, with the *Aly8* sequence types from plants with the same *SRK* alleles being similar, is consistent with linkage disequilibrium between these two loci, but the sample size is small, due to the laborious linkage testing involved in inferring haplotypes. We therefore examined 20 additional individuals from the same population. Plants with known *S*-alleles were tested for *Aly8* sequences with distinctive lengths known from the family data to be present in haplotypes with those particular *S*-alleles; finding the same associations of *Aly8* and *SRK* sequences would support the hypothesis of linkage disequilibrium. For this test, we used two primer pairs, J4F and J4R, which amplify most *Aly8* alleles, plus J4Rc, which amplified the sequence found in S_6 haplotypes. Unlike the family analyses, the sequences were not verified by sequencing; we simply recorded presence or absence of bands of the expected lengths, and thus we do not record the long sequences seen with this set of primers, because they cannot be distinguished without sequencing. In two cases the 477 band found in the initial S_1 plants was absent in a plant carrying S_1. This appears to be genuine heterogeneity among S_1 haplotypes, as other instances with only the 570^{395} band were also found in other populations (see Figure 3 below). Apart from this, 16 of the plants had the expected *Aly8* bands (Table 3). The absence of an expected sequence in two plants suggests recombination in the ancestry of these haplotypes (S_{14} and S_6). Failure to see unexpected bands in the majority of the

TABLE 2

Summary of the *S*-haplotypes found among the parents of the families from all seven Icelandic populations (Mt. Esja and six other populations), and the *Aly8* sequences found, using the numbering system for different sequences explained in MATERIALS AND METHODS

	Aly8 sequences detected			
SRK allele	Long	Short	Populations	Families
S_1	None	477 and 570^{395}	Mt. Esja	12, 14, BM02-H5A, H5B
			1	44
			6	43 and 47
			14	42
			Russia or Sweden	Mapping family
S_1	None	570^{395}	15	49
S_6	None	539	Mt. Esja	23, BM02-H5A, H5B
S_9	L415	575	Mt. Esja	12, 23, BM02-H2A, H2B, H4B
	L419	575	Russia or Sweden	Mapping family
S_{11}	L419f	None	5	47
S_{12}	None	572	Mt. Esja	BM02-H2A, H2B, H4B
			6	47 and 49
S_{14}	None	562	Mt. Esja	12
			14	42
S_{15}	None	570	2	44
			6	43
S_{16}	L419d	None	Mt. Esja	BM02-H2A, H2B, H4B
			5	42
			2	43
S_{18}	L419g	None	5	42, 47
S_{22}	L419e	None	Mt. Esja	BM02-H2A, H2B, H4B
			15	49
S_{25}	L419a	None	Mt. Esja	14, BM02-H5A, H5B
			1	44
S_{27}	L419c	None	Mt. Esja	23
			2	43, 44
S_{38}	L419b	570^{399}	Mt. Esja	12, 14
$S_{unknown}$	L419i	None	6	49

Segregation data for the Mt. Esja families are in Table 1 (detailed data from the other families are not shown).

120 tests supports linkage disequilibrium, since each plant was tested with both primer pairs. The two instances when unexpected bands were seen do not necessarily indicate recombination, as contamination is possible. Alternatively, the 477 band in plant 99R-28/3, in which S_1 has not been detected, may simply be a case of a missed *S*-allele, and the 539-bp band in 99R-19/1 (which has S_1 and S_{25}, but not the S_6 allele), may be another instance of a plant with three *SRK* sequences, like those occasionally found (see above).

Since the individuals described so far all originated from the same population as the parental plants of the families, close relationships may account for some of the linkage disequilibrium (WAKELEY and LESSARD 2003). To test whether the same associations are found in other populations, we determined phases of haplotypes from five additional families, involving parents from different Icelandic populations, most at considerable distances from Mt. Esja (Figure 1). Only one plant did not behave as expected, of 194 progeny chromosomes. One haplotype classified as S_1 had the L419a sequence, normally found with S_{25}; this individual may be triploid, like the plant in family 14 (see above). The results confirm the presence of seven haplotypes with associations of *Aly8* and *SRK* identical to those in Mt. Esja (S_1, S_{12}, S_{14}, S_{16}, S_{22}, S_{25}, and S_{27}), plus four new haplotypes, S_{15} (in two families, derived from different populations), and three with L419 sequences: L419f-S_{11}, L419g-S_{18} (in two different population 5 parents), and a haplotype with an unknown *S*-allele, denoted L419i-$S_{unknown}$ (Tables 2 and 3).

Rigorous tests for linkage disequilibrium between *Aly8* and the *S*-locus require testing whether different *S*-haplotypes always, or generally, carry different *Aly8* alleles, and, even more importantly, whether different examples of the same *S*-haplotype generally carry the same *Aly8* sequence. We therefore cloned and sequenced *Aly8* from as many *S*-haplotypes from our families as possible, including the same *S*-haplotype from different populations, when available (supplemental Table 1 at http://www.genetics.org/supplemental/). Sequencing allowed us to increase our sample size by

