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Impact of polyploidy on fertility variation of Mediterranean *Arundo* L. (Poaceae)

Impact de la polyploïdie sur la fertilité du genre Arundo *L. (Poaceae) en Méditerranée*

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A B S T R A C T

Failure of seed production in the genus *Arundo* L. (Poaceae) is often attributed to polyploidy. This study tested the impact of two ploidy levels ($2n = 12$ and $18x$) on the fertility of four Mediterranean *Arundo*. Viable pollen was screened from its production to its germination, and seed occurrence was monitored in admixture or isolated conditions. In addition, insights on restructuration of polyploid genomes were analysed using molecular cytogenetics. Our results show that high ploidy levels do not automatically induce failure of sexual reproduction. The two ploidy levels are able to produce viable pollen and seed set depending on species and cultural conditions. The sterility of *A. micrantha* ($2n = 12x$) and *A. donax* ($2n = 18x$) is due to the early failures of gametogenesis steps. For $18x$ cytotypes of *A. donaciformis* and *A. plinii*, seed absence for isolated genotype vs. seed production in admixed culture support their auto-incompatibility.

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Mots clés :
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R É S U M É

L'absence de graines chez les espèces d'*Arundo* L. (Poaceae) est souvent attribuée à la polyploïdie du genre. Cette étude teste l'impact de hauts niveaux polyploïdes ($2n = 12$ et $18x$) sur la fertilité de quatre espèces méditerranéennes. La viabilité pollinique est évaluée de la production à la germination pollinique, ainsi que la présence de graine en condition isolée et en mélange génotypique. De plus, des outils de cytogénétique moléculaire renseignent sur la restructuration de ces génomes polyploïdes. Nos résultats montrent que les hauts niveaux polyploïdes n'induisent pas nécessairement la stérilité, les deux niveaux de ploïdie pouvant produire des graines selon l'espèce et les conditions de culture. La

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stérilité d'*A. micrantha* ($2n = 12x$) et d'*A. donax* ($18x$) provient d'anomalies précoces de la gamétogenèse. Enfin, l'auto-incompatibilité des cytotypes $18x$ d'*A. plinii* et d'*A. donaciformis* est suggérée par l'absence de graines en condition isolée vs leur présence en mélange génotypique.
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1. Introduction

Often highlighted, relationships between polyploidy, morphology, mating systems, fertility and ecological adaptations are still under discussion [1–4]. Whole-genome duplication events may provide high levels of heterozygosity in doubling allele number [5], maintaining genetic diversity at intra-genomic scale [6] and allowing subfunctionalization of duplicate genes [7]. Thus, it has been hypothesized that polyploidy could induce new genomic entities possessing higher adaptability and phenotypic plasticity than their progenitors [8]. For these "super-genotypes", the maintenance of sexual reproduction could represent a risk due to outbreeding. In addition, the frequent formation of multivalents could cause strong meiotic irregularities leading to non-viable gametes, which dramatically reduces reproductive success [9]. Thus, clonal species may possess a higher competitive ability to persist in changing environments and marginal habitats without investing energy in sexual reproduction [6].

In Poaceae, the mating system of annual crops is well known, because they have been selected, crossed or even polyploidized (e.g., wheat, oat, rye) to increase seed yields since the Neolithic. In contrast, long-lived and tall reedy grasses are also polyploid, but they mainly show low fertility, auto-incompatibility, anemophily [10] and even rare flowering events (Bamboos). The subfamily Arundinoideae Dum. provides a suitable case to assess the impact of polyploidy on reproduction. After removal of the Danthonioid group, it currently remains the smallest grass subfamily, mainly subtropical, with 15 genera and *ca.* 44 species [11]. All taxa counted are polyploid and the literature suggests $x = 6, 9, 12$ as chromosome base numbers for Arundinoideae [11]. Following Conert [12], $x = 6$ seems the most suitable theoretical base number because:

(i) it characterizes the related subfamily Danthonioideae Barker & H.P. Linder ($2n = 12$, 24, etc.);
(ii) it is the only one compatible with the various numbers found in Arundinoideae ($2n = 36, 42, 48, 54, 72, 90, 108$);
(iii) it avoids the conflictual consideration of $2n = 36$ as a $3x$ in *Phragmites* and as a $4x$ in *Molinia*, two sister genera.

The genus *Phragmites* (five species) has been extensively studied [13], particularly *P. australis* (Cav.) Trin., one of the most widespread taxa. For Lambertini et al. [14], European populations of this common reed "could be considered as member of a single metapopulation" that form monoclonal stands up to 86 ha. Moreover, this polyploid complex [15,16] has everywhere a low mean seed set (< 10%: [17,18]). Eurasian *Arundo* spp. look like *Phragmites* spp., resulting from morphological convergences of rhizomatous taxa living in similar wetland habitats. Nevertheless, *Arundo* spp. are above all ruderal plants growing in waste-lands. Owing to their vast range, dynamism and high biomass, *P. australis* and *A. donax* are currently selected for biofuel production [19,20]. *Arundo* taxa are characterized by tall panicles (up to 60 cm) bearing thousands of protandrous florets, long-time autumnal flowering, pollen and seed dispersion by wind. According to recent phylogenetic studies [21–23], this genus has at least five species across subtropical Eurasia: one endemic to Taiwan, *A. formosana* Hackel, and four living in the Mediterranean area: the Eurasian *A. donax* L., the circum-Mediterranean *A. micrantha* Lam., the Italian-Balkan *A. plinii*, and the narrow French-Ligurian endemic *A. donaciformis* (Loisel.) Hardion et al.

