Polyploidy influences sexual system and mating patterns in the moss

Atrichum undulatum sensu lato

Linley K. Jesson*, Amanda P. Cavanagh and Danielle S. Perley

INTRODUCTION

Over 50% of mosses, 75% of liverworts and 8% of angiosperms exhibit separate sexes (Wyatt, 1982; Geber et al., 1999), and evolutionary transitions between separate sexes and combined sexes have occurred frequently across these plant lineages (Weiblen et al., 2000; Vamosi et al., 2003; Crawford et al., 2009). The conditions that promote the evolution and maintenance of one sexual system over another have been investigated frequently in flowering plants (Charlesworth and Charlesworth, 1978; Lloyd, 1982; Geber et al., 1999), but less attention has been paid to other land plants despite similar evolutionary lability (but see Klekowski and Baker, 1966; Haig and Westoby, 1988; Eppley et al., 2007). Differences in the life cycles of other land plants, such as haploid dominance and the expression of sex organs at the haploid phase, may influence the evolution or maintenance of different sexual systems.

In mosses, dioecy is likely to be the ancestral condition, with frequent reversions to combined sexes (for discussion see Wyatt, 1982). Both mosses and their sister taxa liverworts have chromosomes correlated with sex expression by gametophytes, with female gametophytes associated with an X chromosome and male gametophytes with a Y (Allen, 1935a, b, 1945; Lewis, 1961; Okada et al., 2001; McDaniel et al., 2007). If sex in mosses is indeed determined by sex chromosomes, the proximate mechanism for the evolution of combined sexes can be through chromosome doubling (Allen, 1935a; Lewis, 1961; Crawford et al., 2009). For example, mating between two haploid moss gametophytes will result in a sporophyte that is XY. In diploid gametophytes, the genotype of the sporophyte would be XXYY. Depending on the pairing of X and Y chromosomes during meiosis, the resulting gametophytes could be XX, XY or YY; XY gametophytes could result in the expression of both male and female sexual structures (Allen, 1935a). Indeed, a recent phylogenetic investigation combining multiple bryophyte phylogenies showed a strong correlation between separate sexes and low chromosome numbers, strongly supporting polyploidy as the mechanism for the evolution of hermaphroditism (Crawford et al., 2009).

Even if polyploidy does not cause the evolution of hermaphroditism it could still be associated with changes to the mating system and many subsequent evolutionary effects. For example, selfing could be correlated with polyploidy due to bottlenecks during the formation or establishment of polyploids (Grant, 1956; Stebbins, 1957, 1971; Ramsey and Schemske, 1998). The union of unreduced gametes, resulting in autoploidy, may be more common in selfing species (Grant, 1956; Stebbins, 1957; Ramsey and Schemske, 1998). In addition, selfing would enable the establishment of polyploids if polyploids are reproducively isolated from their diploid progenitors, or if hybrid offspring have lower fitness (Levin, 1975).

© The Author 2010. Published by Oxford University Press on behalf of the Annals of Botany Company. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
The selfing rate evolves jointly with inbreeding depression, and the simplest theoretical models suggest two stable evolutionary endpoints: the transmission advantage to a selfing mutant should select for predominant selfing if inbreeding depression is low, whereas predominant outcrossing should evolve if populations experience strong inbreeding depression (Lande and Schemske, 1985). However, the addition of other ecological factors can predict evolutionarily stable mixed mating (Goodwillie et al., 2005). Extending Lande and Schemske’s (1985) models to mosses would predict the rapid spread of a selfing variant in hermaphroditic populations because the expression of sexual structures at the gametophyte stage can result in hermaphrodites selfing within a gametophyte (intragametophytic selfing) (Klekowski and Baker, 1966; Klekowski, 1969). This contrasts with selfing between sibling gametophytes (intergametophytic selfing), which is what occurs in flowering plants and animals. One generation of intragametophytic selfing should lead to complete homozygosity at all loci expressed in the sporophyte. This can increase the transmission of the selfing mutation, as well as purging most sporophytic inbreeding depression in one or only a few generations. Indeed, in a comparison of levels of inbreeding depression in a moss with separate sexes (Ceratodon purpureus) with a moss with combined sexes (Funaria hygrometrica), Taylor et al. (2007) found significant inbreeding depression in C. purpureus but not F. hygrometrica, consistent with the prediction of purging in highly selfing populations. The rapid purging of genetic load, combined with the automatic transmission advantage to a selfing mutant, would suggest that selfing phenotypes should be evolutionarily stable in hermaphroditic moss populations, and the simultaneous expression of female and male sex organs should be selected.

