

Australian *Senecio macrocarpus* and *S. squarrosus* were suggested as apomictic but are fully sexual: evidence from flow cytometric seed screening analyses

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Abstract

Asexual seed reproduction, apomixis, is a relatively rare reproductive strategy in angiosperms. Its rarity is probably due to evolutionary constraints that require full and synchronous expression of all the steps that modify the ancestral sexual pathway. Therefore, the documentation of any new occurrence of apomixis, especially in taxonomic groups with no previous report, should be based on thorough and solid evidence.

Here we correct the report of apomictic reproduction in two endemic hexaploid species of *Senecio* from Australia. Based on either excess or deficiency of expected heterozygosity across loci, overrepresentation of several multilocus genotypes, and segregation analysis assessed using microsatellite markers, it was suggested that apomixis together with selfing could explain the observed patterns of genetic variation in perennial *S. macrocarpus* and annual *S. squarrosus*.

We used flow cytometric seed screening analyses to verify the mode of reproduction of several widespread multilocus genotypes that are considered to be apomictic. These analyses revealed that all seed progeny arose by the sexual pathway, as they exhibited a constant 3:2 endosperm to embryo ploidy ratio, typical of double fertilization of a reduced embryo sac by two reduced sperm cells. Therefore, both species reproduce sexually. We suggest that the unusual genetic patterns in both allopolyploids are caused by originally low variation in the parental species, which probably also reproduce by selfing, disomic inheritance and probably also the presence of null alleles. We call for a more careful and critical interpretation of the patterns obtained, taking into account all aspects related to apomictic reproduction.

Introduction

Apomixis, defined here as asexual reproduction via seeds, has been reported in ca. 2.2% of genera mostly belonging to three families: Asteraceae, Poaceae and Rosaceae (Gustaffson 1946–1947, Asker and Jerling 1992, Hojsgaard et al. 2014). The cause of its rarity is unknown but it has widely been accepted that apomixis is a complex developmental mechanism which makes its acquisition difficult (Marshall and Brown 1981). Apart from sporophytic apomixis where an embryo arises directly from a somatic cell of the nucellus in addition to normal sexually-derived embryo (adventitious embryony), gametophytic apomixis is a derived state of sexual reproduction and requires full expression and tight synchronization of the following developmental steps: (i) omission of female meiosis, the end product of which is an unreduced female gamete – an egg cell (apomeiosis); (ii) autonomous embryo formation from the unreduced egg cell, and (iii) formation of endosperm nutritive tissue, which can arise autonomously (without fertilization of central nucleus) or pseudogamously (after fertilization of central nucleus by a pollen spermatid cell) (Gustaffson 1946–1947). Given the extremely long evolutionary history of sexual reproduction in eukaryotes (Cavalier-Smith 2010), embryo sac formation and double fertilization in flowering plants (Friedman 2006), a transition to apomixis thus necessitates the modification of well-established reproductive pathways and may be hard to complete.

Apomixis is closely associated with polyploidy, hybridisation, perenniality and usually self-incompatibility in diploid ancestors of apomicts (Asker and Jerling 1992). At the diploid level, apomixis is naturally known only in the genus *Boechea* (Brassicaceae) (Böcher 1951, Aliyu et al. 2010), although extremely rare, and usually unfit, diploid apomicts were produced by haploid parthenogenesis from tetraploid apomictic parents (Bicknell 1997). Nogler (1984) suggested that apomixis is encoded by a dominant allele that is only viable and transmissible in the heterozygous state, thus requiring at least a diploid gamete. Consequently, apomixis can only be expressed in polyploid organisms. An explanation provided by Carman (1997) suggests that apomixis arises from the asynchronous expression of duplicated genes involved in the development of the female gametophyte. This occurs due to a 'miscommunication' between differently adapted genomes acquired from different genotypes within the same species or, more commonly, from divergent species and can be viewed as the effect of changes in regulatory regions. Since hybrids, but also autopolyploids, often exhibit disrupted meiosis, apomixis could be seen as a way of escaping sterility (Darlington 1939). When fully established, apomixis impacts morphological and genetic variation (Gornall 1999, Majesky et al. 2017).

