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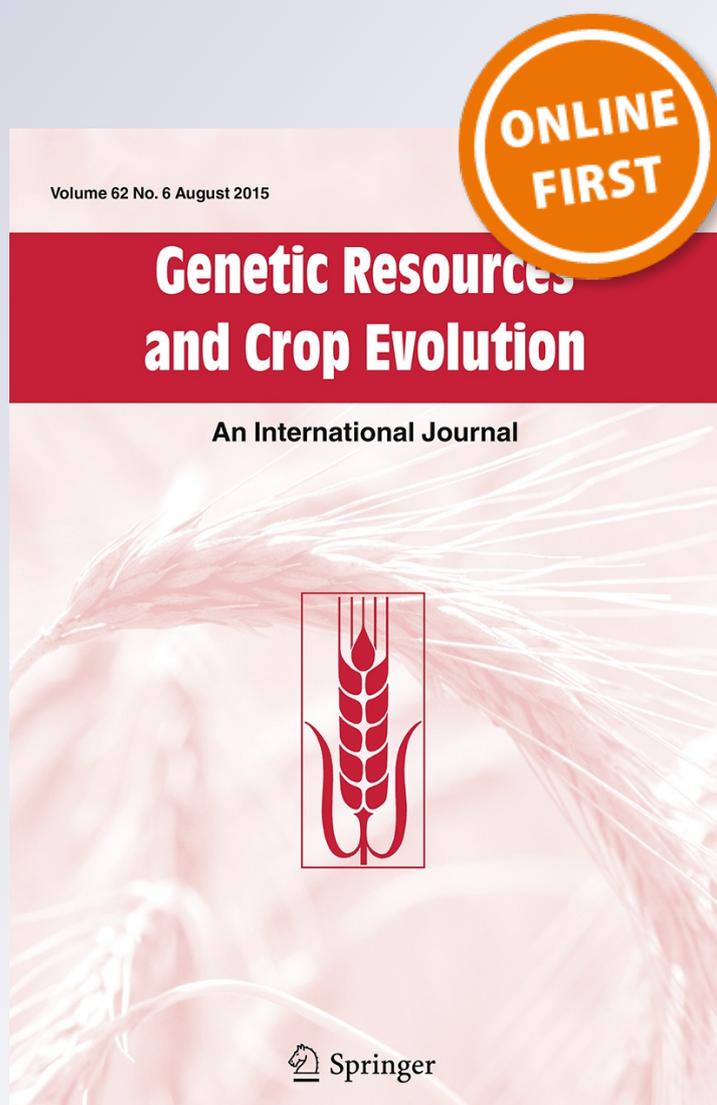
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# Unravelling the ambiguous reproductive biology of *Paspalum malacophyllum*: a decades old story clarified

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**Abstract** In a recent manuscript published by our group we analyzed the reproductive biology of the grass *Paspalum malacophyllum* by using traditional embryological techniques combined with current cytological and molecular methods. Our findings confirmed apparent contradictions regarding the reproductive behavior of *P. malacophyllum* from six independently published reports over the past six decades. Herein we summarize the main findings, conclusions, and validations of all previous studies, highlighting the need for multiple approaches to characterize reproductive systems when using apomictic plants in a breeding program.

**Keywords** Apomixis · Breeding programs · Embryology · Flow cytometry · *Paspalum malacophyllum* · Poaceae · Progeny test · Sexuality

## Introduction

Traditional plant breeding programs are based on the transferability of characters via hybridization by using gametes as genetic vectors during sexual reproduction. However, sexuality breaks up favorable genetic/chromosomal combinations and generates heterogeneous progeny, conditions at odds with the phenotypic homogeneity needed for the development of most commercial varieties. There are only two ways to produce uniform offspring through seeds: (1) by using highly homozygous genotypes (inbred lines, hybrids between two inbred lines or their selfed progeny, or doubled haploids), or (2) via forms of apomixis that bypasses meiosis in the ovule and produce unreduced gametophytes and clonal seeds by developing somatic-like embryos via parthenogenesis. Such apomictic systems occur naturally in polyploids of many angiosperms (Nogler 1984; Asker and Jerling 1992), including several perennial tropical forage grasses (Hanson and Carnahan 1956; Pullaiah and Febulaus 2000).