However, polyploidy could also influence evolution of the mating system through its effect on the expression of inbreeding depression. In mosses with polysomic inheritance (multiple pairing of homologous chromosomes at meiosis in autopolyploids), intragametophytic selfing may not always lead to complete homozygosity in one generation of selfing. Models of inbreeding depression in allo- and autotetraploids by Lande and Schemske (1985) suggest that the equilibrium level of inbreeding depression will be half that expected for diploids if deleterious alleles are completely recessive. By contrast, Ronfort (1999) found that under tetrasomic inheritance of autopolyploids, inbreeding depression depends greatly on the assumptions of dominance of the mutant deleterious alleles; if completely recessive, autotetraploids should have equivalent levels of inbreeding depression to that of diploids. Additionally, some combinations of dominance coefficients of the different alleles can result in inbreeding depression increasing with the selfing rate, or the evolutionary stability of mixed mating. Thus, although most models predict lower inbreeding depression in polyploids (and hence selection for selfing), it is not completely clear how changes in polyploidy influence the selfing rate.

Certain features of the moss life cycle, such as intragametophytic selfing and an independent gametophyte, are also likely to have strong effects on the evolution of the mating system. Here, we contrast selfing rates in 16 populations of the moss Atrichum undulatum sensu lato (s.l.), in which populations consist of individuals that are male, female, simultaneous or sequentially hermaphroditic, and can be haploid, diploid or triploid. In particular we investigate: (1) is there a correlation between hermaphroditism and polyploidy (either diploid or triploidy)? (2) Are selfing rates higher in populations containing hermaphrodites? (3) What are the levels of intra- and intergametophytic selfing in natural populations?

MATERIALS AND METHODS

Study species

We studied 21 populations of Atrichum undulatum s.l. in New Brunswick, Canada. Because Atrichum is considered a difficult genus with much taxonomic confusion, we have chosen to group these populations under the single descriptor, pending the results of future taxonomic and molecular studies. In eastern North America there are four recognized Atrichum species: A. crispum (James) Sullivant, A. undulatum (Hedwig) P. Beauvois, A. altecristatum (Renaud & Cardot) B. B. Smyth & L. C. D. Smyth and A. crispulum Bescherelette (Smith Merrill and Ireland, 2005). Traditionally, A. altecristatum and A. crispulum were considered varieties of A. undulatum s.l. (Grout, 1928; Crum and Anderson, 1981). They were recognized as distinct species by Ireland (1969) on the basis of plant height, capsule morphology and sexual condition (presence of hermaphrodites versus only separate-sexed individuals). A. altecristatum is described as ‘polyoicous’ with some sequationally hermaphrodic plants whereas others are apparently strictly male or female, with chromosome number n = 14; A. crispulum is described as ‘dioicous’, with n = 14, while A. undulatum is ‘polyoicous’ with n = 7, 14 and 21 (Crum and Anderson, 1981; Ireland, 1969, 1982). Our studies suggest that considerable morphological variation exists both within and between populations, making it difficult to categorize plants from individual collections into a clear species. Moreover, our results from flow cytometry studies (see Results) suggest that many populations consist of mixtures of haploid, diploid or triploid gametophytes within a population. Indeed, flow cytometry of determined herbarium specimens shows that many of these consist of shoots with differing ploidy levels (results not shown). However, our preliminary molecular evidence suggests that diploid and triploid individuals from different populations form separate clades (D. S. Perley and L. K Jesson, unpubl. data). Thus, it is likely that populations consist of multiple species, and further taxonomic study is warranted. We have deposited voucher material from each population in the herbarium of the New Brunswick Museum.