Previous morphological and phylogenetic studies have evidenced a failure of seed set in most of these taxa, low intraspecific genetic diversity and two high ploidy levels, $12x$ and $18x$ [21–23]. One non-fructiferous genotype of the common *A. donax* L. (the giant reed) has invaded almost all the world's warm regions [23,24]. For this $18x$-clone, severe alterations of gametogenesis prevent its sexual reproduction [25–27], while seed set occurs in Asia [28,29] where *A. donax* possesses a lower ploidy level $12x$ [30–32].

The present study assesses the potential impact of polyploidy on the reproductive biology of Mediterranean *Arundo*. In particular, we test the hypothesis that polyploidy events induce reproductive failures of gametogenesis and seed set. For this purpose, we compare ploidy levels estimated by chromosome counts and DNA content:

(i) with microscopic observations of male gametogenesis steps, looking for early failures of sexual reproduction;
(ii) with seed sets *in* and *ex situ*, in genotype-isolated and admixture conditions, testing auto-incompatibility phenomena;
(iii) with the number and the activity of rDNA sites, potentially evidencing restructuration of highly polyploid genome.

2. Materials and methods

2.1. Plant materials

This study concerns the four species of the genus *Arundo* occurring in the Mediterranean basin. Caryological and morphological analysis was carried out on the basis of an extensive sampling of 91 localities: 16 for *A. donax*, 27 for *A. micrantha*, 40 for *A. plinii*, and 8 for the endemic *A. donaciformis* (See details in Appendix A). For each locality, five samples (stem with panicle) were deposited in the herbarium of Aix-Marseille University (MARS).

Rhizomes collected *in situ* were rooted in water to provide clean young material; all accessions were treated under standard conditions. After a cold pre-treatment of 4 °C for one day, root tips were fixed with an ethanol–acetic

acid solution (4:1, v/v), kept at room temperature for two weeks, and then stored at −18 °C until used. Similarly, for the gametogenesis study, during the flowering periods (2011 and 2013 autumns), young and mature spikelets were fixed *in situ*, and/or sampled weekly *ex situ*. All materials were stained in 45% aceto-carmine-ferriacetate, boiled for 3 min, and then squashed between slide and cover-slide. Two samples per locality were studied and, for each one, the three best mitotic metaphases were identified by light microscopy (15 × 100, Leitz Dialux 20, equipped with a Canon EOS 550D camera). Chromosomes were counted on photographs and drawn using a camera lucida. To complete this study, flow cytometry analysis was performed using internal standards (*Petunia hybrida* PxPC6, 2C = 2.85 pg) to determine 2C DNA content (Partec CyFlow 532-nm laser cytometer) following the technique described by Fridlender et al. [33].

2.2. Fluorescent in situ hybridization (FISH) of rDNA loci and AgNOR staining

Fixed material was washed in citrate buffer (10 mM, pH 4.6), and macerated for 1 h at 37.8 °C in a solution of 2% (v/v) cellulase (Calbiochem) in citrate buffer, and 20% pectinase in 40% glycerol in citrate buffer. Slides were stained with 4% Giemsa solution diluted with Sörensen phosphate buffer (0.2 M; pH 6.9). The 45S and 5S rDNA multigene families were localised using the pTa71 [34] and pTa794 [35] clones, respectively. The pTa71 and pTa794 probes were labelled with digoxigenin-11dUTP and biotin-16-dUTP, respectively, through a nick translation procedure (Roche, Germany). We followed the *in situ* hybridization protocols of Rosato et al. [36].

Digoxigenin-labelled probe detection was performed with anti-digoxigenin antibodies conjugated to fluorescein isothiocyanate (Roche). Biotin-labelled probe detection was performed with streptavidin conjugated with Texas Red (Vector Laboratories, Peterborough, UK). Finally, DAPI counterstaining was carried out on slides mounted in Vectashield (Vector Laboratories). Fluorescent signals were detected using an Olympus fluorescence microscope with Camedia C-2000-Z digital camera. The images were optimized for best contrast and brightness on Adobe Photoshop v.7.0. Active NORs and maximum number of nucleoli were identified after silver nitrate staining [37].