TABLE 3

***Aly8* sequences present in individuals with known *S*-alleles from the Mt. Esja population**

Individual	*S*-alleles detected	477 (S_1)	539 (S_6)	562 (S_{14})	572 (S_{12})	575 (S_9)	L415 (S_9)
99R-5/4	S_1	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-9/5	S_1 S_{14}	+[a]	—[b]	—[a,c]	—[b]	—[b]	—[b]
99R-10/2	S_1 S_x	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-11/1	S_1	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-11/3	S_6	—[b]	+[a]	—[b]	—[b]	—[b]	—[b]
99R-13/3	S_1 S_x	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-15/2	S_1? S_{25}	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-16/1	S_1 S_6	—[a]	—[a,c]	—[b]	—[b]	—[b]	—[b]
99R-18/1	S_1 S_x	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-19/1	S_1 S_{25}?	+[a]	+[b,c]	—[b]	—[b]	—[b]	—[b]
99R-20/2	S_1 S_{15}	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-25/1	S_1 S_9	+[a]	—[b]	—[b]	—[b]	+[a]	+[a]
99R-28/1	S_1S_{25}	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-28/3	S_{12}	+[b,c,d]	—[b]	—[b]	+[a]	—[b]	—[b]
99R-32/1	S_1 S_{25}	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-32/2	S_1 S_{25}	—[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-36/3	S_1 S_{22}	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-37/4	S_1 S_9	+[a]	—[b]	—[b]	—[b]	+[a]	+[a]
99R-43/1	S_1 S_{25}	+[a]	—[b]	—[b]	—[b]	—[b]	—[b]
99R-44/4	S_{12}	—[b]	—[b]	—[b]	+[a]	—[b]	—[b]

Each column shows results for a given sequence length and the *S*-allele with which the sequence was associated in the families in Tables 2 and 3. "+" denotes cases when a sequence was detected in the individual, while "—" symbols indicate that the sequence in the column was not found.

[a] An *Aly8* sequence was expected in the plant represented by the row.

[b] Sequences that were *not* expected to be present.

[c] All cases that deviate from the expected pattern, except for two plants with S_1 but with no 477 band (see text).

[d] 99R-28/3 has the 477 band, but no S_1 allele has been detected.

inferring sequences from additional plants whose *SRK* sequences were known, but from which progeny were not available, using reasoning similar to that in the previous section. If a plant sampled from nature carries a known *SRK* sequence, and we have determined the phase of haplotypes carrying this allele in one or more families, we can predict the *Aly8* sequence that this haplotype should carry if the associations observed in the families are maintained in unrelated plants. If sequencing reveals the presence in the plant of an *Aly8* sequence identical to the expected one, we can infer that this plant carries the previously observed *SRK-Aly8* haplotype. In total, we sequenced *Aly8* from 16 *S*-haplotypes.

One indication of linkage disequilibrium is that, if *SRK* and *Aly8* alleles are indeed associated over long evolutionary times, haplotypes with different *SRK* alleles should always have different *Aly8* alleles. The two haplotypes from the United States studied by Kusaba *et al.* (2001) were therefore added to the *S*-haplotypes tested. The physically longer Sb is equivalent to allele S_{20} of Schierup *et al.* (2001) and has a long *Aly8* sequence of 824 bp, while the Sa (equivalent to allele S_{13} of Schierup *et al.* 2001) has a short *Aly8* sequence (557 bp). No genomic DNA was available from the plant from which the BAC clones were made, so only the clones containing the *S*-locus were studied, but independent plants with the S_{13} and S_{20} alleles were studied (98E17/10 and BM04-A1/7 provided by Barbara Mable or already available in the lab), originating from the same U.S. population (Charlesworth *et al.* 2003b). These yielded the same band lengths and sequences as those from the BAC clones, indicating that no second *Aly8* allele is present in the genome, outside the cloned region. Thus, only the three *S*-haplotypes already mentioned carry two *Aly8* sequences. The S_{13} and S_{20} haplotypes were found to carry different *Aly8* alleles, each differing at multiple sites from other sequences, and they are again homogeneous in independent individuals. The S_{37} haplotype, corresponding to the *A. thaliana* haplogroup A (Shimizu *et al.* 2004), also differed at several (five) sites from the most similar other *Aly8* sequence.

Nine *S*-haplotypes appeared in families from at least two populations (1, 9, 12, 14, 15, 16, 22, 25, and 27), including S_9 (Kuittinen *et al.* 2004). Three more were found in multiple families from the same population (S_6 and S_{38} from Mt. Esja and S_{18} from population 5); multiple copies of these were not sequenced from the same population, as finding the same associations in different populations is much better evidence of linkage disequilibrium (LD), but the *Aly8* lengths were the same in each of the pairs with the same *SRK* and different between haplotypes with different *SRK* sequences. In

addition, we sequenced S_{11} from two different Icelandic populations (5 and 1, with the phase established only from the former). Despite repeated efforts with the available reverse primers, no full-length S_{22} sequence was obtained. Thus, in total, 9 *S*-haplotypes (Table 2 and Figure 3) could be tested for homogeneous associations between populations, the strongest test for linkage disequilibrium, and 11 could be tested for the presence of different *Aly8* sequences in haplotypes with different *SRK* alleles. Ones with the same *SRK* allele generally had very similar or identical *Aly8* sequences, even when they originated from different populations. Most haplotypes contained only one or two sequence variants, including indels (Figure 3).