After critical re-evaluation of literature records, effective apomixis was confirmed in 22 genera in Asteraceae (Noyes 2007). For the remaining 46 genera for which apomixis was reported, firm evidence is either still lacking (16 genera), or was rejected (30 genera) because of occasional developmental abnormalities or the presence of only one component of apomixis like apomeiosis, i.e. unreduced gamete formation, which is however a normal element of the polyploidization process in angiosperms regardless of the presence of apomixis (Ramsey and Schemske, 1998).

Uncritical acceptance of unconfirmed or unclear records on apomixis and their perpetuation through review papers has inflated the real number of genera with apomixis (Noyes 2007). This suggests that any suspicion of apomixis in those taxonomic groups with no previous firm evidence should be thoroughly scrutinized prior to its acceptance and publication. Such a critical consideration is relevant not only for accurate estimation of the incidence of apomixis in angiosperms, for purposes such as meta-analysis, but also for evolutionary, ecological or conservation biology-oriented studies as reproductive mode influences fitness parameters at the population level. For these reasons, we re-assess the report by Ahrens and James (2015), two co-authors of the present study, of apomixis in two *Senecio* taxa from Australia.

Ahrens and James (2015), assessed genetic variation in 20 populations of *Senecio macrocarpus* L., a declining perennial herb endemic to southeastern Australia, and in one population of *S. squarrosus* A. Rich, a more common and partially sympatric annual species. Both species are hexaploid with $2n = 6x = 60$ and self-compatible (Lawrence 1980, Ahrens and James 2015). By applying thirteen microsatellite markers (SSR) to more than 500 plants, they found low genetic diversity with 100 multilocus genotypes (MLGs thereafter) in total and a strong deviation from HW equilibrium (either excess or deficiency of expected heterozygosity across loci). Importantly, several MLGs were over-represented and were shared among many plants and more than one geographic location. Specifically, in *S. macrocarpus*, MLG002 was found in 108 plants, MLG005 in 55 plants, MLG037 in 9 plants, and in *S. squarrosus*, MLG038 was

found in 25 plants. Moreover, the same MLGs with no sign of recombination/segregation of the alleles were found in offspring of several seed plants belonging to the above listed over-represented MLGs (Ahrens and James 2015). These results led the authors to hypothesise that the genetic patterns could be explained by both species being at least partially (facultatively) apomictic and that apomixis has fixed rare sexual events and further variation accumulated via somatic mutations (Ahrens and James 2013, 2015). A recent study of *Taraxacum* using FCSS and SSR-seq genotyping showed that the mode of reproduction could be inferred from maternal parent-offspring SSR-seq genotypes (Šarhanová et al. 2024). Genotypes of all apomictic progeny were identical to the maternal parent and the authors concluded that SSR-seq was suitable for genotyping seed in apomictic complexes in the family Asteraceae. Despite their interpretation of genotyping results based on SSR allele size for *S. macrocarpus* and *S. squarrosus*, Ahrens and James (2015) were aware, however, that the only genus with a record of apomixis in the Senecionae tribe is *Petasites*, and even this record was based on a lack of direct evidence (see Noyes 2007). Because of the putatively controversial record of apomixis in *Senecio*, the first author of this study requested seeds from over-represented MGLs to determine whether their origin was sexual or apomictic by using flow cytometry seed screening (FCSS). Here, we provide evidence that the two Australian taxa, *S. macrocarpus* and *S. squarrosus* which have recently been suggested to reproduce by apomixis (Ahrens and James 2015) are in fact sexual species.

Material and methods

Seed material

Seed material for FCSS was collected on 23 October 2013 from mature capitulas of five individual plants of *S. macrocarpus* and one plant of *S. squarrosus* growing in one large population in the Messent Conservation Park, the Coorong, southeastern Australia (Table 1, for further details see Ahrens and James 2015). These plants were previously genotyped and attributed to one of several over-represented and shared multilocus genotypes which were considered to reproduce apomictically (Ahrens and James 2015, Table 1).