2.3. Meiosis and pollen study

From spikelet fixations, meiosis and pollen mitosis were observed on stained anthers of young florets, for each taxon. Owing to the pollen uniformity of Poaceae, only the diameter of mature pollen grains (*i.e.*, equatorial axis) was measured (30 grains per sample) to assess the relationships between ploidy level and cell size. In the botanical garden, at anthesis, pollen was collected from freshly opened florets in the morning and placed in Petri dishes containing a germination medium (1% agar, 15% sucrose, 0.01% boric acid, 0.02% calcium nitrate and 0.02% magnesium sulphate), and incubated at 25 °C for 3 h. Pollen grains were considered germinated when the length of the pollen tube exceeds the grain diameter. Alexander's stain was used to distinguish between abnormal (blue-green coloured) and normal grains (magenta-red stained). The percentage of red stained grains was considered as an indicator of pollen viability [38].

2.4. Self-incompatibility and seed set

Rhizomes collected *in situ* were transplanted in the Botanical sites of Aix-Marseille University according to two conditions:

(i) isolated individuals cultivated in greenhouses of Saint-Charles Faculty as control without outbreeding possibilities;
(ii) admixture of the four species and cytotypes planted in the Botanical Garden of Saint-Jerome Faculty, randomly planted 0.5 m apart from one another.

As reported by Ishii and Kadono [18] for *Phragmites*, we did not perform emasculation or manual crossing, due to the small size and induration of florets. Seed set rates were estimated for each taxon, on 20 florets per panicle and five panicles per locality.

3. Results

3.1. Chromosome numbers

The synthesis of our counts in the Mediterranean basin exhibits an unexpected chromosomal variability in *Arundo* (Table 1). This genus is characterized by two ploidy levels and aneuploidy events, except for the French-Ligurian endemic *A. donaciformis* ($2n = 18x = 108$). In the other species, we found: $2n = 18x = 108–110$ in *A. donax*, $2n = 12x = 70–72$ in *A. micrantha*, and five cytotypes ($2n = 72$, 74, 76, 108, and 114) in *A. plinii*. Whatever the base chromosome number chosen, the multiple $2n = 72$ and 108 can be designated as a euploid series, and the others as derived aneuploid numbers. Moreover, only *A. plinii* exhibited a clear geographical structuring of aneuploid cytotypes (Fig. 1): hyper-aneuploids in central and northern Italy ($2n = 76$ and 114), euploid numbers in Balkans ($2n = 72$ and 108), and the strongest

Table 1
Cytogenetic data in Mediterranean *Arundo* taxa, including ploidy level (2*n*), DNA content (2C); numbers of 5S and 45S rDNA sites, and maximum number of Ag-staining nucleoli (NORs) observed.

Taxon	2*n*	2C value (pg)	No. of 5S sites	No. of 45S sites	No. of nucleolus
A. micrantha	12*x*	3.2 ± 0.1	4	4	4
A. plinii	12*x*	3.4 ± 0.2	4	4	4
A. plinii	18*x*	4.8 ± 0.2	6	6	5–6
A. donaciformis	18*x*	4.7 ± 0.1	5	5	3
A. donax	18*x*	4.9 ± 0.1	6	6	6

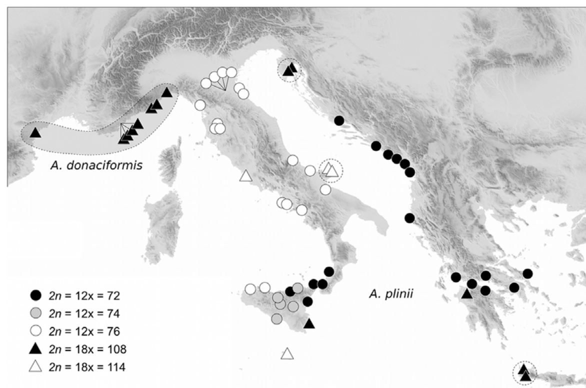

Fig. 1. Geographical distribution of the different cytotypes of *Arundo donaciformis* and *A. plinii*. Dashed lines group same genotypic clone identified from Hardion et al. [22].

diversity in Sicily, with all five cytotypes counted for this species.

3.2. Polyploidy levels and rDNA sites

The number of rDNA sites (or signals) distinguishes the ploidy levels of *Arundo* (Table 1). The two 12*x*-cytotypes, *A. plinii* (Fig. 2A) and *A. micrantha* (Fig. 2C), show four 5S and four 45S rDNA sites, whereas six 5S and six 45S rDNA sites are present in the two 18*x* cytotypes of *A. donax* (Fig. 2B) and *A. plinii*. Only the third high polyploid *A. donaciformis* possesses five 5S and five 45S rDNA (Fig. 2D). AgNOR staining suggests that all 45S rDNA sites are active in both *Arundo* cytotypes because up to four (Fig. 2G) and six nucleoli (Fig. 2E, F) were observed in the 12*x* and 18*x* cytotypes, respectively. As previously, only *A. donaciformis* 18*x* is distinguished from all others exhibiting only three nucleoli (Fig. 2H).