There are two major exceptions to this uniformity of sequences sharing the same *SRK* allele. The S_9 haplotype in the mapping family, like the Mt. Esja S_9 haplotype, has two copies of the *Aly8* gene. The short (pseudogene) sequences are almost identical (two differences), but the lengths and sequences of the long copies differ. The Mt. Esja S_9 haplotype *Aly8* allele differs from the sequence from the mapping family allele by 24 SNP variants, many more than within any other haplotype; the Mt. Esja sequence is also 4 bp shorter than the other L sequences (it has a length of 415, see Figure 4). The S_1-linked *Aly8* sequences also vary. In the Mt. Esja families and the *A. lyrata* mapping family (from distant populations), all haplotypes with the *SRK* S_1 allele carried 477 (pseudogene) and 570^{395} *Aly8* sequence types (Table 2). However, the S_1 haplotype in a family from a different Icelandic population, 15, had no 477 sequence, and other Mt. Esja plants also carry S_1 without this sequence (Table 3 and Figure 3). The S_1-linked sequences of the 570-bp band also differ, mainly because of differences between S_1 haplotypes with two *Aly8* copies *vs.* only one (Figure 3), but there were also 6 variants among the four lacking it; 4 of these variants are shared with *Aly8* sequences from other *S*-haplotypes, suggesting exchange events (see also below). The S_1 haplotypes with the 477 sequence (from five different populations) include no variants and may therefore be derived from the haplotype lacking it.

Homogeneity within haplotypes with a given *SRK* is thus not complete. Clusters of *Aly8* sequences according to the haplotype's *SRK* allele are nevertheless clearly seen in the NJ tree (Figure 4). There is often good bootstrap support, even among the short sequences, which are only ~500 bp long, but less often among the long sequences, which differ less. In either case, resolution is quite surprising. The numbers of differences between *Aly8* sequences from the same *S*-haplotype are overestimates. Some variants within *S*-haplotypes are confirmed by sequencing multiple clones, but, for some haplotypes only one or a few clones were sequenced, for example, L419a-S_{25}, and the variation detected may be due to PCR error (as was frequently observed when several clones from the same individual were sequenced).

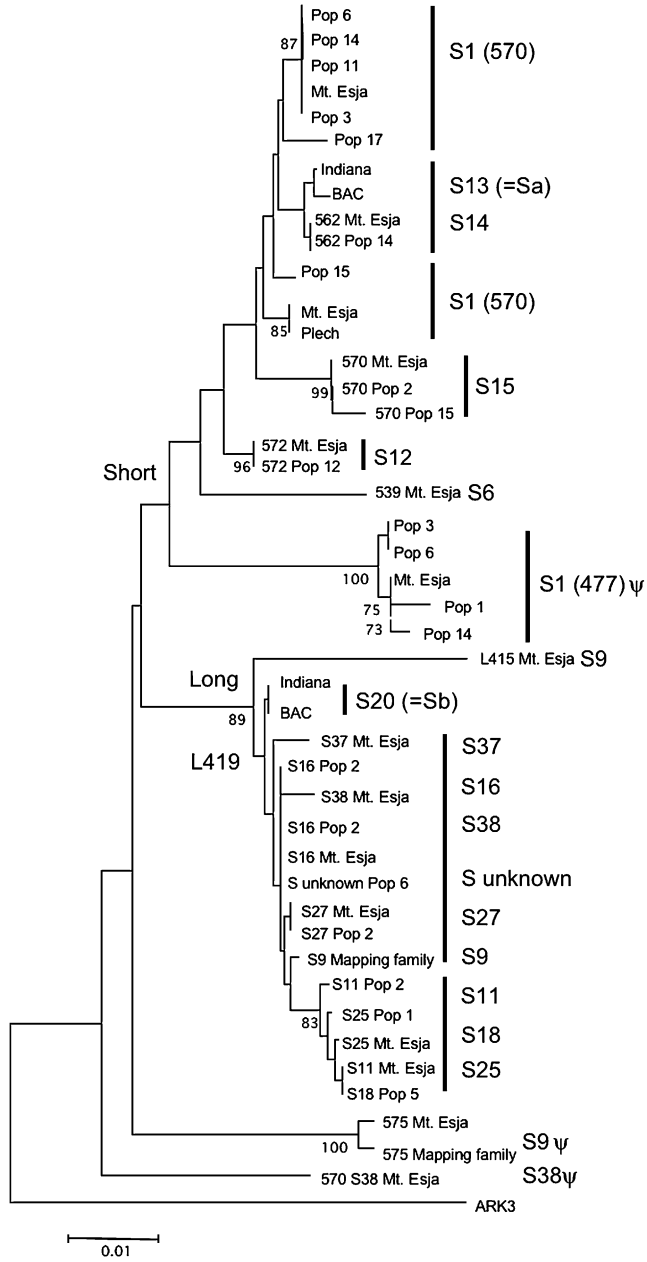


FIGURE 4.—NJ tree including all sequenced *Aly8* alleles, with the sequence of the *A. thaliana* ortholog, *ARK3* from the *Col-0* strain of *A. thaliana* (GenBank accession no. AL161555) as an outgroup. The populations from which the sequences were derived are indicated, and the *Aly8* sequence lengths and the *SRK* allele numbers are shown. The S_1, S_9, and S_{38} haplotypes all have two copies of *Aly8* (BECHSGAARD 2002). The analysis used all single-nucleotide variants in the alignment to estimate Kimura two-parameter distances, using the pairwise deletion option. Bootstrap values (based on 1000 bootstraps) are shown for nodes with values >66%. The BAC clones of alleles S_{13} and S_{20} were kindly provided by June Nasrallah (see MATERIALS AND METHODS), and the other plants carrying these two *SRK* alleles were from plants grown from a natural population from Indiana (see MATERIALS AND METHODS).