Sex ratio

We refer to populations in this study as with or without hermaphrodites, as all populations with hermaphrodites also had shoots that were female for many consecutive years, and many populations also had some male-only shoots. In flowering plant terminology this is gynodioecy, or perhaps trioecy if males are genetically male. We have no evidence that females or males reproduce only through one sex function, and so cannot apply a more specific functional descriptor.
We sampled shoots from 21 populations around New Brunswick. Populations were defined as contiguous areas where distances between patches of *Atrichum* were closer than 2 m. Each population was separated by at least 50 m. We collected at least 100 shoots per population, and when possible at least 20 shoots with sporophytes; otherwise we collected all sporophytes in the population — two populations had no sporophytes in 2007. We dissected shoots under a microscope and recorded the presence of male and female organs. All fertile shoots in each sample were counted. Shoots without sex organs were considered sterile. Hermaphroditic shoots were recorded as either a single hermaphrodite, with old male sex organs (apparently from a previous year) in a leaf bract far below the archegonia which were terminal to the shoot, or simultaneous (paroicous, with both male and female sexual structures found in the same inflorescence but separated by a bract; synoicous, with sex organs intermingled; or autoicous, with males and females on separate branches). We determined a shoot to be female or male if only one year of sex organs was present, but we recorded the presence of two or more innovations that were both the same sex. It is important to note that many of the shoots that were recorded as male or female may produce alternate sexual structures in future years, although this is unlikely to affect our estimation of selfing rates.

**Ploidy determination**

We performed flow cytometry on gametophytes from each sample of the 21 populations with known sex ratio (above). Ten to 20 shoots were sampled at random, and the sex of the shoot was unknown. Four populations could not be resolved in 2007 (coefficients of variation >10 or numbers of nuclei <300), probably due to the age or storage conditions of the sample. These populations were re-run in 2009, using dried shoots of known sex (n = 10 for each population; with the exception of population 16 which was sampled more extensively for another study). In 2007, shoots were stored refrigerated for 1–6 months before processing; in 2009, we used air-dried samples which generally produced better results.

Flow cytometry was performed on a BD Biosciences FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) at the University of Guelph, Ontario. For each sample, we finely chopped approx. 1 cm of green leaf material from one shoot and a similar amount of leaf standard (to correct for variation in machine function or sample preparation) in a Petri dish containing 1.5 ml of ice-cold nucleus isolation buffer (LBO1; Dolezel *et al.*, 1989). We initially used a radish standard (*Raphanus sativus* L. ‘Suxa’, DNA content of diploid nuclei = 1.05 pg/2C; Dolezel *et al.*, 1992), but changed to *Epilobium hirsutum* (0.83 pg/2C; Kron and Husband, 2009) as the *R. sativus* standard overlapped with the peak obtained by triploid shoots. Samples repeated in 2009 were run using the radish peak as the samples were of good quality, making it easier to distinguish the triploid peak from the radish. We filtered the sample at 30 μm, centrifuged at 1200g for 15 min at 4 °C, and resuspended the pellet in 0.5 mL of LB01 buffer with 50 μg mL⁻¹ propidium iodide and 50 μg mL⁻¹ RNase for 20–60 min. We used an FL2 detector (585/42 nm) to measure fluorescence. We used a measure of integrated fluorescence signal (the parameter FL2-Area) as a correlate of DNA content. Samples were run on ‘low’ for up to 7 min with the goal of obtaining 1000 nuclei per moss 1C and standard 2C peak. We used the CellQuestPro software to acquire data (Becton Dickinson and Co., 1996), and Modfit LT software (Verity Software House, Inc., 2000; http://www.vsh.com/products/mflt/index.asp) to measure mean peak positions, coefficients of variation and nuclei number per peak.

All coefficients of variation ranged between 1.58 and 7.50. Individuals with coefficients of variation >10.0 or <300 events were excluded from the ploidy dataset and for calculating DNA content we only used samples that had a coefficient of variation <5. It should be noted that the numbers of events were lower than are typically used for genome size determination. Because we ran flow cytometry on gametophytes, we refer to individuals as haploid, diploid or triploid at the gametophyte stage. *Atrichum* shoots exhibited endopolyplody, with significant populations of 1C and 2C nuclei. Endopolyploidy probably occurs when DNA replication is not followed by a mitotic division, and is apparently nearly ubiquitous in mosses (Bainard and Newmaster, 2010). Relative DNA content of shoots was therefore expressed as the ratio of the mean position of the lowest peak to the mean peak position of the standard. We calculated 1C values as peak position of the sample/peak position of the standard × 2C standard DNA content (pg DNA).