Apomixis can be an impediment or a valuable tool in the improvement of C<sub>4</sub> grasses. Historically, apomictic breeding was limited to selecting, evaluating, and releasing naturally occurring apomictic

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ecotypes that were more vigorous and productive than existing ones. Many cultivars of apomictic tropical grasses used today were obtained in this manner (e.g. the cultivars ‘Hartley’, ‘Bryan’, and ‘Rodd’s Bay’ of *P. plicatulum*, and cultivars ‘Argentine’, ‘Paraguay’, ‘Competidor’, and ‘Wilmington’ of bahiagrass (*P. notatum*) (Oram 1990; Blount and Acuña 2009). The success of this approach is completely dependent upon whether or not superior apomictic ecotypes of a species exist in nature (Burson and Young 2001). Breeding of apomictic grasses utilizing hybridization became a reality when it was determined that (1) plants with some degree of sexual seed production exist within predominately apomictic species, and (2) apomixis is genetically controlled and the methods of reproduction (sexuality vs apomixis) often segregate similar to other traits (Bashaw et al. 1970; Hanna and Bashaw 1987). The success of breeding apomicts then depends on the availability of sexual germplasm that is cross-compatible with its apomictic counterparts. The sexual plants are used as maternal parents to be pollinated with apomicts, which then produce new gene combinations. Most apomicts are highly heterozygous because of their hybrid origin (e.g. Hörandl and Paun 2007), and this heterozygosity also is expressed in the  $F_1$  hybrids resulting in an array of progeny that segregate for method of reproduction (Burson and Young 2001). The apomictic  $F_1$  hybrids are true breeding with heterosis fixed and genotypes unchanged in future generations. This breeding system is very efficient since seed from the fertile, superior apomictic  $F_1$  hybrids can be increased and immediately evaluated as potential cultivars (Bashaw and Funk 1987; Savidan 2000; Burson and Young 2001; Miles 2007).

*Paspalum* L. is primarily a New World genus of warm-season grasses (Zuloaga and Morrone 2005; Morrone et al. 2012; Scataglini et al. 2014). In a few *Paspalum* species, traits such as forage yield and quality, cold tolerance, photoperiod response, rapid stand establishment, and seedling vigor have been improved by breeding (e.g. Burton 1982; Acuña et al. 2007, 2011). Bahiagrass, *Paspalum notatum* Flügge, is the most extensively cultivated species of the genus in different areas of the world, especially in the southern and southeastern United States (Burton et al. 1997; Blount and Acuña 2009). The species has two botanical varieties: the typical one which is tetraploid and apomictic, and includes several different

cultivated types, and *P. notatum* var. *saurae* Parodi which is diploid and reproduces sexually (Gates et al. 2004). This diploid cytotype is extensively cultivated in USA as Pensacola Bahiagrass and has been improved using recurrent restricted phenotypic selection (Burton 1982).

*Paspalum malacophyllum* Trin., also known as “ribbed paspalum” because of its distinctively ribbed seed, is another *Paspalum* species that reproduces primarily by apomixis. Its natural distribution extends from Mexico to Argentina. In South America, the species is found in northeastern and southern Brazil, Bolivia, Paraguay, and central and northern Argentina (for details on localities see Morrone et al. 2000), ranging from sea level to 3000 meters (Morrone et al. 2000; Zuloaga and Morrone 2005). Because of the diversity of its biotypes in southern Brazil and Paraguay, this area is considered the center of diversity of the species (Morrone et al. 2000; Hojsgaard et al. 2008; Zilli et al. 2014). The species has three reported ploidy levels, i.e. diploid ( $2n = 2x = 20$ ), tetraploid ( $2n = 4x = 40$ ), and hexaploid ( $2n = 6x = 60$ ). Only tetraploids occur throughout its entire area of distribution (e.g. Burton 1942; Honfi et al. 1990; Burson and Hussey 1998; Hojsgaard et al. 2008, 2009) while diploids are restricted to southern Brazil (Hojsgaard et al. 2008; Pozzobon et al. 2008), and only one hexaploid has been reported (Gould 1975). This plant was an escape from a nursery that was planted by Professor R. L. Hensel in College Station, Texas in the 1930s and the site of the nursery is known as Hensel Park (Gould 1975). Although the source of the original *P. malacophyllum* accession planted by Hensel remains unknown, it could be a progeny of one of the first germplasm introductions into the USA (see next paragraph).

