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Published in:
Annals of Botany

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

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Download date: 13. May. 2021
Cryptic introgression into the Kidney saxifrage (*Saxifraga hirsuta*) from its more abundant sympatric congener *Saxifraga spathularis*, and the potential risk of genetic assimilation

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Running title: Cryptic introgression in *Saxifraga hirsuta*

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• **Background and Aims**  Although hybridization can play a positive role in plant evolution, it has been shown that excessive unidirectional hybridization can result in replacement of a species’ gene pool, and even the extinction of rare species via genetic assimilation. In the present study, we have examined levels of introgression between the common *Saxifraga spathularis* and its rarer congener *S. hirsuta*, which have been observed to hybridize in the wild where they occur sympatrically.

• **Methods**  Seven species-specific single nucleotide polymorphisms (SNPs) were analyzed in 1025 plants representing both species and their hybrid *S. × polita* from 29 sites across their ranges in Ireland. In addition, species distribution modelling was carried out to determine whether the relative abundance of the two parental species is likely to change under future climate scenarios.

• **Key results**  *S. spathularis* individuals tended to be genetically pure, exhibiting little or no introgression from *S. hirsuta*, but significant levels of introgression of *S. spathularis* alleles into *S. hirsuta* were observed, indicating that populations exhibiting *S. hirsuta* morphology are more like a hybrid swarm, consisting of backcrosses and F2s. Populations of the hybrid, *S. × polita*, were generally comprised of F1s or F2s, with some evidence of backcrossing. Species distribution modelling under projected future climate scenarios indicated an increase in suitable habitat for both parental species.

• **Main conclusions**  Levels of introgression observed in this study in both *S. spathularis* and *S. hirsuta* would appear to be correlated with the relative abundance of the species. Significant introgression of *S. spathularis* alleles were detected in the majority of the *S. hirsuta* populations analysed and, consequently, ongoing
introgression would appear to represent a threat to the genetic integrity of *S. hirsuta*, particularly in areas where the species exists sympatrically with its congener and where it is greatly outnumbered.

**Key words:** genetic assimilation; hybridization; introgression; population genetics; *Saxifraga hirsuta; Saxifraga spathularis*; single nucleotide polymorphisms; species distribution modelling
Hybridization is a common phenomenon in plant taxa, and can result in a wide range of potential evolutionary consequences, both positive and negative (Barton 2001). The process can give rise to the evolution of new species (Anderson & Stebbins 1954; Seehausen 2004), and introgression can act as a source of potential adaptive variation (Lewontin & Birch 1966), but excessive unidirectional hybridization can result in replacement of a species’ gene pool (Beatty, Philipp & Provan 2010) and has even been implicated in the extinction of rare species via genetic assimilation (Levin, Francisco-Ortega & Jansen 1996). It is thus clear that no single evolutionary trajectory results from the process of hybridization, and the unpredictability of the final outcomes has led to ongoing interest from researchers.

In recent years, global climate change has led to shifts in the distribution ranges of many species (Parmesan & Yohe 2003; Hickling et al. 2006; Kelly & Goulden 2008). One consequence of these range shifts is the creation of novel species assemblages. Such ecosystem reorganizations offer new opportunities for hybridization between species that would otherwise remain isolated from each other, both ecologically and reproductively (Garroway et al. 2010; Muhlfeld et al. 2014; Potts et al. 2014). In addition to observational studies on emergent hybridization, species distribution modeling approaches are now being employed to determine the role of future range dynamics in the potential creation of new hybrid zones (Sanchez-Guillen et al. 2014). To date, however, these models have not been applied to assess the possible impacts of climate change on existing known hybrid zones in the context of potential genetic assimilation.
Saxifraga spathularis Brot. and S. hirsuta L. are both members of the Lusitanian flora, whose disjunct distribution between Ireland and northern Spain has been of great intrigue to botanists (Webb 1983; Beatty & Provan 2013, 2014). Both species belong to the Saxifraga Section Gymnopera, which also includes two other members, S. umbrosa L. and S. cuneifolia L. S. umbrosa readily hybridizes with both S. spathularis, resulting in S. × urbium, an artificially induced/propagated hybrid commonly referred to as London Pride, and with S. hirsuta, resulting in S. × geum L., a wild naturally occurring hybrid, found where the ranges of the two parental species overlap in western Pyrenees (Webb & Gornall 1989). S. cuneifolia, however, does not form hybrids with any of its congener species, either in the wild or artificially. The two Lusitanian species exhibit contrasting patterns of occurrence throughout their divided ranges. In the Iberian reaches of their range, S. hirsuta is more commonplace, stretching from the mountains of Galicia and Andalusia in the northwest, across northern Spain to the Pyrenees, compared to the more limited range of S. spathularis, which is restricted to the northwestern corner of Iberia. In Ireland, however, levels of abundance are reversed: S. spathularis is significantly more abundant and wide-ranging, being found throughout the south and west of the country, primarily Counties Galway, Kerry, Cork and Waterford, whilst S. hirsuta is limited to the extreme southwest in Counties Cork and Kerry. Both species have similar habitats although S. spathularis reaches a greater altitude and S. hirsuta is less tolerant of exposed sites. A putative hybrid between the two species, S. × polita (Haw.) Link, has been identified in both the Irish and Iberian parts of their range. Records of S. × polita are rare in Iberia compared to Ireland, most likely due to the relatively limited area of overlap in the parental species’ ranges in Iberia, and the fact that where they do, they tend to occupy
different environmental niches, (McGregor, 2008). In Ireland, however, the hybrids are
common where both species co-occur in Counties Cork and Kerry and, interestingly, also
in Co. Galway, where only one of its parental species, namely S. spathularis, occurs
(Webb 1951).