Note, however, that most such variants are also seen in other samples.

Tests for recombination or gene conversion: If the *SRK* and *Aly8* loci recombine, even occasionally, the same *Aly8* sequence should occur in haplotypes with entirely different *SRK* alleles. We found only one clear instance of this. Among the set of *S*-haplotypes with similar long *Aly8* alleles S_{11}, S_{18}, and S_{25} (Figures 3 and 4), the sequence from the S_{18} haplotype of population 5 was identical to the S_{11} haplotype sequence from Mt. Esja; the population 5 S_{11} *Aly8* was sequenced from a single clone, and the single difference may be a PCR error. The S_{11} and S_{18} *Aly8* sequences may differ elsewhere in the gene, but the similarity between them in the portion studied suggests recombination between these two haplotypes, whose *SRK* sequences are not closely similar and belong to different dominance classes (Mable *et al.* 2004). S_{22} may be another case, but is not shown in Figure 4, because only part of the S_{22}-linked *Aly8* was sequenced (with the J4Rb primer); this was identical to the *Aly8* sequence from the S_{18} and S_{25} haplotypes, but it must differ in the primer site, at least, since it does not amplify with the J4R primer.

Small-scale exchanges may also have occurred between haplotypes. We tested for gene conversion between haplotypes with different *S*-alleles, using the test of Betran *et al.* (1997) implemented in DNAsp. Even between the long and short sequence types, the test detects several conversion events (boxed in Figure 3; the tests require at least three individuals in each sequence set, so few individual haplotype pairs could be tested). GeneConv detects a single event spanning a region of 218 bp, from the first to the last of the regions detected above ($P = 0.0324$, after correction for multiple tests).

Assigning *Aly8* sequences to loci and estimating diversity: The *S*-locus is subject to frequency-dependent selection, leading to high polymorphism. Given that *Aly8* loci are close to the *S*-locus, probably with a very low recombination rate, we expect its diversity to be high, as explained in the Introduction. One goal of our work was to estimate diversity of the *Aly8* genes, to test this prediction. However, to estimate diversity we need to establish how many loci are present and which sequences are allelic and which paralogous (*i.e.*, to assign alleles to their respective loci) or else quantify the rate of genetic exchange of sequences between the two loci (Innan 2002).

It is unclear whether the long and short *Aly8* sequence types represent different loci or whether the nonpseudogene short and long sequences are allelic. The *A. thaliana ARK3* sequence of the *Col*-0 strain shares most of the large insertion (~270 bp in the first intron) with the long *Aly8* alleles, suggesting that the long state is ancestral and the short state derived within *A. lyrata*. Three smaller indels, plus a single fixed nucleotide difference, also distinguish the long sequences from all the short ones, and these are not found in *ARK3* (in the region containing the third indel, the *ARK3* sequence has a larger deletion with respect to the *Aly8* sequences). We cannot obtain any information about the ancestry of the long and short sequences from the putatively orthologous *B. oleracea SFR2* sequence, because only exon sequence could be aligned, precluding analysis of the indels, which are in the first intron.

To try to determine whether the nonpseudogene short and long *Aly8* sequences are allelic, we estimated F_{ST} between the two sequence types. The values are high (0.67 for all sites and 0.55 for silent sites or slightly less if the duplicate, pseudogene sequences are included). However, these values are not based on random samples from the populations, as our sample includes at least two of many *S*-haplotypes; F_{ST} values estimated from just one of each nonpseudogene *S*-haplotype are much lower (<0.125 for all site types), but the NJ gene tree nevertheless clearly clusters the long sequences (Figure 4). In contrast, sequences from haplotypes with two copies, S_{38} (L419b and 570^{399}), S_9 (L415 and 575), and S_1 (570^{395} and 477) do not suggest a distinct clade of haplotypes with these sequences, although they share the small deletion noted above, near the start on intron 1.

The long/short separation does not, however, necessarily imply that these sequences represent different loci. As in other cases with paralogous genes where there is diversity (Bosch *et al.* 2004), it is difficult to draw firm conclusions about which *Aly8* sequences are allelic. The synonymous site divergence between the two functional sequence types in our sample is 5.4% (average number of single-nucleotide differences 17.9); excluding the pseudogene sequences, only 5 are fixed differences, plus at most three indels (Figure 3). However, the lack of fixed differences is largely due to high polymorphism within each set of sequences. Synonymous site diversity (π_s) estimated from all the nonpseudogene sequences in our sample is 3.9% (3.7% within the short set of sequences and 1.4% for just the more uniform long ones); a similar, although smaller, difference is seen for all silent sites. The distinctiveness of the two types is thus overestimated by considering fixed differences, because, with our fairly small numbers of sequences, some polymorphisms will be missed. We tentatively conclude that they probably represent highly diverged alleles (with the pseudogene sequences being generated by occasional duplications in diverse *S*-haplotypes). Unequal crossing over generating haplotypes with different copy numbers after an initial duplication seems to be excluded, because the duplicates are each very distinctive in sequence (see Figure 4).