3.3. Meiosis and pollen study

Microgametogenesis would appear to be regular in most *Arundo* taxa (Fig. 3A and B), therefore the observation of cytomixis events (i.e. chromosome exchange between two mother cells) in *A. micrantha* (12*x*; Fig. 3C) reveals the presence of severe disturbances. Pollen mitoses generally appear regular (Fig. 3E, F, G), but sometimes in the 18*x* cytotypes deviant haploid numbers ranging from 50 to 62 could be observed. Large size grains (macro-pollen) were found for each cytotype, assumed to correspond to unreduced gametes (2*n* gametes; Fig. 3J).

Whereas pollen stainability values reached 80.8, 84.6% and 86.8% in *A. donaciformis* 18*x* and the two cytotypes 12*x* and 18*x* of *A. plinii*, respectively, their pollen germination rates dropped to 10.6%, 7.8%, and 8.0%, respectively (Fig. 3D). In these taxa, pollen stainability was not closely related to germination rate (Table 2). In contrast, both *A. micrantha* 12*x* (Fig. 3 J) and *A. donax* 18*x* (Fig. 3I) showed similar very low pollen stainability (3.7% and 6.2%, respectively), numerous collapsed grains and no pollen germination. In addition, at the anthesis, these two species show a high frequency of sclerified and empty anthers (84.1 and 91.3%, respectively).

3.4. Seed production

In situ, seed set was strictly restricted to the *A. plinii* 12*x*, with an average of 9.2%, and was never found in

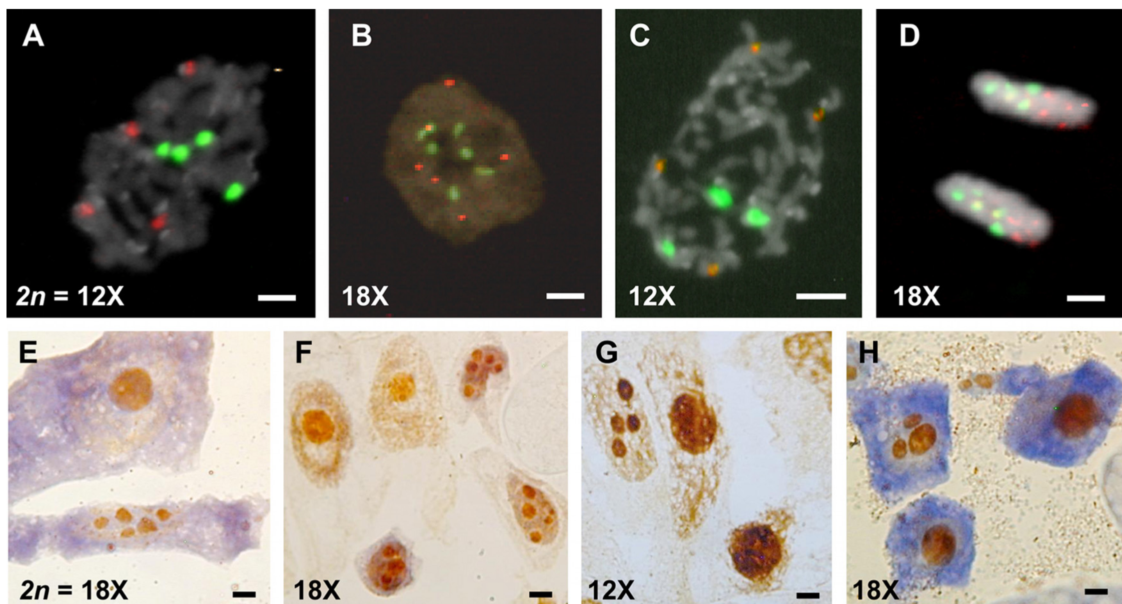

Fig. 2. (Colour online.) Fluorescent *in situ* hybridization (FISH) of 5S rDNA (red) and 45S rDNA (green) probes on cells counterstained with DAPI (A, B, C, D), and number of nucleoli using Ag-staining on somatic cells (E, F, G, H). **A**. Prophase of *A. plinii* 12*x*: four 5S and four 45S rDNA sites. **B**. Interphase of *A. donax* 18*x*: six 5S and six 45S sites; **C**. Metaphase of *A. micrantha* 12*x*: four 5S and four 45S sites. **D**. Telophase of *A. donaciformis* 18*x*: five 5S and five 45S sites. **E**. *A. plinii* 18*x*: up to six nucleoli. **F**. *A. donax* 18*x*: up to six nucleoli. **G**. *A. plinii* 12*x*: up to four nucleoli. **H**. *A. donaciformis* 18*x*: up to three nucleoli. Scales: white bars = 2 μm, black bars = 1 μm.