Given the previously documented threats of genetic assimilation by a more abundant
congener in other plant species, and the relative abundances of S. hirsuta and S.
spathularis, along with the occurrence of the hybrid S. × polita in Ireland, the questions
addressed in the current study were: (1) Is there evidence of introgression of S.
spathularis alleles into S. hirsuta? (2) If so, to what level is the introgression occurring
and is it placing the gene pool of the rare S. hirsuta under threat of genetic assimilation?
(3) What is the genetic composition of S. × polita hybrid populations? (4) Is the
distribution of the two parental species is likely to change under future climate scenarios,
and thus increase or decrease the likelihood of hybridization?
MATERIALS AND METHODS

Study species

*Saxifraga spathularis* (St. Patrick’s cabbage) and *S. hirsuta*, (Kidney saxifrage) are perennial stoloniferous herbs. The two species and their hybrid (*S. x polita*) can be distinguished by relative leaf shape, pubescence toothing, and the width of the cartilaginous margins of the leaf lamina (Gornall 1998). *S. spathularis* leaves are extremely distinctive, with smooth, thick, waxy, spathulate leaves, each of which has a sharply toothed margin. The leaves of *S. hirsuta* are soft and hairy, and are fringed with a crenate toothing, with petioles that are long thin and hairy. The leaves of *S. x polita* exhibit an intermediate form, both in terms of relative leaf shape and toothing of the leaf margin. The width of the cartilaginous margins of the leaf lamina in *S. spathularis* is 0.1mm, for *S. hirsuta* it ranges between 0.1-0.2mm, while for *S. x polita*, an intermediate range of between 0.1-0.15mm has been recorded (Gornall, 1998). The leaves of all three congers form a basal rosette/turf, from which the erect flowering stem projects. All three taxa flower from May through to July, when the stem bears panicles of star-shaped, five-petaled white/pink flowers. The flowers are insect pollinated and seeds are primarily wind dispersed.

Sampling and DNA extraction

Samples were collected from 29 locations across the ranges of *Saxifraga spathularis*, *S. hirsuta* and *S. x polita* in Ireland (Figure 1; Table 1). These included two sites in Co. Galway, where both *S. spathularis* and *S. x polita* were present, but where no verified
records of *S. hirsuta* exist (Stelfox 1947), as well as samples of *S. spathularis* from Co. Waterford, where neither *S. hirsuta* nor *S. × polita* are present. DNA was extracted using the CTAB method of Doyle and Doyle (1987).

*Single nucleotide polymorphism (SNP) ascertainment and genotyping*

Species-specific single nucleotide polymorphisms (SNPs) were developed from an ascertainment set containing four individuals of each of the two parent species using the ISSR cloning method outlined in Beatty, Philipp & Provan (2010). Allele-specific PCR (AS-PCR) primers were designed as described in Provan *et al.* (2008), and SNP analysis carried out as described in Beatty, Philipp & Provan (2010). In total, 1025 individuals were genotyped for seven SNP loci (Table 2).

*Data analysis*

As populations studied comprised a mixture of parental species and hybrids, and thus were unlikely to be close to Hardy-Weinberg equilibrium, the commonly used software package STRUCTURE (Pritchard, Stephens & Donnelly 2000) was not used. Instead, the percentage of alleles from each parental species found in each individual was represented as a stacked histogram as suggested by Hauser *et al.* (2012). To further investigate the genetic composition of hybrid individuals, we used the program NEWHYBRIDS (V 1.1b; Anderson & Thompson 2002). As suggested by the authors, the number of generations was restricted to two, which gave six classes of genotypes (both parents, F1, F2, BC₅ *S. spathularis* and BC₅ *S. hirsuta*). The program was run with the nuclear SNP data using 50,000
burn-in iterations followed by 500,000 Markov Chain Monte Carlo iterations using
default priors for allele frequencies and mixing proportions.