Consistent with the long and nonpseudogene short sequences being alleles, the evidence from diversity supports the conclusion above that both sequence sets are subject to selective constraint. π_a/π_s values are low (0.169 for long and 0.103 for short sequences or 0.125 for both sets of sequences combined). These are similar to the K_a/K_s value for divergence from *ARK3* (see

above) or between long and short sequences (0.117 using nonpseudogenes and 0.173 for all short sequences). These values suggest selective constraint, not balancing selection that could account for the high diversity. Tajima's *D*-values (not shown) were negative, but did not differ significantly from zero for the long or the short sequence sets or the combined set, so there is also no evidence for a selective sweep that could have been caused by recent spread of a duplicate copy. Fu and Li's tests (using *ARK3* as an outgroup) were also nonsignificant. Haplotype tests were also nonsignificant and are discussed below. The pseudogene sequences had higher polymorphism, with π_a- and π_s-values of 0.064 and 0.027, and an elevated π_a/π_s of 0.42 (for all site types, π was 0.051 for the pseudogene sequences *vs.* 0.024 for the others), suggesting that they arose long ago, consistent with their wide distribution in the tree (Figure 4), and have accumulated sequence differences in addition to those causing loss of function (see above).

DISCUSSION

Tight linkage of *Aly8* gene copies: Absence of recombination between the pollen and pistil expressed *S*-genes is a prerequisite for keeping the incompatible *S*-allele pairs together in functional haplotypes (*e.g.*, Casselman *et al.* 2000). Given the considerable rearrangement in the *S*-locus region (Kusaba *et al.* 2001), it is plausible that recombination is repressed across the whole region, but no independent evidence has so far been obtained. No recombination was detected between several genes close to *SRK* in *B. oleracea* families (Casselman *et al.* 2000). However, the four completely linked genes were located within only ~40 kb, which, with the estimated recombination rate in *A. lyrata* of between ~400 and 600 kb/cM (Wright *et al.* 2003) or even with the higher rate in *A. thaliana* (~200 kb/cM for noncentromere regions, see Copenhaver *et al.* 1998), would require a family larger than the 250 plants studied to accurately estimate crossing over. A population genetic approach may be more likely to detect recombination, especially in the adjoining genome regions (Takebayashi *et al.* 2004).

To use such an approach, however, we must understand the genetic situation of *Aly8* sequences. Previous work on *Aly8* (Charlesworth *et al.* 2003b) did not establish the number of copies or whether more than one is linked to *SRK*, although there was some evidence of linkage disequilibrium between *Aly8* and *SRK*. We now conclude that *Aly8* is closely linked to the *S*-locus and, furthermore, that some haplotypes exist with two copies and there is no unlinked copy in the genome. The situation for *Aly8* is thus similar to that sometimes found for *S*-loci; duplicates of both *SCR* and the S-linked *SLG* loci have been found in some *S*-haplotypes (Cabrillac *et al.* 1999; Kusaba *et al.* 2001). Interestingly, the three instances of haplotypes with two copies seem to have arisen independently. Whether the long and short sequences are allelic or not, we can use the *Aly8* sequence types to test for long-term associations with *SRK* alleles.

Linkage disequilibrium between *Aly8* and *SRK*: Our association results suggest that recombination between *Aly8* and the *S*-locus is rare over evolutionary time (as well as in the single generation tested in our families). Previous studies of the *SRK* gene sequences of a set of different *S*-haplotypes (either typed by pollination tests or inferred from the *S*-domain sequences to represent different *S*-alleles) showed extreme homogeneity of the kinase domains within each *S*-haplotype, whereas different alleles differ greatly. This suggests that little or no recombination occurs within the *S*-locus, across a physical distance of up to 3 kb (Charlesworth *et al.* 2003a). This was confirmed by the finding that linkage disequilibrium does not decrease with increasing distances between sites in the sequence and also that silent site diversity is as high at sites in the kinase domain as in the *S*-domain, which is the presumed location of the sites under balancing selection (Charlesworth *et al.* 2003a).

The results presented here for the more distant *Aly8* gene, showing very similar *Aly8* sequences in independent haplotypes with the same *SRK*, across several different *A. lyrata* populations, are similar to those for the *SRK* locus itself, but across a distance of at least several kilobases from *SRK* (Kusaba *et al.* 2001). Only occasional variants were found within haplotypes. Evidently the same *S*-haplotypes are maintained in different Icelandic populations and also in the few populations from other geographic regions from which we have samples. The fact that new *Aly8* sequences are found, whenever we apply our typing method to *A. lyrata* plants with *S*-alleles different from ones already sequenced, also strongly suggests fairly long-term linkage disequilibrium between *Aly8* and the *S*-locus. Thus *Aly8* and the *S*-locus are not only linked, but also in linkage disequilibrium. Note that using *SRK* sequences in lieu of knowing that the haplotypes carry functionally different *S*-alleles is conservative. If *SRK* sequences differ among alleles of the same *S*-allele type, associations will be obscured.

It will be interesting to study the geographic scale of *SRK-Aly8* associations further, to better estimate the timescale over which they are maintained. It is also not yet possible to compare our results with LD elsewhere in the *A. lyrata* genome, because no good data yet exist on genetic and physical maps for this species. Results from five nuclear loci within the Mt. Esja population alone suggested LD higher than expected with recombination rates per base pair as high as in *A. thaliana* (Wright *et al.* 2003). This could be due to low recombination in *A. lyrata*, but, until additional populations have been studied, we cannot exclude other plausible causes of LD, such as a bottleneck in the population's history. Data on recombination rates on four *A. lyrata*

chromosomes suggest a recombination rate per megabase for noncentromere regions similar to that in *A. thaliana* or slightly lower (Hansson *et al.* 2006).