Table 1

The basic statistics of flow cytometric seed screening analyses of bulk seed samples of *Senecio macrocarpus* (ID #1428-#1471) and *S. squarrosus* (ID #1491-#1534). MLG – code of over-represented shared multilocus genotypes (Ahrens and James 2015). S – the mean position, i.e. mean fluorescence intensity of G1 nuclei of the standard plant *Pisum sativum*, em – the mean position of G1 embryonic nuclei of the seed sample (bulk of 5 seeds), en – the mean position of G1 endospermic nuclei of the seed sample. CV – coefficients of variation of peaks corresponding to the standard plant and the seed sample (em – embryo, en – endosperm). em / S – the ratio of the relative fluorescence intensity of G1 embryonic nuclei of seed sample to the relative fluorescence intensity of G1 nuclei of standard, en / em – the ratio of the relative fluorescence intensity of G1 endospermic nuclei to the relative fluorescence intensity of G1 nuclei of embryonic nuclei of the seed sample.

ID	MLG	S	em	en	CV-S [%]	CV-em [%]	CV-en [%]	em / S	en / em
#1428	MLG002	168.73	198.44	293.77	1.64	2.56	2.04	1.18	1.48
#1452	MLG002	168.84	197.3	295.96	2.04	2.84	2.49	1.17	1.5
#1471	MLG037	170.22	199.05	297.72	1.87	2.75	1.93	1.17	1.5
#1491	MLG038	166.78	193.45	289.88	1.79	2.14	1.61	1.16	1.5
#1532	MLG038	148.52	171.96	259	1.9	2.44	2.36	1.16	1.51
#1534	MLG038	167.9	195.19	291.18	2.16	2.6	1.99	1.16	1.49

Flow cytometric seed screening analyses (FCSS)

In July 2015, five fully developed seeds from each seed family were analysed (Table 1). The samples were prepared using a two-step procedure (Otto, 1990; Doležal and Göhde 1995) and *Pisum sativum* cv. Ctirad (2C = 9.09 pg DNA, Doležal et al. 1998) as an internal standard. Briefly, a seed sample was chopped together with a small piece (~ 0.5 cm²) of leaf of the standard in 0.1 mL of Otto I buffer (0.1 M citric acid and 0.5% Tween 20) with a subsequent addition of 0.5 mL of Otto I. The suspension was then filtered through a 42-µm nylon mesh. After filtration, 0.6 mL of the Otto II buffer (0.4 M Na₂HPO₄·12H₂O, β-mercaptoethanol and 4',6-diamino-2-phenylindole (DAPI) as an AT-specific fluorescent stain) was added to the suspension. The intensity of fluorescence of samples and standard nuclei was measured using a Partec Cyflow instrument equipped with a HBO lamp. Histograms were accumulated at a flow rate of ca. 10–30 particles s⁻¹ for a total count ranging between 1,300 and 3,500 nuclei per analysis. The resulting histograms were analysed with FloMax software (v.2.4d, Partec, Munster, Germany) using the automatic peak analysis tool which calculated mean positions of the peaks of the internal standard and embryonic and endospermic tissues of the sample corresponding to their relative DNA content and the coefficients of variation of these peaks.

Interpretation of flow cytometric seed screening patterns

FCSS makes it possible to discriminate between sexual and apomictic pathways involved in seed formation due to differences in the ratio of ploidy in the embryo and corresponding endosperm (Matzk et al. 2000). Because sexual reproduction in angiosperms is associated with a double fertilization, when one reduced pollen spermatid cell fertilizes a reduced egg cell, and the second pollen spermatid cell

fertilizes a central nucleus which arises by fusion of two reduced polar nuclei, the ploidy of endosperm is 1.5× larger than the ploidy of corresponding embryo (Matzk et al. 2000). However, this ratio is valid only under certain conditions. First, the central nucleus must arise from a fusion of two polar nuclei. Such a composition is typical for a monosporic 8-nuclei embryo sac of the *Polygonum*-type that has been recorded in ca. 70% of angiosperms (Davis 1966) and has been confirmed multiple times in *Senecio* (Winge 1914, Carano 1921, Banchetti 1961, Rad and Hajisadeghian 2014). In contrast, the endosperm to embryo ploidy ratio will be larger than 2 in polysporic ES with more than two polar nuclei. Second, only reduced gametes of the same ploidy can be involved in fertilization to produce a ploidy ratio of 3:2 endosperm to embryo, otherwise this ratio will differ from 1.5, but will always be smaller than 2 (e.g. Mráz and Zdvořák 2019). In the case of autonomous apomixis, where both embryo and endosperm develop from unreduced cells of an ES without fertilization, the ploidy ratio will be equal to 2. Importantly, the 2:1 ratio of detected peaks suggesting autonomous apomixis can equivocally be interpreted as G1 and G2 phases of embryonic tissue since endosperm in mature seeds of many Asteraceae species might be reduced or even undetectable (Bonifácio et al., 2019). In the case of pseudogamous apomixis, the endosperm arises after fertilization of the central nuclei and therefore the ploidy ratio will be larger than two and the pattern can be the same or very similar to that produced by a fertilized polysporic ES. The exact value of the ploidy ratio will depend on the ploidy of contributing pollen.