**Allozyme electrophoresis**

We screened for variation in 20 allozyme loci. Samples were ground in a drop of grinding buffer (20 mg dithiothreitol, 10 mg EDTA, 25 mg bovine serum albumin, and one drop of Tween 80 dissolved in 20 mL of 0.05 M sodium phosphate, pH 7.0). The samples were loaded onto cellulose acetate plates (Titan III Helena Laboratories, Beaumont, TX, USA) that had been pre-soaked in the running buffer for a minimum of 20 min. Gels were run at 200 V for 15 min. All enzymes were stained according to the procedures given by Herbert and Beaton (1993).

From this screen, four enzymes showed variation and consistent banding patterns (*ADH, G6PDH, MDH, PGI*). We used a continuous Tris-maleate buffer to separate *ADH* and *G6PDH*, a continuous CAAPM buffer system for *MDH*, and a sodium phosphate buffer to resolve *PGI* (Herbert and Beaton, 1993; Soltis *et al.*, 1983). To develop a model of inheritance, we screened ten gametophytes from 5–10 sporophyte families from one haploid, one diploid and one triploid population. Spores were grown on agar containing Bold’s basic medium (Carolina Biological Supply Company, 1987), and once germinated individual sporlings were transplanted to separate agar plates, and later transplanted into plastic containers filled with sand. *ADH, G6PDH* and *MDH* exhibited apparently disomic inheritance in the three populations (with two variable alleles per locus), although the low numbers of alleles mean that polysomic inheritance may not have been detected. All gametophytes in each triploid family exhibited fixed heterozygosity for *PGI*; this pattern was also seen in all gametophytes in every triploid-only population. It is unknown if this pattern is due to separate cytosolic
and nuclear loci for PGI or due to some other process such as hybridization. Because this pattern was consistent for all triploids measured, we used PGI in gametophytes as a marker for triploidy in populations with mixed ploidy.

**Population-level selfing rates**

Selfing rates were estimated using allozyme electrophoresis of sporophytes from 16 populations – two populations did not have any sporophytes and so could not be screened, and three populations did not have sufficient genetic diversity for estimation of selfing rates. We scored a minimum of ten sporophytes from each population collected in 2007 for each of the four allozyme loci (sample numbers in Table 1). For each sample we loaded both sporophyte and gametophyte tissue in adjacent lanes – data for ADH, G6PDH and MDH from sporophytes were used to estimate selfing rates, and fixed heterozygotes in PGI for gametophytes were scored as triploids.

**Data analysis**

To test the association between the presence of triploids and the presence of hermaphrodites in a population, we conducted a Fisher’s exact test using the sex ratio and the numbers of haploids, diploids or triploids in each population.

To estimate population-level selfing rates, we first estimated $f$, the Bayesian estimate of $F_{IS}$ (the deficiency of heterozygotes in a population relative to that expected under random mating), using the software Hickory (Holsinger and Lewis, 2001–2009). An $f$ value of 0 would indicate that the numbers of heterozygotes in the population are equal to that expected under random mating; increases in $f$ indicate a deficiency of heterozygotes relative to expectation, which can occur as a result of selfing. We assumed uniform [0,1] priors on $f$ and so the 95% credible intervals associated with the estimate can never overlap 0. To determine if our estimates of $f$ were different from zero we compared the deviance information criterion (DIC) to that of a model that assumes no inbreeding. When models differ by only one parameter, a significant difference is approximately 2 log-likelihood units. DIC involves an average log-likelihood, and so if the difference between the two models was less than 3 DIC units we could not reject the null hypothesis that $f = 0$. In contrast, DIC values of $3 – 7$ or $DIC > 7$ were considered moderate or strong support that $f > 0$, respectively. In populations that contained both diploid and triploid gametophytes, we considered gametophytes heterozygous for PGI triploid, and all other gametophytes diploid. We ran analyses on the sporophytes from these two groups separately. We could not do this for populations with co-occurring haploid and diploid gametophytes, but excluding these populations from the analyses did not affect the results in any significant way.