We did not attempt to analyze linkage disequilibrium explicitly or to quantify it, for several reasons. First, the duplication prevents our determining which *Aly8* sequences are allelic. The distances between the *Aly8* loci are also unknown. It is not reasonable to estimate LD between sites that may not be in alleles (they might fail to recombine simply because they are at different positions in the chromosome, yet the region around them might recombine, and it is recombination in the region, not within the *Aly8* gene, that we wish to test). Second, the length of sequence is short, after excluding sites that are absent from the short sequences, plus gaps in the alignment between these very different sequences; this means that we have low ability to detect a decline in LD with distance between sites and could not conclude that recombination is absent, merely because no decline is detected.

Third, even just within *SRK*, we previously found low LD within either the *S*- or kinase domains, despite very clear evidence (reviewed above) for distinctive differences maintained by different *S*-alleles throughout the *SRK* sequences, over long evolutionary times (Charlesworth *et al.* 2003a). LD may be obscured by gene conversion between different *S*-alleles or because divergence times between alleles are very long, so that identical mutations may have occurred in different haplotypes; in either case, variants are expected to be shared between haplotypes, and this is indeed seen in *SRK* sequences (between 12 alleles analyzed, over a length of 782 bp of the highly polymorphic *S*-domain, 222 of the 496 variable sites are shared variants found in 2 or more *S*-alleles) and in *Aly8* (between 16 *S*-haplotypes analyzed, of length 525 bp, excluding indels, 27 of the 50 variable sites were shared between different haplotypes).

A final, more subtle, reason for not quantifying linkage disequilibrium is that we have at present sequenced only a few instances of each *S*-haplotype. To establish *Aly8* sequences of haplotypes and to have material for testing for associations, we aimed to get as many different haplotypes as possible, as well as to obtain sequence *Aly8* from multiple haplotypes with the same *SRK* sequence. Thus our present sample is not a random population sample. The same is true for many sequence data sets from the *S*-loci themselves, where the emphasis has generally been on sequencing as many different functional allelic types as possible. Diversity is thus overestimated, although there may also be a possibility of underestimation due to failure of PCR primers to amplify some particularly divergent sequences. In our sample of *Aly8* alleles, the number of haplotypes is consistent with neutral expectations assuming no recombination, given the number of SNPs in our nonpseudogene sequences (using coalescent simulations implemented in DNAsp), but is not significantly lower than expected with even a small amount of recombination (R per gene = 1); thus this test does not exclude a low recombination rate. However, if we include two copies of each *S*-haplotype (*i.e.*, assuming that our observed low diversity within *S*-haplotypes would hold for haplotypes for which we currently have only one sequence), the data exclude even a low recombination rate. Clearly, therefore, more *S*-haplotypes must be investigated in the future.

A different type of LD test is to compare estimates of nucleotide diversity within and between *S*-haplotypes, defined by their *SRK* sequences. A very small recombination rate will lead to diversity within allelic classes being very similar to the diversity in the full sample (Charlesworth *et al.* 1997; Innan and Tajima 1999), just as rare migration in an island model of migration between demes leads to within-deme diversity being almost the same as total diversity (reviewed in Charlesworth *et al.* 2003). For the 11 *S*-haplotypes for which we have multiple *Aly8* sequences, the mean within-haplotype diversity (π_A of Charlesworth *et al.* 1997) is 0.0036 for all site types, including the two very different S_9 haplotypes, while the diversity for our full *Aly8* sequence sample (excluding the pseudogene sequences), π_T, is six times higher, 0.022. We estimated the analog of F_{ST} between all pairs of such haplotypes, and the value is 0.965 for all site types. F_{ST} is 0.865 for all short nonpseudogene sequences and 0.798 for just the long ones, excluding S_9 haplotypes, or 0.346 including these sequences (in all cases, the haplotypes are highly significantly differentiated using the K_s test of Hudson *et al.* 1992). The high isolation between different *S*-haplotypes is evidence that LD is strong, and the F_{ST} value estimates a quantity σ_d^2 (Charlesworth *et al.* 1997; Innan and Tajima 1999) that is closely similar to the LD measure r^2 (McVean 2002).

Do exchanges occur between different *S*-haplotypes? Among all the sequences from different haplotypes, we found only one instance of the same *Aly8* sequence associated with different *S*-haplotypes (an *Aly8* sequence found in an S_{18} haplotype is identical with one of the very similar sequences found in an S_{11} haplotype). More such cases would be expected if the *SRK* and *Aly8* loci recombine, even occasionally. However, there is some evidence for smaller-scale exchanges (see Figure 3). Taken together, our results suggest that linkages of particular *Aly8* sequences and *SRK* alleles are maintained over long enough periods of time for migration to occur between different populations. Because (as mentioned above) associations will not persist unless recombination is very infrequent, this suggests that low recombination may extend to a wide region surrounding the *S*-locus. The *S*-region is, however, known to vary in both length and gene order between the only two haplotypes yet studied in detail (our S_{13} and S_{20}, equivalent to Sa and Sb of Kusaba *et al.* 2001). It will be important in the future to determine physical distances in the *S*-locus

region and also to further develop the necessary theory to predict the extent of the region in which LD is expected and the expected magnitude of F_{ST} between haplotypes at different recombination distances.