The first agronomic characterization of this species was made on a Brazilian and an Argentinian accession, both introduced into the USA in 1921 (Stephens 1941; Bennett 1953). One accession was determined to be a tetraploid by Burton (1940), and later was confirmed by Burson and Hussey (1998). It is probable that the other introduced genotype was also a tetraploid based on Stephens’ (1941) morphological description, which does not match the morphology of known diploid accessions (Hojsgaard et al. 2008; Pozzobon et al. 2008; Zilli et al. 2014). Due to significant morphological dissimilarities, the diploids

together with a tetraploid biotype with exceptionally wide leaf blades had been proposed to be segregated as a new species (for details see Hojsgaard et al. 2008; Zilli et al. 2014). Although *P. malacophyllum* has good palatability as a forage grass, it does not persist well under grazing (Burton 1940; Stephens 1941). Nevertheless, the species is potentially useful for breeding because it is a desirable source of disease resistance and high seed fertility (Burton 1940; Stephens 1941). *Paspalum malacophyllum* has high seed set and is not susceptible to ergot (*Claviceps paspali* Stevens et Hall), a fungus that reduces seed quality in dallisgrass, *P. dilatatum* Poir. and produces toxins harmful to cattle (e.g. Cawdell-Smith et al. 2010). For that reason, the species was used to produce interspecific hybrids before its method of reproduction was known (Bennett 1953; Bennett and Bashaw 1960). Later studies elucidating the reproductive biology of tetraploid *P. malacophyllum* resulted in conflicting findings (detailed in the next section), indicating that tetraploids were (1) highly or obligate sexual (Bennett and Bashaw 1960; Bashaw et al. 1970), (2) facultative apomicts (Burson and Hussey 1998; Hojsgaard et al. 2008, 2013), or (3) obligate apomicts (Hanson and Carnahan 1956; Brown and Emery 1958; Hojsgaard et al. 2013).

### The reproductive discrepancy: single techniques in independent analyses

The first method used to determine the reproductive behavior of *P. malacophyllum* was reported by Hanson and Carnahan (1956). These authors reported, based on the observations of H.W. Bennett (B.L. Burson, personal communication), that the species was an apomict after observation of morphological uniformity of its F<sub>1</sub> progeny (sexual progeny is morphologically heterogeneous). In a later analysis, concordant with the Hanson and Carnahan report, Brown and Emery (1958) embryologically studied the structure of mature embryo sacs in 15 ovules of a single *P. malacophyllum* plant and concluded it was an obligate apomictic after observing only 4-nucleated female gametophytes that lacked antipodal cells (a feature of embryo sacs of apomictic grasses; see, for example, Warmke 1954).

Contrary to these findings, Bennett and Bashaw (1960) reported a *P. malacophyllum* accession to be

sexual because F<sub>2</sub> progeny from a hybrid between a pentaploid apomictic *P. dilatatum* biotype and this *P. malacophyllum* accession were sexual based on their variable phenotypes and aneuploid chromosome numbers. They hypothesized that this *P. malacophyllum* accession had the capacity to overcome factors controlling apomixis and restore sexuality in the F<sub>1</sub> hybrid. Later Bashaw and co-workers (Bashaw et al. 1970) arrived at the same conclusion by embryologically examining embryo sacs in this tetraploid *P. malacophyllum* accession.

Finally, Burson and Hussey (1998) determined from embryological analyses that 14 tetraploid accessions collected in geographically diverse areas of Bolivia and Argentina had high levels of apospory initial cells at early ovule developmental stages and variable levels of functional meiotic (from 64.7 to 96.4 % of reduced gametophytes) and apomictic (10.0–83.3 % of unreduced gametophytes) embryo sacs at anthesis (see Table 3 in Burson and Hussey 1998). Thus, the authors concluded the species must reproduce by facultative apomixis, a conclusion shared by Hojsgaard et al. (2008) after analyzing embryo sac developmental patterns in two other South American genotypes.