Results and discussion

Flow cytometric analyses of seeds revealed that all analysed plants of *Senecio macrocarpus* and *S. squarrosus*, produced seeds with embryos showing a relative genome size that was ca. 1.16-1.17-times larger than that of the internal standard (Table 1, Fig. 1). These results thus correlate with the same chromosome number reported for both species, i.e. $2n = 60$ (Lawrence 1980, Ahrens and James 2015) and with the taxonomic treatment of both taxa being classified as members of the the informal 'Erechthitoid' taxonomic group of Australian *Senecio* (Lawrence 1980).

The ratio between ploidy of endosperms to ploidy of corresponding embryos in all bulk samples and one individual seed analysis was always equal or very close to 1.5 (Table 1). These data thus indicate that all 30 analysed seeds arose by a sexual pathway, i.e. through double fertilization and involvement of solely reduced male and female gametes formed by meiosis. Consequently, the plants which produced these seeds and belonged to over-represented MLGs with suggested apomictic reproduction (Ahrens and James 2015) must have reproduced sexually. In contrast, we did not find any indication of apomictic seed formation in these plants, i.e. 2:1 ratio of ploidy of endosperm to ploidy of embryo which is characteristic for autonomous apomixis in Asteraceae as has been shown, for example, in two large scale studies in the genus *Hieracium* (Mráz et al. 2019, Mráz and Zdvořák 2019), in *Pilosella* (Krahulec and Krahulcová 2011, Šingliarová et al. 2011, Sailer et al. 2020), and in *Taraxacum* (Mártonfiová et al. 2010). The current flow cytometrical results thus contradict the interpretations based on population genetic data by Ahrens and James (2015).

Before discussing this discrepancy, let us to briefly provide further indications which support the flow cytometric evidence for sexual reproduction in both *Senecio* species. First, despite very intensive biosystematics research in *Senecio*, one of the largest genera in Asteraceae, there is no evidence for functional apomixis in the genus, nor in the whole tribe Senecionae (Noyes 2007). Second, taxonomic evaluation in *Senecio* does not reflect any discrete morphological patterns that may have been stabilized by apomixis (Thompson 2015) and are typical for apomictic complexes (arrays of microspecies or infraspecific taxa, see Majeský et al. 2017). Third, apomixis is tightly associated with perenniality with one remarkable and known exception – the *Erigeron annuus* agamic complex (Noyes 2000). Although *S. macrocarpus* is perennial, *S. squarrosus* is annual, and therefore apomixis should not be expected in this species. Finally, several embryological studies on different representatives of the genus found normal reduced embryo sac formation (Winge 1914, Carano 1921, Banchetti 1961, Rad and Hajisadeghian 2014). Although the last cited study reported the occurrence of normal sexual embryogenesis in *Senecio vulgaris* in 70% of ES, they also described 'exceptional embryogenesis' consisting of early embryo formation from antipodal cells in 30% of ES. However, Rad and Hajisadeghian (2014) who called this process 'antipodal apogamy' did not specify the ploidy of the examined material, whether or not such 'antipodal' embryos were fertilized, or their fate. Such 'exceptional apomixis' has never been described or discussed in compendia devoted to apomictic reproduction (Gustaffson 1946-47, Asker and Jerling 1992, Carman 1997). Furthermore, the proliferation of antipodal cells in ES has been observed in many species, including *Senecio vulgaris*, but has been interpreted as a haustorial organ with a nutritive function, not as an embryo (Small 1919) in contrast to Rad and Hajisadeghian's (2014) interpretation of apomixis.