Analysis of variance was used to test the effect of the presence of hermaphrodites on a population’s estimate of sporophytic $f$. We weighted each estimate of $f$ by the inverse of its variance (Quinn and Keough, 2002). To test the effect of

<table>
<thead>
<tr>
<th>Population</th>
<th>Ploidy</th>
<th>$n$</th>
<th>$f$</th>
<th>s.d.</th>
<th>95% Credible interval</th>
<th>Difference in deviance information criteria</th>
<th>$S$</th>
<th>$S = 0.51$</th>
<th>$S = 0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Lacking hermaphrodites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1,2</td>
<td>11</td>
<td>0.26</td>
<td>0.17</td>
<td>0.01–0.64</td>
<td>−0.98</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>23</td>
<td>0.44</td>
<td>0.19</td>
<td>0.07–0.79</td>
<td>1.57</td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>28</td>
<td>0.52</td>
<td>0.21</td>
<td>0.08–0.88</td>
<td>2.22</td>
<td>0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>22</td>
<td>0.18</td>
<td>0.15</td>
<td>0.01–0.53</td>
<td>−1.93</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>31</td>
<td>0.66</td>
<td>0.16</td>
<td>0.28–0.92</td>
<td>9.20</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>33</td>
<td>0.18</td>
<td>0.14</td>
<td>0.01–0.52</td>
<td>−1.77</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>0.37</td>
<td></td>
<td></td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>(B) Containing hermaphrodites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1,2,3</td>
<td>19</td>
<td>0.53</td>
<td>0.24</td>
<td>0.07–0.92</td>
<td>1.64</td>
<td>0.14</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>19</td>
<td>0.46</td>
<td>0.22</td>
<td>0.05–0.86</td>
<td>1.15</td>
<td>0.08</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>13</td>
<td>0.37</td>
<td>0.24</td>
<td>0.02–0.87</td>
<td>−0.94</td>
<td>0.02</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>13</td>
<td>0.25</td>
<td>0.18</td>
<td>0.01–0.69</td>
<td>−1.33</td>
<td>0.06</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>2</td>
<td>23</td>
<td>0.76</td>
<td>0.15</td>
<td>0.39–0.97</td>
<td>12.15</td>
<td>0.31</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>34</td>
<td>0.49</td>
<td>0.22</td>
<td>0.06–0.88</td>
<td>1.30</td>
<td>0.11</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>3</td>
<td>40</td>
<td>0.67</td>
<td>0.13</td>
<td>0.37–0.89</td>
<td>17.28</td>
<td>0.24</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>45</td>
<td>0.58</td>
<td>0.23</td>
<td>0.11–0.94</td>
<td>3.99</td>
<td>0.17</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>3</td>
<td>16</td>
<td>0.51</td>
<td>0.29</td>
<td>0.02–0.98</td>
<td>−0.02</td>
<td>0.12</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>29</td>
<td>0.44</td>
<td>0.19</td>
<td>0.06–0.78</td>
<td>1.83</td>
<td>0.07</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>28</td>
<td>0.32</td>
<td>0.19</td>
<td>0.02–0.71</td>
<td>−0.32</td>
<td>−0.02</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>0.51</td>
<td></td>
<td></td>
<td>0.51</td>
<td></td>
</tr>
</tbody>
</table>

$n =$ number of sporophytes sampled. Five populations (mixed haploid and diploid) had insufficient sporophytes or insufficient genetic diversity to measure population selfing rates. The difference in deviance information criteria were estimated from a model including a parameter from selfing (full model) and one which assumed no inbreeding ($f = 0$). Values in bold represent a model where $f$ is estimated strongly preferred to one where $f = 0$. $S$ is the proportion of the progeny in a population produced by intergametophytic selfing and $S_I$ is the proportion produced by selfing within gametophytes. $S$ was estimated assuming $S_I = 0$ or 0.51.
ploidy on the estimate of \( f \), we again used weighted analysis of variance, but focused only on populations with hermaphrodites to remove the confounding effects of sex. Additionally, we only considered single-ploidy populations or populations for which the ploidy of all individuals could be inferred using allozyme data from gametophytes. We also used Hickory to assess support for differences in \( f \) between ploidies within a population. For each population with co-occurring diploid and triploid gametophytes, we compared the difference between paired random samples of \( f \) from the Bayesian posterior distribution (see Holsinger and Wallace, 2004). If the 95% credible interval for the difference included zero, we concluded there was no evidence for differences between the diploids and triploids within that population.