The apparent discrepancies in these findings, which categorized different tetraploid accessions as being sexual, facultative apomicts, or obligate apomicts, can be related to the different methodologies used for evaluations (Table 1). Each technique used to examine and characterize *P. malacophyllum* was based on different reproductive stages (i.e. at meiosis and embryo sac stages—beginning and end of gametogenesis—during ovule development, and adult offspring; Table 1). As levels of functional sexuality in apomicts vary between genotypes and at different developmental stages within a single genotype (e.g. Hojsgaard et al. 2013, 2014a), the levels of sexuality and/or apomixis observed in these early studies probably varied because of the different genotypes evaluated and different developmental stages observed (e.g. at flowering and at surviving offspring; see details in the next sections).

Although 8-nucleated *Hieracium*-type embryo sacs are rare aposporous embryo sacs in apomictic grasses (see for example Brown and Emery 1958), Caponio and Quarin (1987) reported this type of female gametophyte in *P. simplex* Morong, a species closely related to *P. malacophyllum*. The presence of

**Table 1** Methodologies employed and reproductive classifications for tetraploid *Paspalum malacophyllum* genotypes evaluated in different studies during the last six decades

Study	<i>N</i>	Accession codes <sup>a</sup>	Origin <sup>b</sup> (vouchers <sup>c</sup> )	Methodology	Plant's life cycle stage analyzed	Plant reproductive classification
Hanson and Carnahan (1956)	1	(nd)	(nd)	Morphology	F <sub>1</sub> progeny (progeny test)	Obligate apomict
Brown and Emery (1958)	1	PI (nd)	(nd)	Embryology	Ovules (flowering stage)	Obligate apomict
Bennett and Bashaw (1960)	1	Miss-1 (FC03490 or FC04240)	Argentina or Brazil	Interspecific crossing	Variable F <sub>1</sub> progeny and aneuploidy in F <sub>2</sub> progeny	Sexual
Bashaw et al. (1970)	1	Miss-1	Argentina or Brazil	Embryology	Ovules (flowering stage)	Sexual
Burson and Hussey (1998)	14	Miss-1; PI508794-508802; PI508804; PI508805; 263-79; 51-82	Argentina (PI, 263-79, 51-82); Argentina or Brazil (Miss-1); Bolivia (PI)	Embryology	Ovules (early ovule and flowering stages)	Facultative apomict
Hojsgaard et al. (2008)	2	TK2449; V5095	Argentina (CTES, MO); Brazil (CEN)	Embryology	Ovules (early ovule and flowering stages)	Facultative apomict
Hojsgaard et al. (2013)	5	DH374; GR564; Q4286; TK2449; V5095	Argentina (CTES, UTEP); Bolivia (CTES, MO); Brazil (CEN); Paraguay (CTES, BAA)	Embryology FCSS Molecular markers	Ovules (early ovule and flowering stages) Seeds F <sub>1</sub> progeny (progeny test)	Facultative apomict Facultative apomict Obligate apomict

*N* number of *P. malacophyllum* genotypes under evaluation, *nd* non determined in the study, *FCSS* flow cytometric seed screening

<sup>a</sup> Accession numbers of plant materials storage on USDA and FCA gene banks (US National Plant Germplasm System, and the Faculty of Agro Sciences, University of Northeast, Argentine, respectively), and/or cultivated at different Experimental Stations of the USDA and the Botanic Garden of IBONE-FCA, UNNE-CONICET

<sup>b</sup> see details about collection sites on each specific referenced manuscripts, accession codes and/or plant introduction databases

<sup>c</sup> Herbarium codes where vouchers were deposited follow the Index Herbariorum (<http://sciweb.nybg.org/science2/IndexHerbariorum.asp>)

antipodal cells in unreduced *Hieracium*-type embryo sacs would make them indistinguishable from normal meiotically reduced *Polygonum*-type embryo sacs, thus introducing the possibility of embryo sac misclassification. This would make precise embryological categorizations a very challenging task and would overestimate the level of sexuality at flowering. Nevertheless, this methodological inconvenience may be solved by analyzing proportions of functional megaspores and aposporous initials at early ovule developmental stages, when both reproductive pathways are clearly distinguishable during the transition

between the end of megasporogenesis<sub>2</sub> and the beginning of megagametogenesis (e.g. Burson and Hussey 1998; Hojsgaard et al. 2014a).