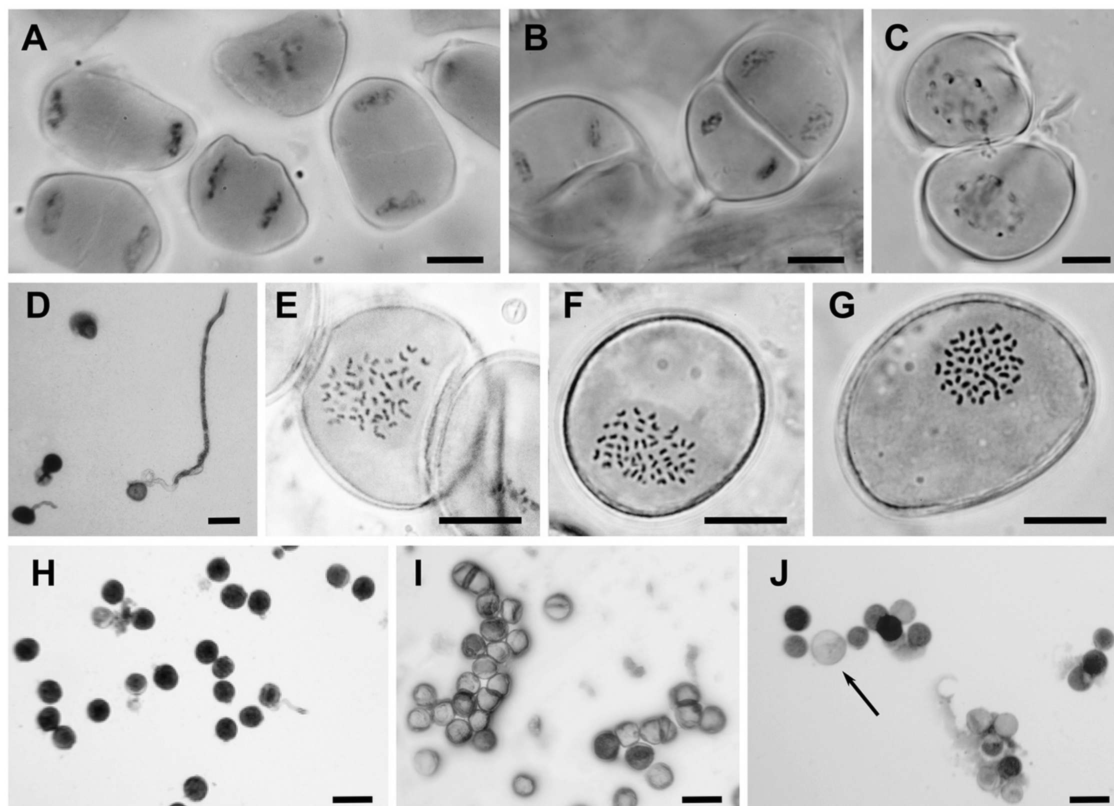

Fig. 3. Observations of meiosis (A, B, C), mitosis (E, F, G), viability (H, I, J) and germination (D) of pollen. **A**. Telophase I of *A. plinii* 12*x*. **B**. Anaphase II of *A. plinii* 12*x*. **C**. Cytomixis of *A. micrantha* 12*x*: chromosome exchange between two mother cells. **D**. Pollen germination of *A. plinii* 18*x*. **E**. Normal pollen and pollen mitosis of *A. donaciformis* 18*x*: $n = 54$. **F**. Pollen mitosis of *A. plinii* 18*x*: $n = 56$. **G**. Pollen mitosis of *A. donax* 18*x*: $n = 54$. **H**. Normal pollen of *A. plinii* 12*x* (homogeneous size and staining). **I**. Collapsed and uncoloured pollen grain of *A. donax* 18*x*. **J**. Abnormal pollen of *A. micrantha* 12*x* (heterogeneous in size and colour; arrow showing the diplogamete). Scales: **A** to **H**, black bars = 10 μm; **H**, **I** and **J**, black bars = 10 μm.

A. micrantha 12*x*, or within all 18*x* cytotypes: *A. donax*, *A. donaciformis* and *A. plinii* (Table 2). However, *ex situ* genotypic admixture in our botanical garden induced the formation of some fructiferous florets (5 to 7%) in the sister species *A. donaciformis* 18*x* and *A. plinii* 12*x* and 18*x*, whereas *A. micrantha* 12*x* and *A. donax* 18*x* remained non-fructiferous. In addition, all plant controls grown in isolation never produced seed.

4. Discussion

4.1. Cytogenetic study

Chromosome counts exhibited two ploidy levels in *Arundo* taxa, 12 and 18*x*, with aneuploid variants around each euploid number. Measurements of DNA content support these two ploidy levels, but fail to detect aneuploid events as it was found in other taxa with small genome sizes [39]. Our results confirm previous reports in the Mediterranean area for *A donax*: $2n = 110$ [40,41], 112 [42], and $2n = 72$ in *A. micrantha* from Portugal [43] (under the name *A. plinii s.l.*) and in *A. plinii* from southern Italy and Sicily [40]. Generally aneuploids are randomly distributed, often due to difficult counts of these numerous and small chromosomes (0.9–1.9 μm). On the other hand, chromosomal variations show a clear geographical structuring only in the Italian-Balkan *A. plinii* (Fig. 1). The more ancestral cytotype remains $2n = 72$, from which were derived aneuploids ($2n = 74–76$) in Italy and high polyploid

Table 2
Cytogenetic data including ploidy levels ($2n$) and haploid numbers from meiosis or pollen mitosis (n); pollen viability and *in vitro* germination percentages; seed set percentages *in situ* and *ex situ* (in isolated or admixed conditions) in Mediterranean *Arundo* taxa.