Linkage across the *S*-locus region is, however, clearly not complete on an evolutionary timescale, since silent site diversity in the kinase domain of *SRK* is much higher than at *Aly8* or the other flanking loci studied (Kamau and Charlesworth 2005), including the *B80* gene (another gene in the region flanking the *S*-loci; see Kusaba *et al.* 2001). Recombination is also suggested by the results for the *S*-locus region in *A. thaliana*. Although this species is self-compatible, the orthologous region is still present (Kusaba *et al.* 2001). High diversity was found for the homologs of both *Aly8* (*ARK3* in *A. thaliana*) and *B80* (a U-box gene also located in the region flanking the *S*-loci; see Kusaba *et al.* 2001). These genes appear to mark the boundaries of a region affected by a selective sweep at the pollen *S*-locus, *SCR1*, which may have been the site of the mutation causing loss of incompatibility in this species (Shimizu *et al.* 2004). On this interpretation, crossing over must be frequent enough to allow *SCR1* to recombine with *ARK3* and the U-box gene, so that allelic diversity at these loci remained very high.

Occasional recombination or other exchange events may also account for why the *Aly8* sequence is not completely uniform within *S*-haplotypes (Figure 3). Even if crossing over is infrequent near the *S*-locus, gene conversion might still occur, as in low-recombination regions of *Drosophila melanogaster* (Langley *et al.* 2000; Jensen *et al.* 2002), and could homogenize parts of the alleles' sequences. However, the large fixed insertion in intron 1 may limit the frequency of gene conversion, allowing the long and short sequences to diverge. Our observation (see above) that the apparently derived short *Aly8* sequences (even just those that are not pseudogenes) have at least as high diversity as the ancestral long ones supports the possibility that exchanges occur, and explicit tests for gene conversion are consistent with this (see Figure 3).

However, the same *S*-haplotype is rarely found associated with different *Aly8* sequences. The S_1 haplotype, where some variants are seen, corresponds to a very recessive *S*-allele (Mable *et al.* 2004). Recessive *S*-alleles are expected to persist in populations for longer than more dominant ones (Schierup *et al.* 1998; Uyenoyama 2000), and so there will have been more time during which mutation and recombination events can have occurred in their ancestry, compared with other alleles. Our result that the S_1 haplotype is less homogeneous in its *Aly8* sequences than other haplotypes is therefore consistent with this theoretical expectation, but recombination is not necessary to generate this variability. However, the S_9 haplotypes, which differ much more in their *Aly8* sequences, are in a higher *S*-allele dominance class (Mable *et al.* 2004).

Another expectation under our interpretation of long-term LD between the *Aly8* and *SRK* loci is that *Aly8* sequence clades should be congruent with those of *SRK*. Four different groups of *SRK* sequences have been distinguished (Mable *et al.* 2004), corresponding to *SRK* alleles with different dominance levels. However, we find no strictly congruent clustering of *Aly8* sequences with the four *SRK* groups. There is also no correspondence between *SRK* sequence types and the number of *Aly8* copies and therefore no suggestion of different physical arrangements of the *S*-loci and *Aly8*, although this should be studied explicitly in the future. Overall, these results suggest that *Aly8* is sufficiently distant from *SRK* and *SCR* that exchanges have occurred over the long evolutionary times during which the *S*-alleles have been maintained.

***Aly8* diversity:** Our results show that the high diversity of *Aly8* sequences is largely, but not entirely, due to the presence of the two different sequence types. It seems clear that there are at most two loci, probably due to a tandem duplication in some haplotypes. We cannot definitively exclude the possibility that all haplotypes have two copies and that the second copy does not amplify with the primers we used; this, however, seems unlikely, because three different primer combinations all failed to amplify any new sequence types in plants that seem to have just one gene copy. If many haplotypes carry copies undiscovered due to variation in our primer sites, we should also score some haplotypes with no copies amplifying, which never happened. Moreover, Kusaba *et al.* (2001) found only single *Aly8* copies in the two haplotypes they studied, and we confirmed this for the genomes of independent plants with these *SRK* alleles.

The uncertainty about the allelic status of the *Aly8* genes does not affect our inferences about associations with *SRK* alleles, but it does impede our ability to estimate diversity. Unequal crossing over between tandem duplicates might generate haplotypes with different copy numbers, and exchanges of sequence may occur. Many such cases are known, including *A. thaliana* disease resistance gene clusters (Baumgarten *et al.* 2003) and the human Rhesus blood group D and E genes (Innan 2003), and there is evidence that exchanges between duplicates are common in yeast (Gao and Innan 2004). If allelic and nonallelic sequences can be reliably distinguished, as is often the case, diversity can be estimated. If, however, a distinction is not possible, rates of exchange can sometimes be estimated, if they are not high, and used to obtain diversity estimates (Innan 2002).