Species distribution modelling
Species distribution modelling was carried out to determine suitable future climate
envelopes for both species using the maximum entropy approach implemented in the
MAXENT software package (version 3.3.3; Phillips & Dudik 2008). Species occurrence
data in Ireland between 1950 and 2000 (330 and 181 occurrences for *S. spathularis* and *S.
hirsuta*, respectively) were downloaded from the Global Biodiversity Information Facility
data portal (http://www.gbif.org/). Current-day climatic data (1950–2000; Hijmans *et al.*, 2005) at 2.5-minute resolution were clipped to encompass the island of Ireland (i.e.
10.75° W to 5.20° W, and 51.35° N to 55.55° N) to reduce potential problems associated
with extrapolation. Models were generated using cross-validation of 10 replicate runs
under the default MAXENT parameters. Model performance was assessed based on the
area under the receiver operating characteristic curve (AUC). Models were projected
onto climate data for the years 2050 and 2080 generated under the UKMO-HADCM3
model based on three different emissions scenarios (A1b, B2a and A2a; www.ccafs-
climate.org). Outputs from the three models were averaged to give a single consensus
model for each species at each time period.
RESULTS

Genetic analyses

Analysis of 1025 individuals from all three species, including potential hybrid S. × polita populations, indicated a broad spectrum of intra-individual allele frequencies, ranging from 100% S. spathularis to 100% S. hirsuta, with all levels of intermediate allelic composition and hybrid classification evident (Figure 2). S. spathularis individuals tended to be largely genetically pure, exhibiting little or no introgression from S. hirsuta. Most individuals were either 100% S. spathularis, and less than 5% of individuals exhibited more than two S. hirsuta alleles. This was reflected in the NEWHYBRIDS analysis, which assigned the majority of the individuals clearly to S. spathularis, and only four as having a majority chance of falling onto the BC_S.spathularis class. In total, S. hirsuta alleles only accounted for 4.7% of the S. spathularis gene pool of the populations analysed. There was much greater evidence for introgression of S. spathularis alleles into S. hirsuta populations, with less than 15% of individuals exhibiting 100% S. hirsuta alleles. Overall, the level of introgression of S. spathularis alleles into S. hirsuta populations was 19.2%, significantly higher than the opposite scenario (Mann-Whitney test; z = -14.37, P < 0.0001). Six individuals exhibited S. spathularis alleles at a frequency greater than 0.5. The NEWHYBRIDS analysis showed high levels of assignment to the BC_S.hirsuta class, indicating that populations exhibiting S. hirsuta morphology are more like a hybrid swarm, also containing some F2s. Populations of the hybrid, S. × polita, were generally comprised of F1s or F2s, with some evidence of backcrossing. All
hybrid individuals exhibited a mixture of alleles from both parental species. In total, *S.*
spathularis alleles were slightly predominant, at a frequency of 0.536.

Species distribution modelling

The mean AUC values (0.881 [sd = 0.034] and 0.957 [sd = 0.037] for *S.* spathularis and
*S.* hirsuta respectively) indicated a prediction that was far better than random. The
present-day (2000) models for both species were an accurate representation of their actual
distributions, and projected future distributions indicated an increase in suitable habitat
for both species (Figure 3). A comparison of the area in southwest Ireland, where both
species are currently found sympatrically, indicated an increase in suitable cells of ca.
18% for *S.* spathularis between 2000 and 2080, and an increase of ca. 91% for *S.* hirsuta
over the same time period (Figure 4). The proportion of suitable cells for *S.* hirsuta that
were also suitable for *S.* spathularis rose from 94% in 2000 to 100% in 2050 and 2080.
DISCUSSION

The present study further highlights the utility of species-specific SNPs in studies into hybridization in plant taxa, indicating the occurrence of cryptic introgression into both *S. spathularis* and *S. hirsuta*, but particularly the latter. Nevertheless, despite developing primer pairs from the ISSR libraries to amplify 96 sequence-tagged sites (STS), we only managed to develop seven sets of AS-PCR primers that could consistently be used to genotype SNPs. This was mainly due to difficulties in amplifying the orthologous locus in *S. hirsuta* using primers developed from the *S. spathularis* ISSR library, even after relaxing PCR conditions by lowering the annealing temperature. This was surprising, given the fact that they are sister species (Sanna 2013), and given that cross-genus amplification of orthologous loci was possible using the same ISSR-based approach in *Pyrola* (Beatty, Philipp & Provan 2010). Furthermore, it should be borne in mind that genetically more similar orthologues might be more likely to undergo recombination, thus facilitating introgression.