Taxon	$2n$	n	Pollen viability (%)	Pollen germination (%)	Seed set (%)		
					In situ	*Ex situ* isolated	*Ex situ* admixed
A. micrantha	12*x*	36	3.7	0	0	0	0
A. plinii	12*x*	–	84.6	7.8	9.2	0	7.3
A. plinii	18*x*	56	86.8	8.0	0	0	5.2
A. donaciformis	18*x*	(50) 54 (62)	80.8	10.6	0	0	6.4
A. donax	18*x*	54–55	6.2	0	0	0	0

($2n = 108$–114) numbers. This geographical pattern did not yet corresponds to previous taxa recently fallen in synonymy of *A. plinii*, as *A. collina* Ten. or *A. hellenica* Dainin et al. [21,44,45]. As in many other groups [46], all 18*x* cytotypes *Arundo* probably resulted from an intraspecific fusion of reduced and unreduced gametes, and not from hybridization as suggested [40,41] for the monophyletic *A. donax* [23]. The rate of diplogametes generally increases with thermal shocks [47], and thus we found *A. plinii* 18*x* mainly on the northern and southern edges of the species' distribution range. According to previous genetic studies, the origin of *A. donaciformis* 18*x* was included in the phylogeny of *A. plinii* [22]. This euploid cytotype ($2n = 108$) cannot be derived from the nearest northern Italian aneuploids 12*x* (Fig. 1), but rather from a euploid lineage ($2n = 72$) conserved in southern Italy and Sicily.

If the most frequent numbers of 45S rDNA sites were two and four in diploid flowering plants [48], in the Poaceae this number is generally two [49,50]. The presence of four functional 45S rDNA sites in 12*x* *Arundo* shows a drastic turnover by their physical elimination from ancestral genomes. Except for *A. donaciformis*, the mathematical increase of 45S and 5S rDNA sites evidenced from 12*x* to 18*x* *Arundo* cytotypes (four and six sites respectively) suggests that this rDNA pattern is evolution-conserved. According to Roa and Guerra [48], these 18*x* *Arundo* cytotypes may have a more recent origin than *A. donaciformis* 18*x* that shows a trend to reduce the number of rDNA sites (five) and nucleoli (three).

4.2. Gametogenesis and pollen

The cytological impact of polyploidy (e.g. multivalent formation and segregation irregularities) is often designated as a probable cause of reproductive failure in *A. donax* [26]. If meiosis and pollen mitosis observations seem to show a correct pattern of behaviour, in fact cytomixis events found in *A. donax* 18*x* [40] and *A. micrantha* 12*x* (in this study) explain male anomalies. This phenomenon is responsible for asymmetric segregation, chromosome laggards and diplogametes that produce aneuploid and polyploid numbers [51,52]. In addition, these two species with distinct ploidy levels showed a very low rate of viable pollen and no germination. Whatever their ploidy levels, only taxa capable of pollen germination can produce seed set *ex situ*: 12*x* and 18*x* cytotypes of *A. plinii*, and *A. donaciformis*. These results suggest that microgametogenesis steps may explain the differential reproductive success of *Arundo* taxa, and discredit the hypothesis of the predominant role of polyploidy in the failure of meiosis and pollen germination.

These features must be related to previous studies on megagametogenesis, with distinct results according to species. *A. plinii* showed embryo sacs developing in mature ovules, then seeds [26]. In contrast, early megagametophyte degeneration has been reported in *Phragmites* [53] and *A. donax* [26,27]. On the basis of 36 666 florets, Johnson et al. [25] found only 43 ovules apparently matured, but only five displaying dehydrogenase activity. In fact, the low seed set found in rhizomatous Poaceae may be mainly due to callose deposition around embryo sacs, producing abnormal pistils with a low capacity to capture pollen grains [54].

4.3. Breeding systems and polyploidy in Arundo

The absence of seed set in isolated plant controls shows that *Arundo* spp. are not autogamic or agamospermic, conversely to the rule for many polyploids [10]. Knowing that *A. donax*, *A. micrantha* and *A. donaciformis* are monoclonal in the Mediterranean area [21–23], the more probable reason to explain seed set absence *in situ* is auto-incompatibility processes in the genus, such as evidenced in *Phragmites* [18]. This feature also explains why only *A. plinii* 12*x* produces seeds *in situ*, whereas related taxa *A. plinii* 18*x* and *A. donaciformis* 18*x* were only able to produce seeds under genotypic admixture conditions. Thus, polyploidy is not entirely responsible for seed set absence due to self-incompatibility phenomena, geographical distances between populations become preponderant (Fig. 1). This mechanism is reinforced by cytogenetic barriers between 12*x* and 18*x*, geographical isolation of all 18*x* cytotypes and the higher genetic diversity of 12*x* *A. plinii* [22]. In this complex, we did not find aneuploidy impact. On the other hand, the low seed set ($< 10\%$) of *Arundo* is also observed in many other long-lived or rhizomatous plants, such as *P. australis* [16,18]. Within these taxa, the low seed set generally linked to ovule abortion is compensated by thousands of florets per panicle, allowing resource saving and sufficient regeneration [55].