Selfing rates were calculated from values of \( f \) using the following formula:

\[
F_e = \frac{(S + 2S_1)}{(2 - S)}
\]

where \( F_e \) is the equilibrium inbreeding coefficient, \( S \) is the proportion of the progeny in a population produced by intergametophytic selfing and \( S_1 \) is the proportion produced by selfing within gametophytes. We used our estimate of \( f \) for \( F_e \) under the assumption that populations were at inbreeding equilibrium. This will be true for populations undergoing intragametophytic selfing. In populations that contained only separate-sexed individuals, intergametophytic selfing was estimated as:

\[
S = 2F_e/(1 + F_e).
\]

Because females and hermaphrodites have a very similar morphology, we made the assumption that the amount of selfing between gametophytes is similar for both groups. Hence, to estimate \( S_1 \) in populations containing hermaphrodites, we used the estimate of intergametophytic selfing obtained for females.

RESULTS

Sex ratios and flow cytometry

Flow cytometry revealed three distinct groups. One group had an estimated 1C value of 0.56 (range = 0.53–0.57, CV = 1.5), the second group had an estimated 1C value of 1.28 (range = 1.21–1.37, CV = 2.8) and the third group had an estimated 1C value of 1.91 (range = 1.82–2.07, CV = 3.5). We considered these groups to be haploid, diploid and triploid, respectively.

Gametophytes of different ploidies were frequently found in the same populations (Fig. 1A, Table 2). Five populations had both haploid and diploid gametophytes, one population had diploid and triploid gametophytes, and two populations had co-occurring haploids, haploids and triploids. Eight populations included only diploid gametophytes, and in five populations we observed only triploid gametophytes (Fig. 1A, Table 2). No pure haploid populations were sampled.

The ratios of genome size between haploids and diploids, and between diploids and triploids were consistent between populations. In 2007, the haploid/diploid genome size ratio was 0.43–0.44 within any single population (population 2 = 0.44, s.e. = 0.014; population 4 = 0.43; s.e. = 0.0042; population 5 = 0.44, s.e. = 0.012). Both populations with mixtures of diploid and triploid gametophytes had genome size ratios of 0.67 (s.e. = 0.018 and 0.022, respectively).

In 2007 there was a strong association between the number of triploids and the number of hermaphrodites in a population (Fig. 1B; Fisher’s exact test, \( P < 0.001 \)). By contrast, there was no significant association between the number of hermaphroditic shoots in a population and the number of haploids (Fisher’s exact test, \( P = 0.991 \)) or diploids (Fisher’s exact test, \( P = 0.543 \)). Five populations had mixtures of haploid (\( n = 43 \)) and diploid (\( n = 23 \)) individuals; none of these had hermaphrodites. However, one population where only diploid individuals were sampled also had a low frequency of hermaphrodites (population 14).

Using the 2009 data consisting of individuals of known sex, a Fisher’s exact test pooled over all populations found a significant association between sex and ploidy (Table 2; two-sided test, \( P = 0.026 \)); some hermaphrodites were diploid (Table 2; populations 2, 13 and 16), but most hermaphroditic gametophytes were triploid. None of the seven sexed haploid individuals was hermaphroditic, consistent with the association found by randomly sampling individuals from populations with a known sex ratio. Using a binomial probability distribution, if males, females and hermaphrodites were equally likely, the probability that seven haploid plants sampled at random would be female is 0.058. If separate sexes and hermaphrodites are equally likely, the probability that all seven haploid plants would be separate-sexed is 0.0078.

Selfing rates

In 2007, estimates of sporophytic \( f \) in populations with only male or female gametophytes ranged from 0.18 to 0.66 (the mean of the population estimates was 0.37, Table 1). This would correspond to an average level of intergametophytic selfing of 0.3–0.8 (with mean of the population estimates = 0.51). Only one population had estimates of sporophytic \( f \) strongly supported as different from zero, suggesting that intergametophytic selfing rates are generally low. In contrast, in four of the 12 populations that contained hermaphroditic gametophytes, a model in which \( f \) was estimated was strongly preferred to one where \( f \) was zero. If we assume a level of intergametophytic selfing in hermaphrodites equivalent to that of females (for most separate-sexed populations, intergametophytic selfing rates were not different from zero), in populations with hermaphrodites, intragametophytic selfing would be 0.51 (Table 1).