The findings of this study would seem to indicate that levels of introgression are primarily density-dependent. This is most apparent in Cleanderry Wood (Site 17, Figure 2), which is the only location that harboured a large proportion (15/22) of genetically pure *S. hirsuta*, and where no populations of either *S. spathularis* or *S. x polita* were found in the vicinity. Similarly, the majority of *S. hirsuta* collected from Site 10 in Glengariff Forest Park, where only two *S. spathularis* plants were found, exhibited 100% *S. hirsuta* alleles. Further evidence of this is seen at sites where hybrids are found with only one of the parental species. Significantly different frequencies of *S. spathularis*
alleles were found in hybrid populations from Connemara National Park (Site 27, Figure 2), where *S. hirsuta* has never been recorded (0.629), compared to Dromagowlane (Site 14, Figure 2), where *S. spathularis* is absent (0.347; Mann-Whitney test; \( z = -5.53, P < 0.0001 \)). Such density-dependent introgression has previously been cited as a threat to the persistence of a rare species sympatrically with a more abundant congener (Ellstrand & Elam 1993; Burgess *et al.* 2005; Chan *et al.* 2006; Lajbner *et al.* 2009; Beatty, Philipp & Provan 2010).

Individuals identified as *S.* × *polita* all contained a mixture of parental alleles, with *S. spathularis* allele frequencies ranging from 0.214 to 0.928. The majority were assigned in the NEWHYBRIDS analysis to F1 or F2 classes, with some backcrosses to both *S. spathularis* and *S. hirsuta*. This is consistent with the observation that wild hybrids within Section Gymnoper (S. × polita in Ireland and S × geum in the western Pyrenees) are fertile, and form hybrid swarms that display a full range of intermediate morphologies between the relevant parental species (Webb 1951; McGregor 2008). Given the apparent fertility of hybrids, and indications of density-dependent introgression, there is thus the real chance that, over time, the rarer *S. hirsuta* could be under threat of genetic assimilation by the more abundant *S. spathularis* (Levin, Francisco-Ortega & Jansen 1996; Beatty, Philipp & Provan 2010). Indeed, over 50 years ago, Webb had already highlighted the fact that *S. hirsuta* was rarer than the hybrid *S. x polita*, and that “… *S. hirsuta* could not be maintained as a distinct species” (Webb 1951; Page 204).

The occurrence of populations of *S. x polita* in Co. Galway, where only one of the parental species – *S. spathularis* – is present, is unusual. The extremely limited number of historical records of the occurrence of *S. hirsuta* in the region have been subsequently
attributed to misidentification of the hybrid (Stelfox 1947). An examination of herbarium
samples held at the National Botanic Gardens of Ireland, Glasnevin, found a single sheet
of *S. hirsuta* samples from the early 20th Century, labelled as a “garden escape” and
collected in the vicinity of Letterfrack, Co. Galway, which is on the periphery of the
current distribution of both *S. spathularis* and *S. × polita*. It is possible that this – or other
– garden plants might have contributed to the formation of populations of the hybrid.

Although *S. hirsuta* is generally far less common than *S. spathularis* in southwest
Ireland, where both species occur sympatrically, species distribution modelling under
future climatic scenarios indicated an increase in suitable habitat for the former relative to
its congener. Assuming the asymmetric introgression observed in the present study is
density-dependent, this would suggest that future introgression of *S. spathularis* alleles
into *S. hirsuta* may not be as pronounced as at present. However, two potential caveats
should be borne in mind. Firstly, it is well documented that such species distribution
models only take into account climatic factors and tend not to incorporate other biotic and
abiotic variables, such as species-species interactions (Pearson & Dawson 2003; Araújo
& Guisan 2006). Secondly, it is very possible that introgression of *S. spathularis* alleles
may lead to a shift in the ecological and/or climatic niche of *S. hirsuta*, thus
compromising these modelled potential future range expansions. Nevertheless, it is likely
that ongoing environmental change will lead to changes in ecosystem function, and this
could also be a factor at the level of the genome.