### Multiple techniques to characterize *P. malacophyllum* reproductive strategies

Recently, simpler and more powerful molecular and cytological tools have been developed to analyze the reproductive behavior of plants. DNA-fingerprinting progeny tests based on molecular markers (e.g. RAPD,

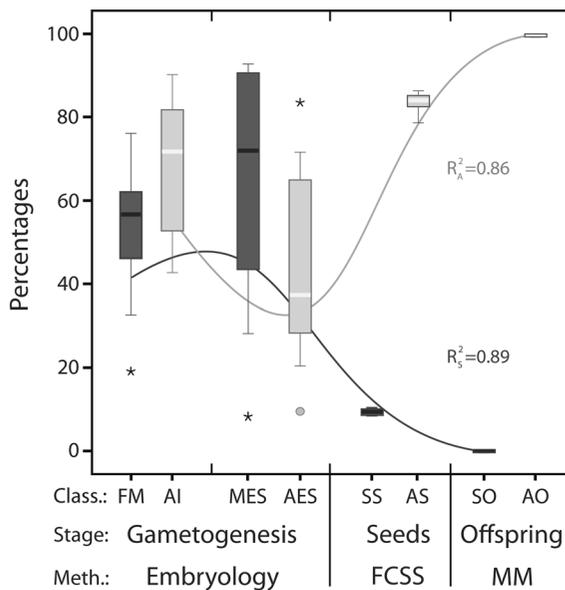
AFLP, ISSR, SSR) and flow cytometric seed screenings (FCSS) allow for the evaluation of up to several hundred samples per day (e.g. Matzk et al. 2000) and are now standard procedures in evaluating reproductive strategies and the assessment of other important characteristics, such as ploidy levels and patterns of genetic variation (e.g. Krejčíková et al. 2013; Liu et al. 2015). Recently, both molecular and cytological techniques were combined with classical embryological approaches to analyze the functionality of reproductive pathways in *P. malacophyllum* throughout the plant's life cycle (two embryological stages plus the reproductive origin of seeds and progeny tests) (Hojsgaard et al. 2013). Five *P. malacophyllum* accessions from Argentina, Bolivia, Brazil, and Paraguay were evaluated. During ovule development, wide variation was observed in the proportion of functional meiotic and aposporous pathways among accessions, but totals at the beginning and end of gametogenesis showed non-significant differences (i.e. between end of meiosis/functional megaspore and embryo sac stages;  $\chi^2 = 3.07$ ;  $P = 0.08$ ; Table 3 in Hojsgaard et al. 2013). However, pathways to seed formation after embryo-to-endosperm tissue-ploidy ratio analyses based on FCSS showed rather uniform proportions of sexual and apomictic seeds (11.1 % average for sexually-derived seeds, 10.8–11.7 % range; Hojsgaard et al. 2013:342). But no sexually derived offspring were found among 245 F<sub>1</sub> individuals from the five sampled progenies grown from open-pollinated seeds. Each progeny-group shared an identical AFLP-genetic profile with its respective maternal genotype, and hence were all clonal (Hojsgaard et al. 2013:342–343). Thus, apomixis out-competed functional sexuality during successive developmental stages (from the end of female meiosis till mature progeny) in intra-individual pollinations among *P. malacophyllum* genotypes. Although the pollen source could influence reproductive output, whether the level of functional sexuality is significantly different or not between intraspecific and interspecific pollinations among apomictic genotypes still needs to be determined.