Finally, there is a paradox concerning the ranges of distribution and reproduction biology of *Arundo* species: native and potentially fertile taxa, such as *A. plinii* and *A. donaciformis* possess smaller ranges of distribution than robust widespread and non-fructiferous clones, such as *A. micrantha* and *A. donax*. As already evidenced for *A. donax* [23], the Mediterranean-wide distribution and the genetic uniformity of these two taxa could be explained by their ancient anthropogenic dispersion due to their countless uses during Antiquity (e.g. arrow and vineyard reeds respectively), as listed by Pliny the Elder. However, the failures of their gametogenesis remain poorly understood. In contrast to speculation in the literature, polyploidy events do not seem to influence their reproductive problems. Related to human impact, these failures could be linked to drastic founder effects and genetic impoverishment of degenerated clones cultivated for millennia, forming a clonal patch without reproductive effort. These early failures of sexual reproduction could also be due to the bioclimatic disequilibrium of these transplanted clones in the Mediterranean [10]. In the context of global change, further investigations should test these environmental *vs* genetic factors regarding the current sterility of these clonal and invasive species.

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Appendix A

Taxon	Specimen	*2n*	DNA content (pg)
A. donax	Croatia, Pag, Fridlender, MARS00047	108–110	–
A. donax	France, Bonifacio, Verlaque, MARS00048	108–110	–
A. donax	France, Marseille, Verlaque, MARS00049	108–110	4.5
A. donax	Greece, Kolimpari, Vila, MARS00050	108–110	4.7
A. donax	Greece, Kavassilas, Hardion & Vila, MARS00051	108–110	–
A. donax	Italy, Volterra, Hardion & Vila, MARS00052	108–110	–
A. donax	Italy, Naples, Hardion & Vila, MARS00053	108–110	–
A. donax	Italy, Cannitello, Hardion, MARS00054	108–110	–
A. donax	Italy, Carloforte, Fridlender, MARS05001	110	–
A. donax	Lebanon, Jadra, Abdel Samad, MARS00055	108–110	–
A. donax	Malta, Marsaskala, Médail, MARS00056	108–110	–
A. donax	Morocco, Rabat, Hardion, MARS00057	108–110	–
A. donax	Portugal, Figueira da foz, Engels, MARS00058	108–110	–
A. donax	Spain, Bonares, Hardion, MARS00059	108–110	–
A. donax	Spain, Amposta, Hardion, MARS00060	108–110	4.8
A. donax	Tunisia, Sidi bou said, Hardion, MARS00061	108–110	4.7
A. donaciformis	France, Fréjus, Hardion, MARS00062	108	4.7
A. donaciformis	France, Puget, Hardion, MARS00064	108	4.7
A. donaciformis	France, Les Arcs, Hardion, MARS00065	108	4.7
A. donaciformis	France, Saint Raphaël, Hardion, MARS00063	108	4.7
A. donaciformis	France, Lespignan, Hardion, MARS00066	108	4.6
A. donaciformis	Italy, Cervo, Hardion, MARS00067	108	–
A. donaciformis	Italy, Andorra, Hardion, MARS00068	108	–
A. donaciformis	Italy, Finale Ligure, Hardion, MARS00069	108	–
A. micrantha	Algeria, Tipasa, Baumel, MARS00070	70–72	3.3
A. micrantha	Algeria, Tizi Ouzou, Ait-Saïd, MARS00071	72	–
A. micrantha	Algeria, Tlemcen, Youssef, MARS00072	72	–
A. micrantha	Algeria, Sidi Aich, Vela, MARS00073	72	–
A. micrantha	France, Golfe Juan, Hardion, MARS00075	72	–
A. micrantha	France, Ste Lucie, Argagnon, Michaud & Molina, MARS00076	72	–
A. micrantha	Greece, Kissamos, Vila, MARS00077	70–72	3.1
A. micrantha	Greece, Mires, Vila, MARS00078	72	–
A. micrantha	Greece, Mirtos, Vila, MARS00079	72	–
A. micrantha	Greece, Vassiliko, Hardion & Vila, MARS00080	72	–
A. micrantha	Greece, Messolonghi, Hardion & Vila, MARS00081	72	–
A. micrantha	Greece, Itea, Hardion & Vila, MARS00082	72	–
A. micrantha	Italy, Trieste, Hardion, MARS00083	72	–
A. micrantha	Italy, Las Plassas, Fridlender, MARS00084	72	3.2
A. micrantha	Lebanon, Nahr el Kalb, Abdel Samad, MARS00085	72	–
A. micrantha	Morocco, Tanger, Hardion, MARS00086	72	–
A. micrantha	Morocco, Rabat, Hardion, MARS00087	72	–
A. micrantha	Israel, Nahal Sorek, Danin, MARS0007	72	–
A. micrantha	Portugal, Figueira da Foz, Engels, MARS00089	72	–
A. micrantha	Spain, Amposta, Hardion, MARS00090	72	–
A. micrantha	Spain, Deltebre, Hardion, MARS00091	72	–
A. micrantha	Spain, Jaen, Hardion, MARS00092	72	–
A. micrantha	Spain, Cordoba, Hardion, MARS00093	72	–
A. micrantha	Spain, Algeciras, Fridlender, MARS03840	72	3.2
A. micrantha	Spain, Huelva, Hardion & Sánchez-Gullón, MARS00094	72	–
A. micrantha	Spain, Huelva, Hardion & Sánchez-Gullón, MARS00095	72	–
A. micrantha	Spain, Bonares, Hardion, MARS00096	72	–
A. plinii s.s.	Croatia, Brsec, Hardion, MARS00099	108	–
A. plinii s.s.	Greece, Sirili, Vila, MARS00100	108	4.6
A. plinii s.s.	Greece, Lauzakies, Vila, MARS00101	108	–
A. plinii s.s.	Greece, Paralia Kimis, Hardion & Vila, MARS00102	72	–
A. plinii s.s.	Greece, Sicyone, Hardion & Vila, MARS00103	72	–
A. plinii s.s.	Greece, Kavassilas, Hardion & Vila, MARS00104	108	–
A. plinii s.s.	Greece, Patras, Hardion & Vila, MARS00105	72	3.4
A. plinii s.s.	Greece, Messolonghi, Hardion & Vila, MARS00106	72	–
A. plinii s.s.	Greece, Levadia, Hardion & Vila, MARS00107	72	–
A. plinii s.s.	Greece, Agios Stephanos, Hardion & Vila, MARS00108	72	–
A. plinii s.s.	Italy, Napoli, Hardion & Vila, MARS00109	76	3.3