An analysis of variance comparing estimates of \( f \) between populations with or without hermaphrodites suggested that \( f \) was significantly higher in populations containing hermaphrodites (\( F_{1,16} = 4.99, P = 0.04 \)). However, there was no difference in estimates of \( f \) between diploid and triploid populations that contained hermaphrodites (\( F_{1,8} = 3.57, P = 0.095 \)). Within a population there was no or only weak support for differences in \( f \) between diploids and triploids (population 15: difference in \( f = 0.51 \), 95% credible
intervals $= -0.02$–$0.88$; population 16: difference in $f = 0.005$, 95% credible intervals $= -0.52$–$0.51$).

**DISCUSSION**

Hermaphroditic *Atrichum* gametophytes can be diploid or triploid, while male and female gametophytes were haploid, diploid or triploid. This is consistent with polyploidy as a proximate mechanism for the evolution of hermaphroditism, although hermaphroditism is not a necessary outcome of polyploidy. Hermaphroditism also results in changes to the mating system. Most populations with strictly separated-sexed individuals have estimates of sporophytic $f$ that are not supported as being different from zero. In contrast, there is strong support that one-third of populations with hermaphrodites undergo selfing, probably as a result of intragametophytic selfing, or through changes in population structure. Below we discuss the implications of changes in ploidy for moss populations, and discuss factors influencing mating system evolution in mosses.

**Polyploidy is correlated with the sexual system**

Allen (1935a) suggested that, in mosses, X and Y chromosomes have separate sex-determining alleles, and polyploidy could result in female (XX), male (YY) or hermaphroditic (XY) gametophytes. The link between polyploidy and hermaphroditism has been noted repeatedly, but few studies have investigated intrageneric variation in these traits. In the populations of *A. undulatum s.l.* studied there was a strong association between the presence of hermaphrodites and the presence of triploids in a population, consistent with this expectation. However, we did find differences in sex expression between diploids and triploids. It is likely that diploid and triploid individuals are different species, and so the genetic mechanism of sex expression may differ in these groups. Interestingly, most diploid populations did not

---

**FIG. 1.** (A) Percentage hermaphrodite (H), female (F) and male shoots (M) and ploidy determination of gametophytes from 21 populations of *Atrichum undulatum s.l.* around New Brunswick sampled in 2007. Pop. = population number; $n$ is the number of fertile shoots, sampled either for sex ratio (sterile shoots were not counted) or for ploidy. (B) The association between percentage hermaphrodites in a population and percentage triploid shoots. All fertile shoots in the sample were included in the estimate of sex ratio, and ploidy was determined from a random sample of these shoots. Five populations were not included as plants were not randomly sampled for ploidy determination. Eight populations have no hermaphrodites and no triploids.
contain hermaphrodites. This is apparently contrary to Allen’s (1935a) genetic model of sex expression as fertilization of an XX female by a YY male should result in XY progeny. It is possible that the absence of hermaphrodites in the diploid populations may be due to interactions of genes determining sex, and dominance effects of alleles on X and Y. For example, Allen (1935b) suggested that, in Sphaerocarpos, the strong female bias of polyploid individuals may be due to dominance of the female-determining allele. Additional factors, such as size or environmental sex determination, may also influence sex expression (and see Ricca and Shaw, 2010), and indeed many hermaphrodites were sequential hermaphrodites, with male and female structures presented on different years. In summary, these results suggest that changes to ploidy can, but do not always, result in hermaphroditism.

Because of sex-associated chromosomes in Atrichum (Tatuno and Kise, 1970), we assumed that haploids can never be hermaphrodites. Indeed, in five populations with only separate-sexed individuals, shoots were either haploid or diploid. Furthermore, in the two populations with co-occurring haploids and hermaphrodites, the hermaphrodites were always diploid or triploid (populations 12 and 16). Flow cytometry of individuals of known sex revealed no haploid hermaphrodites, although this sample size is low (n = 7), and more sampling is required to completely rule out this association.