### Resolving the reproductive discrepancy

Historically, studies of *P. malacophyllum* have yielded contradictory information regarding its mode of

reproduction (Hanson and Carnahan 1956; Brown and Emery 1958; Bennett and Bashaw 1960; Bashaw et al. 1970; Burson and Hussey 1998; Hojsgaard et al. 2008). Today, approaches combining classical embryological with cytological and molecular methodologies in a comprehensive study (Hojsgaard et al. 2013) permit a reinterpretation of prior findings and indicate that former studies essentially were not contradictory. The observed variation in levels of functional sexuality and apomixis in *P. malacophyllum* genotypes from several earlier studies can be arranged by specific life-cycle stages, in relation to each technique used (Fig. 1). Hanson and Carnahan (1956) reported that tetraploid *P. malacophyllum* reproduced by obligate apomixis through direct observation of morphological characteristics in well-established offspring, a result concordant with complete clonality as observed by Hojsgaard et al. (2013) in genetic profiles of progenies obtained from tetraploid genotypes. In the same manner, but after an embryological evaluation of fifteen ovules, Brown and Emery's (1958) conclusion of obligate apomixis could be associated to one *P. malacophyllum* genotype with a high proportion of unreduced female gametophytes (Fig. 1). An accession analyzed by Hojsgaard et al. (2013) had a unique 4-nucleated embryo sac in 91.4 % of examined ovules, which supports a high level of apomixis at anthesis (accession TK2449, Table 2 in Hojsgaard et al. 2013).

Bennett and Bashaw (1960) inferentially classified *P. malacophyllum* as sexual. Possibilities here are speculative. It is worth mentioning that even if sexuality is still functional in *P. malacophyllum*, the possibility of having intraspecific sexual offspring is very low or even null (Hojsgaard et al. 2013). Alternatively, the sexual pathway may be functionally restored in interspecific crosses, as observed in other genera and species (e.g. Chapman and Bicknell 2000). By complementing genetically altered factors, interspecific hybridizations involving apomicts may partially re-establish functionality of sexual programs, as likely occurred in the heptaploid F<sub>1</sub> hybrid between *P. dilatatum* (5×) and *P. malacophyllum* (4×), described as sexual by Bennett and Bashaw (1960). Similarly, the conclusion by Bashaw et al. (1970) that the tetraploid *P. malacophyllum* accession that they evaluated embryologically was highly sexual is plausible considering the observed variation in embryo-sac ratios among *P. malacophyllum* genotypes (Fig. 1). Since plants with a large number of ovules containing



**Fig. 1** Box plots showing functional rates of sexuality and apomixis at four developmental stages in *Paspalum malacophyllum* accessions. Trend lines were obtained from a third-order polynomial regression showing fittest coefficient of determination ( $R^2$ ) on independent datasets ( $R_s^2$  for sexuality;  $R_a^2$  for apomixis) and superimposed on a box plots graphic. Data were collected from Burson and Hussey (1998), Hojsgaard et al. (2008), Hojsgaard (2010) and Hojsgaard et al. (2013). Reproductive classifications: sexual pathway (FM, MES, SS, SO; dark grey box plots and trend line); apomictic pathway (AI, AES, AS, AO; grey box plots and trend line). Gametogenesis reproductive stages include the end of female meiosis/beginning of gametogenesis stage (FM and AI), and the end of gametogenesis stage (MES and AES). FM functional megaspores, AI aposporous initial cells, MES meiotic embryo sacs, AES aposporous embryo sacs, SS sexual seeds, AS asexual seeds, SO sexual offspring, AO asexual offspring. Methodologies used to assess reproductive pathways at each stage: Embryology; FCSS, flow cytometry seed screening; MM, DNA-fingerprint with molecular markers. Outlier and extreme values are represented by circles and stars, respectively

a single meiotic embryo sac are not uncommon (e.g. accession PI 508805, Table 3 in Burson and Hussey 1998), the material evaluated by Bashaw et al. (1970) probably was a genotype with a high frequency of gametophytes with antipodal cells. Alternatively, if *Hieracium*-type embryo-sac development occurs (hiding apomictic development), then the conclusion by Bashaw et al. (1970) was misleading, as the plant would have actually been a facultative apomictic (in agreement with later studies, see below).