Now that we have evidence that *S. macrocarpus* and *S. squarrosus* produce seed sexually, it is necessary to provide alternative explanations for the genetic patterns observed in both population genetic analysis and mother-progeny analysis. Australian *Senecio* species reach sexual maturity within a year of germination (Lawrence 1985a) and all are polyploid, mostly hexaploid followed by tetraploid (Lawrence 1980). Different polyploid *Senecio* taxa have arisen via complex pathways of hybrid evolution (Kadereit et al. 2005). Hexaploids, such as *S. macrocarpus* and *S. squarrosus* are assumed to have arisen as allopolyploids by hybridisation either between diploid and tetraploid parents followed by chromosome doubling, or between closely related tetraploid and octoploid species if the hybrids were fertile and self-compatible (Lawrence 1980). Species belonging to the informal taxonomic Erechthitoid group, including *S. macrocarpus* and *S. squarrosus*, are self-compatible. The main reason Ahrens and James (2015) invoked apomixis as an explanation was the combination of high levels of apparently fixed heterozygosities, the distribution of some multilocus genotypes (MLGs) across large geographic distances and the recovery of identical MLGs in seed parents and their progeny. High levels of selfing, methodological artefacts such as null alleles or more complex inheritance patterns resulting from allopolyploidy may have influenced the results and their interpretation. In polyploids, some genotypes will be phenotypically indistinguishable, even in the absence of null alleles or methodological errors, due to an inability to identify differences in allele dosage between individuals (De Silva et al 2005). It is possible that low genetic variation combined with selfing may have resulted in fewer genotypes being

distinguishable phenotypically in field-sampled *S. macrocarpus* and *S. squarrosus* but a further process is required to explain the identical MLGs found in seed parents and their offspring which suggests an absence of recombination, in turn dependent on inheritance patterns within the species.

Allopolyploids contain distinct genomes and the homologous pairs from each genome form bivalents at meiosis and therefore exhibit disomic inheritance (De Silva et al 2005). The events that led to speciation in *S. macrocarpus* are unknown but as multivalents were not observed and a maximum of two alleles per locus was observed in individuals, disomy was inferred as the mode of inheritance. However, allopolyploidy is considered a restrictive factor in genetic recombination systems (Lawrence 1985b) and the proportion of recombinant progeny produced by polyploids is considerably less than for diploids (Grant 1958, 1975). In addition, allopolyploid species can exhibit genetic heterozygosity that cannot be recombined if each chromosome pair is homozygous but duplicate pairs are heterozygous (Lawrence 1985b). Therefore, low genetic variation, extensive selfing, repetitive subgenomes and a reduced chance of producing recombinant phenotypes may have resulted in the apparent widespread dispersal observed for some MLGs whereas instead, they have arisen from different zygotes producing phenotypically indistinguishable MLGs.

We have discussed several possibilities that could have contributed to the patterns observed, including allopolyploidy, low genetic variation, extensive selfing, repetitive subgenomes and a reduced chance of producing recombinant phenotypes, but as we have no definitive evidence, these ideas are speculative. To improve our understanding of the evolution of *S. macrocarpus* and *S. squarrosus* and their modes of inheritance patterns, additional information is required at both the evolutionary level through phylogenetic analysis and at the population level via hand crosses using biparentally-inherited markers to uncover the biological reasons for the observed genotypic patterns in these species. Irrespective of the underlying reasons, the genetic patterns observed in *S. macrocarpus* are not a result of apomixis and instead are the culmination of other evolutionary processes yet to be understood.

Declarations

Author's contribution PM conceived the study, performed flow cytometric measurements, interpreted the data and wrote the most of part of the first draft of the manuscript. CWA and EAJ provided seed material, suggested explanations of genetic patterns, and contributed to writing. All authors read and approved the final manuscript.

Declarations competing interests The authors declare no competing interests.

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Author Contribution

PM conceived the study, performed flow cytometric measurements, interpreted the data and wrote the most of part of the first draft of the manuscript. CWA and EAJ provided seed material, suggested explanations of genetic patterns, and contributed to writing. All authors read and approved the final manuscript.