Appendix A (*Continued*)

Taxon	Specimen	2n	DNA content (pg)
A. plinii s.s.	Italy, Pozzuoli, Hardion & Vila, MARS00110	76	–
A. plinii s.s.	Italy, Candela, Hardion & Vila, MARS00111	76	–
A. plinii s.s.	Italy, Rodi Garganico, Hardion & Vila, MARS00112	114	4.6
A. plinii s.s.	Italy, Termoli, Hardion & Vila, MARS00113	76	–
A. plinii s.s.	Italy, Lanciano, Hardion & Vila, MARS00114	76	–
A. plinii s.s.	Italy, Ostia, Hardion & Vila, MARS00115	114	–
A. plinii s.s.	Italy, Pisa, Hardion & Vila, MARS00116	76	–
A. plinii s.s.	Italy, Montegabbro, Hardion & Vila, MARS00117	76	–
A. plinii s.s.	Italy, San Giminiano, Hardion & Vila, MARS00118	76	–
A. plinii s.s.	Italy, Volterra, Hardion & Vila, MARS00119	76	3.1
A. plinii s.s.	Italy, Bologna, Hardion & Vila, MARS00120	76	3.3
A. plinii s.s.	Italy, Castel Maggiore, Hardion & Vila, MARS00121	76	3.2
A. plinii s.s.	Italy, Sasso Marconi, Hardion & Vila, MARS00122	76	3.2
A. plinii s.s.	Italy, Pianoro, Hardion & Vila, MARS00123	76	3.2
A. plinii s.s.	Italy, Forli, Hardion & Vila, MARS00124	76	–
A. plinii s.s.	Italy, Ravenne, Hardion & Vila, MARS00125	76	–
A. plinii s.s.	Italy, Santo Stefano, Hardion, MARS00126	72	–
A. plinii s.s.	Italy, Oliveto, Hardion, MARS	74	–
A. plinii s.s.	Italy, Orto Liuzzo, Hardion, MARS00127	72	–
A. plinii s.s.	Italy, Cannitello, Hardion, MARS03395	72	–
A. plinii s.s.	Italy, Dinami, Hardion, MARS00128	72	3.6
A. plinii s.s.	Italy, Siracusa, Hardion, MARS00129	108	4.8
A. plinii s.s.	Italy, Enna, Hardion, MARS00130	74	–
A. plinii s.s.	Italy, Agrigente, Hardion, MARS00131	74	3.5
A. plinii s.s.	Italy, Scilliato, Hardion, MARS00132	74	–
A. plinii s.s.	Italy, Castellamare, Hardion, MARS00133	76	3.6
A. plinii s.s.	Malta, Girgenti, Gambin, MARS00134	114	5.0
A. plinii s.s.	Italy, Salerno, Fridlender, MARS03806	76	3.3
A. plinii s.s.	Italy, Petralia, Fridlender, MARS05011	76	3.4

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