Finally, Burson and Hussey (1998) and Hojsgaard et al. (2008) classified *P. malacophyllum* as a

facultative apomict based on observations of both aposporous and sexual pathways, as well as embryo sac types in mature ovules of 16 different tetraploid accessions. These results also are confirmed in Hojsgaard et al. (2013) by embryological and FCSS analyses showing that both reproductive pathways are functional and produce viable seeds (see Fig. 1), even though the sexual seeds did not germinate, or if they germinated, seedlings did not develop into mature plants.

Indeed, divergent reproductive classifications were correct despite apparent contradictions.

### The knowledge of functional reproductive pathways and their potential impact in plant breeding

Because the level of functional sexuality (and/or the potential for sexuality) can vary during a facultative apomictic plant's life cycle (i.e. ovule developmental stages, seed formation, offspring establishment; see Fig. 1), this can influence the efficiency of a breeding program as well as field-management strategies for forage cultivars. The higher the expression of sexual reproduction in a facultative apomictic cultivar, more variation will occur in each succeeding generation which negates the advantage of fixed heterosis and uniformity that occurs in progeny of highly apomictic cultivars. This also complicates seed production because production fields will need to be isolated from other genotypes of the same species, whereas this is not an issue with obligate or highly apomictic cultivars because out crossing does not occur. Thus the reproductive behaviour, including the level of sexuality and apomixis expressed in facultative apomictic germplasm needs to be thoroughly understood before a potential cultivar is released.

Apomicts usually produce less seed than do their naturally occurring diploid and induced sexual autotetraploid relatives (see for example, Quarin and Norrmann 1990; Kojima and Nagato 1992; Naumova et al. 1999; Quarin et al. 2001; Acuña et al. 2007, 2009; Hörandl 2008). This may be related to the temporal and spatial gene deregulations associated with apomixis in various genera, including *Paspalum* (Polegri et al. 2010) and *Boechera* (Sharbel et al. 2010), and to observed developmental heterochrony in both monocot and dicot genera (e.g. *Tripsacum* sp.,

Grimanelli et al. 2003; *Sorghum* sp., Carman et al. 2011; *Paspalum* sp., Hojsgaard et al. 2013; *Ranunculus* sp., Hojsgaard et al. 2014a). Furthermore, it is probable that once apomixis is activated, it has a progressive, genotype-specific detrimental effect on the sexual reproductive program by altering the level of functional sexuality during ovule developmental stages (as observed in *P. malacophyllum* genotypes, Hojsgaard et al. 2013). Additionally, because apomixis-types probably had diverse genetic controls despite having or not an ancient phylogenetic origin (conserved pathway or *de novo* hypotheses) (Ozias-Akins and van Dijk 2007; Carman et al. 2011; Hojsgaard et al. 2014b), it is anticipated that the species in question and the type of apomixis will further influence natural rates of functional sexuality.

Prior to understanding its method of reproduction, *P. malacophyllum* was used in an interspecific hybridization program attempting to transfer ergot (*Claviceps paspali* Stevens et Hall) immunity factors and high seed set into dallisgrass, a desirable forage grass (Bennett and Bashaw 1960). Although ergot resistance was transferred into the F<sub>1</sub> hybrid that was recovered, the program was unsustainable because of the extremely low crossability between the species (with an F<sub>1</sub> formation rate of  $2.5 \times 10^{-4}$ ) (Bennett and Bashaw 1960).

Sexual reproduction is the most important, simple, and inexpensive pathway to combine important agronomic traits. However, completely sexual genotypes in natural polyploid facultative apomicts are often scarce or absent, and, hence, comprehensive evaluations of residual levels of functional sexuality are valuable for: (1) determining potential rates of hybridization to be exploited when breeding apomictic plants, a critically relevant information to forecast the success capacity and time required to introgress genetic or phenotypic features in breeding programs, and (2) evaluating the potentially expected proportions of non-maternal progenies in commercial cultivars (of selected outstanding natural ecotypes and/or hybrids) in order to estimate natural turn-over rates of apomictic and segregating progenies in the field, and better manage stands to avoid decreases in forage productivity.

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