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Figures

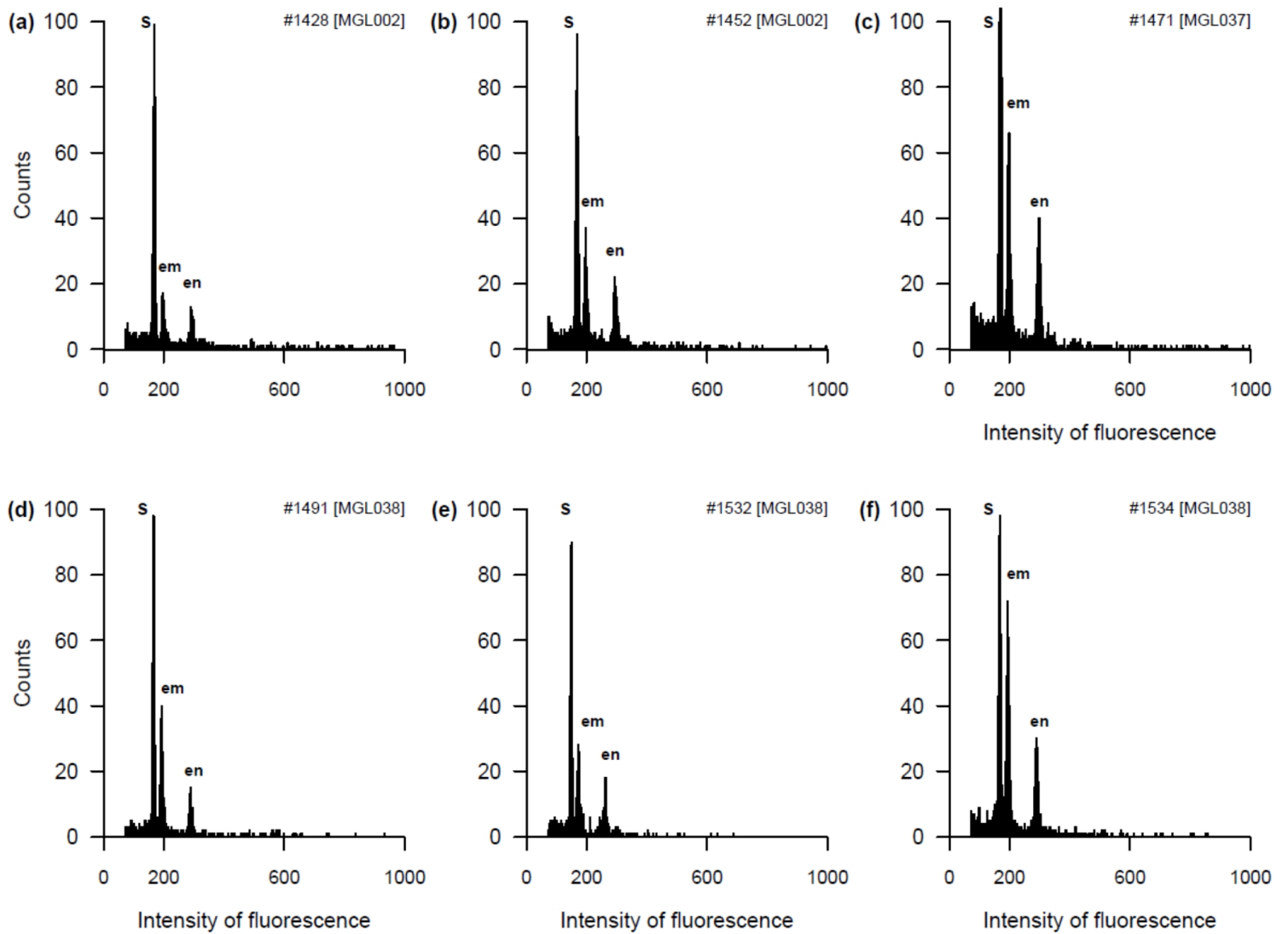


Figure 1

Flow cytometric seed screening analyses of six bulk samples of *Senecio macrocarpus* (a-d) and *S. squarosus* (e-f). Sample from individual maternal plant consisted of five mature seeds which were analysed simultaneously with a standard plant *Pisum sativum*. S – peak corresponding to the G1 phase of nuclei of *Pisum sativum*, em – peak corresponding to the G1 phase of embryonic nuclei of the sample, en – peak corresponding to the G1 phase of endospermic nuclei of the sample.

In the right corner of each plot the plant (sample) ID ('#') and the corresponding multilocus genotype (square brackets) according to Ahrens and James (2015) are depicted.