In the case of *Aly8*, exchanges may be frequent, relative to the age of the *S*-haplotypes, and the haplotypes do not all carry two copies, so that the exchange parameters cannot be estimated. If the short and long nonpseudogene sequences are allelic, nucleotide diversity can be estimated. As explained above, we have

not yet studied random samples of *S*-haplotypes from natural populations, since our main goal was to study associations with *SRK* alleles. Given that there are many *S*-alleles in *A. lyrata* populations (Charlesworth *et al.* 2003b; Mable *et al.* 2003), such samples will consist largely of different alleles. Diversity estimated from the nonpseudogene *Aly8* sequences of one of each *S*-haplotype is 5% for synonymous sites (4% for silent sites); a somewhat lower value is obtained if we use our sample with multiple instances of each *S*-haplotype ($\pi_s = 3.9\%$, $\pi_{silent} = 3.7\%$). If the sequences are two distinct loci, not alleles, this overestimates the diversity at each locus. A crude underestimate, assuming free "migration" of sequences between two loci, *i.e.*, taking the effective size for each locus to be at most twice that for other loci in the species, would halve our diversity estimates. If one sequence type is much more abundant than the other, this overcorrects the effect on diversity; however, our data include roughly equal numbers of the two sequence types. Even this *Aly8* diversity estimate above is about twice as high as that for reference loci in this species (Wright 2003; Ramos-Onsins *et al.* 2004), suggesting that diversity is elevated due to linkage to the *S*-loci (consistent with our evidence for long-term associations). All diversity values are higher if the pseudogene copies are included as alleles (see results).

Overall, *Aly8* thus shows some signs of elevated diversity compared with results from other loci in this species, which average ~1% for silent sites (Wright *et al.* 2003). It is also consistent with a high *Aly8* diversity that there are several indels among the different *Aly8* sequences in the exon part of the sequence, but no fixed indel differences between *A. thaliana* and the *Aly8* consensus sequence. *Aly8* diversity is not, however, extremely high and is much lower than that for the *A. lyrata SRK* locus (Charlesworth *et al.* 2003b). This adds support to the evidence from the apparent gene conversion events in our sequences that some form of genetic exchange between haplotypes occurs in the region. This conclusion is similar to that for other genes in the region (Kamau and Charlesworth 2005).

If the high *Aly8* diversity is caused by its linkage to the *S*-loci, we would expect *ARK3* in *A. thaliana*, a plant lacking SI responses, to be much less polymorphic. Diversity of *ARK3* has recently been studied, and its synonymous site diversity was estimated to be 0.0316 (Shimizu *et al.* 2004). This is much higher than the average for *A. thaliana* loci, for which mean intron or fourfold degenerate site specieswide diversity is estimated to be ~0.008, with a large range (Nordborg *et al.* 2005), and, surprisingly, it is almost as high as that for the *A. lyrata Aly8* loci or even slightly higher if our correction for duplicate gene copies is appropriate. However, the regions of the *ARK3* sequence (5′ noncoding plus part of exon 1) studied do not overlap that studied in *A. lyrata*, so the comparison is only rough. Another large set of *ARK3* sequences does partially overlap (C. Tang, personal communication), and these are also polymorphic. This is probably explained by recent loss of incompatibility in *A. thaliana* (Shimizu *et al.* 2004). It will be interesting in the future to compare the complete region in the two species, to understand whether *A. thaliana* has high diversity throughout the region or whether diversity is highly heterogeneous and is high only in the 5′ region. There is a large disease resistance gene cluster near the *S*-loci on *A. thaliana* chromosome 4 (Baumgarten *et al.* 2003), and it is possible that this may include highly polymorphic loci that affect diversity at genes in this region in *A. thaliana*.

Conclusions: If linkage disequilibrium between *Aly8* and *SRK* genes is indeed maintained for long enough to be consistent in different *A. lyrata* populations, it may be possible to use *Aly8* typing to estimate frequencies of *S*-haplotypes, rather than using the *S*-locus sequences themselves, which are so polymorphic that obtaining reliable PCR amplification from all alleles present in populations, and getting sequences, is very difficult, and errors are frequent. Some *SRK* alleles do not amplify with currently available *SRK* primers, yielding alleles classified as "unknown"; with flanking loci, these can potentially be distinguished from one another, allowing haplotypes to be classified without obtaining *SRK* or *SCR* sequences, which currently requires screening genomic libraries for clones containing the *S*-loci (Kusaba *et al.* 2001). *Aly8* typing can also help to confirm hypotheses from PCR–RFLP results, currently used to suggest which allele-specific primers are most promising for attempting to determine *S*-allele sequences present in individual plants (Schierup *et al.* 2001; Bechsgaard 2002; Mable *et al.* 2004). This approach should be helpful for testing the predictions of theoretical models about allele frequencies in natural populations (*e.g.*, Schierup 1998; Uyenoyama 2000; Muirhead 2001), which requires ascertaining the haplotypes of large numbers of plants. For example, *SRK* alleles show slight population structure and low F_{ST} (Charlesworth *et al.* 2003b), as predicted for a locus under balancing selection (Schierup *et al.* 2000b), and our results suggest that this is also true for *Aly8*. Current data cannot yet test this rigorously, but it should be tested in the future.

This approach may not be limited to the species studied here or to the sporophytic incompatibility system of Brassicaceae. Genes closely linked to the *S*-loci have now been identified in many self-incompatible plants. Diversity studies of *S*-alleles and flanking loci genes will be a first kind of valuable evidence; if there is linkage disequilibrium, these loci should have higher diversity than other loci in the genome (Charlesworth *et al.* 1997; McVean 2002), because both the linkage disequilibrium and the diversity in these situations reflect divergence between alleles associated over long evolutionary times with functionally different *S*-alleles. More data from natural population studies of diversity in *S*-locus regions of other plant genomes are thus needed.

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