In conclusion, the levels of introgression observed in this study in both *S. spathularis*
and *S. hirsuta* would appear to be correlated with the relative abundance of the species.
This is reflected in the genetic composition of populations of the hybrid, *S. × polita*, and
its sympatric occurrence with one or both parental species. From a conservation viewpoint, although significant levels of introgression of S. spathularis alleles were detected in the majority of the S. hirsuta populations analysed, there were only a few individuals in which S. hirsuta alleles were in a minority. This suggests that the S. hirsuta morphology is being retained, despite varying degrees of introgression. Nevertheless, ongoing introgression of S. spathularis alleles into S. hirsuta would appear to represent a threat to the genetic integrity of the latter, particularly in areas where the species exist sympatrically and where S. spathularis outnumbers S. hirsuta.
ACKNOWLEDGEMENTS

The authors are grateful to Robert Beatty for assistance with sampling. This project was funded by British Ecological Society Research Grant number 4309-5281 to G.E.B.
LITERATURE CITED


Hybridization between *Pyrola grandiflora* and *Pyrola minor* in West Greenland: a tension zone maintained by clonality. *Botany* 90:1036-1047


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<td>26</td>
<td>Conor Pass</td>
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<td>10.2078</td>
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<td>9.9458</td>
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<tr>
<td>28</td>
<td>Clare Island</td>
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<td>10.0431</td>
<td>10</td>
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<tr>
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<td>Mahon Falls</td>
<td>52.2324</td>
<td>7.5470</td>
<td>22</td>
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<td>-</td>
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</table>

TOTAL                      | 353     | 310      | 362              |
### TABLE 2  *Saxifraga spathularis / S. hirsuta* AS-PCR primers

<table>
<thead>
<tr>
<th>Locus</th>
<th>SNP</th>
<th>Flanking primers</th>
<th>SNP primers</th>
</tr>
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<tr>
<td>3A09</td>
<td>G → A</td>
<td>AATATGTACTTTACCGTCCTC</td>
<td>CAAGTCAGGGAGGGGAG</td>
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<td></td>
<td>TGTGGAAGTTCAGCATTG</td>
<td>TGCACTACGTAAGTACCCT</td>
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<tr>
<td>3C09</td>
<td>4 bp indel</td>
<td>AATCTAAAACAAACCCTAGAAAAAC</td>
<td>CATTCCAGATAAAATATGGCTAC</td>
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<tr>
<td></td>
<td></td>
<td>AAGTCCAATATTTAAAAAATATATTTG</td>
<td>AATTATGCTAGACGAACCTTG</td>
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<tr>
<td>3G12</td>
<td>C → T</td>
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<td>AATTTTTAATTTACACTAAAAACAGG</td>
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<td></td>
<td></td>
<td>GTATCTATAAACACATAATTTATGAAA</td>
<td>TGTTAGTATATGAAATTGGAGAGTTT</td>
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<td>4 bp indel</td>
<td>CATGCCATATAAATTTGATAATAC</td>
<td>GGTACGAATCAAATCAAAATGG</td>
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<td>GGTATGCTAATCAACATTGA</td>
<td>GGTCACGGATTTTTTTTTTTTTTTCTGCCACA</td>
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<tr>
<td>4D05</td>
<td>C → T</td>
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<td>ATCTCAACGGTAAAAATTTATTTC</td>
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<td>ATCTCAACGGTAAAAACTTTATTTC</td>
<td>ATCTCAACGGTAAAAACTTTATTTC</td>
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<tr>
<td>4F03</td>
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<td>GATGTCTGATTTTGGAGAGAAG</td>
<td>TTTTTCTCCTCATTCTTCCTGTTCCACAC</td>
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<td>CTTCACCTTTTCTGCGCG</td>
<td>CTTCACCTTTTCTGCGCG</td>
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<td>4H06</td>
<td>16 bp indel</td>
<td>CTAGATCGCGCGCGCAGTC</td>
<td>CCTCTGTTATCTCAGACCGGGAATGGCG</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ACACACAGAACAAGCTCTCTCTTCCT</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

FIG. 1  Maps showing locations of populations sampled in this study. Numbers refer to populations listed in Table 1.

FIG. 2. Allele frequencies (above) and results of the NEWHYBRIDS analysis (below) for each of the three taxa studied. In all diagrams, each column represents a single individual. The length of each coloured segment in the NEWHYBRIDS is proportional to the Bayesian posterior probability of assignment to the corresponding genotypic class. Numbers refer to populations in Figure 1 and Table 1.

FIG. 3. Species distribution models for *S. spathularis* (a-c) and *S. hirsuta* (d-f). (a,b) 2000; (c,d) 2050; (e,f) 2080.

FIG. 4. Number of climatically suitable cells predicted by the species distribution models for *S. spathularis* and *S. hirsuta* in southwestern Ireland for each of three time periods.
Number of suitable cells

- **S. spathularis**
- **S. hirsuta**

Years:
- 2000
- 2050
- 2080