The influence of polyploidy on sex expression may also depend on the degree of allo- or autoploidy. Hybridization will influence chromosomal inheritance through preferential pairing of homologues. The X and Y chromosomes may not pair at metaphase if they are sufficiently dissimilar from each other, which could occur as a result of divergence in the two parent species. Instead, X would pair with X and Y with Y. This would lead to more frequent segregation of X and Y to one gametophyte, potentially resulting in hermaphroditism (Lewis, 1961). The history of hybridization and the mechanism for determination of sex expression have not been investigated in polyploid species of Atrichum, and fixed heterozygosity at PGI in triploid individuals could have resulted from hybridization, although other explanations are possible. We are currently growing half-sibling families from haploid, diploid and triploid populations to investigate sex ratio effects that would be expected under different patterns of chromosome segregation.

Hermaphroditism increases the selfing rate, probably due to intragametophytic selfing

Most populations with separate sexes have selfing rates not different from zero, suggesting negligible intergametophytic selfing. This is consistent with findings from Eppley et al. (2007), who found that in ten species of mosses with separate sexes, 16 of the 18 populations surveyed had selfing rates not different from zero. Similarly, Szövényi et al. (2009) measured selfing rates in a population of Sphagnum lescurii (which has separate sexes) and found equal proportions of inbred and outcross sporophytes, resulting in a mean population-level selfing rate of zero. In contrast to this, intragametophytic selfing in populations with hermaphrodites could be high (S1 ranged from 0 to 0.76). Because intragametophytic selfing reduces heterozygosity completely in one generation, increases in f will be greater than would occur with the same amount of intergametophytic selfing.

It is interesting that four hermaphroditic populations showed levels of selfing strongly supported as being different from zero, but considerably less than one. This is apparently contrary to theory. In the absence of a cost to selfing, selfing should be favoured and could be expected to increase to fixation. Intragametophytic selfing in hermaphrodites should reduce or eliminate the cost to selfing, as deleterious alleles should be purged in one or only a few generations (Klekowksi, 1979; Hedrick, 1987; Holsinger, 1987). Indeed, in many other species of mosses with combined sexes (including Atrichum flavisetum), sex structures are synoicous, with male and female organs intermingled. This arrangement would suggest a reliance on selfing, as a continuous film of water or other unpredictable vectors would not be required for fertilization (Stark, 1983). Only very occasionally did we find male and female organs intermingled in the Atrichum individuals studied here. Generally, male and female sexual organs were separated by at least one leaf bract. Thus, the advantage to hermaphroditism in Atrichum seems not to assure reproduction in times when transport of sperm is restricted.

Table 2. Numbers of individuals of known sex and ploidy (1n = 1, 2 or 3x) of gametophyte material from five populations of Atrichum undulatum s.l. in New Brunswick sampled in 2009

<table>
<thead>
<tr>
<th>Population</th>
<th>Ploidy</th>
<th>H</th>
<th>F</th>
<th>M</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Haploid</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diploid</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Triploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>Haploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Diploid</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Triploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Haploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Triploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>Haploid</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Triploid</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>Haploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diploid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Triploid</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>All</td>
<td>Haploid</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Diploid</td>
<td>11</td>
<td>22</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Triploid</td>
<td>53</td>
<td>74</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

H, hermaphrodite; F, female; M, male; S, sterile shoot.
as other life histories, such as an independent haploid stage, can influence the expression of inbreeding depression. Although most homosporous ferns apparently exhibit extreme outcrossing, mixed mating has been demonstrated in some populations of *Droteris expansa*, *Hemionitis palmata* and *Blechnum spicant* (Soltis and Soltis, 1992). Epplle et al. (2007) also found significant estimates of mixed mating in one population of *Breutelia pendula*. Thus, the paradox of mixed mating seems to extend to land plants generally. It could be argued that the evolutionary stability of mixed mating is especially enigmatic in hermaphroditic mosses, where the benefits of purging to alleviate inbreeding depression can occur in a very few generations. An extension of current theoretical models to a moss life cycle (including intragametophytic selfing) would clearly provide interesting insights.

ACKNOWLEDGEMENTS

We thank Paul Kron and Brian Husband for access to and support using the flow cytometer and for running additional samples, Dave Kubien and Peter Szövényi for comments on the manuscript, and Katie Friars and Jessica Moore for laboratory assistance. This study was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant and a New Brunswick Museum F. M. Christie Research Fellowship to L.K.J.

LITERATURE CITED


