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ANALYSIS OF CHROMOSOME NUMBER AND SPECULATIONS ON THE ORIGIN OF *ARUNDO DONAX* L. (GIANT REED)

Arundo donax (commonly called Giant Reed) is a perennial rhizomatous grass native to Asia, nowadays diffused all over the world. Due to its high biomass production and great adaptability to marginal land, interest in this species is increasing. In fact A. donax could represent an important and promising energy crop for heat and bioethanol second generation production. The propagation of A. donax is strictly agamic by rhizome fragmentation and cane node germination, strongly limiting the possibility of genetic improvement by breeding. The sterility could be caused by the fact that A. donax is a hybrid with uneven ploidy or a triploid species. It is difficult to propose an explanation for its sterility, because the chromosome number of A. donax is still a matter of debate, due to the high number and small size of the chromosomes; in the bibliography different counts ranging from 40 to 110 are reported. With the aim of establishing the chromosome number of A. donax we selected and counted 17 metaphase plates prepared from root tips obtained by hydroponic cultivation of cane nodes; our counts showed that A. donax most probably has 110 chromosomes. Our results suggested us two possible hypotheses, also based on SSR molecular marker results, concerning the evolutionary processes involved in the origins of A. donax.

Keywords: *Arundo donax, Energy crops, Chromosome number, Polyploidy, SSR.*

Introduction. *A. donax* (*Poaceae: Arundinoideae*) is a perennial rhizomatous plant growing spontaneously all over the world as an invasive plant reaching more than 8 m in height [1–3]. It is a sterile plant which reproduces itself only agamically, through rhizomes and cane fragments which are transported by water or through human action [4, 5] and as such, genetic variability found among *A. donax* clones is small [6, 7]. In the past it was used industrially for the production of musical instruments, textile fibers and cellulose [1]. On a domestic scale, it is still used to make reeds for wind instruments, fences, baskets, mats, fishing canes and as stakes to support vines and other horticultural plants [1]. *A. donax* is considered one of the most promising energy crops, as it is characterised by high energy balance [8]. In fact, great quantities of biomass are produced with minimum input needed during cultivation [9]: the growth, starting from rhizomes in the early spring (Fig. 1, *a*), produces several canes (Fig. 1, *b*) and continues until autumn when after flowering the plant dries, turning yellow (Fig. 1, *c*). In fact different authors reported a yield of about 40 tonnes of dry matter ha^{-1} in the Lower Po Valley and Central Italy [8]. Moreover, it is highly suitable for marshy marginal lands, not competing with food crops. However *A. donax* also represents a big problem in many areas where it has become invasive: after being planted in riparian zones, with the aim to control erosion (e.g. Florida, California, Southern Europe), it invaded all the habitat, overwhelming the native plants [10]. Furthermore even if it produced a big yield in plot experiments, the cultivation of *A. donax* at a full field scale is still a problem because of the very expensive (about one Euro per plant) costs of rhizome transplantation [11]. Regarding the origin of *A. donax*, this is still an open question due to its sterility and the uncertainty concerning the chromosome number. However data obtained in the work [7] indicated a monophyletic origin of *A. donax* which supported the suggestion of the origin in Asia and a later spread into the Mediterranean area and rest of the world. In this work we propose a chromosome number for *A. donax* and a couple of hypotheses regarding its origin, involving two closely related species, *A. plinii* and *Phragmites australis*.

Material and methods. *Plant material. A. donax* plants used in this work were cultivated in the experimental field of the University of Milan located in Landriano (N 45°18', E 9°15') where there is a collection of Italian *Arundo donax* clones.

Hydroponic cultivation. With the aim to obtain root tips (fresh meristem tissue rich in metaphase plates) we used hydroponic cultivation starting from cane nodes. Samples of flowering canes were cut into smaller fragments containing 1 node (about 10 cm long) and put into a box (26 × 20 × 40 cm) filled with tap water, at a density of about 1 node

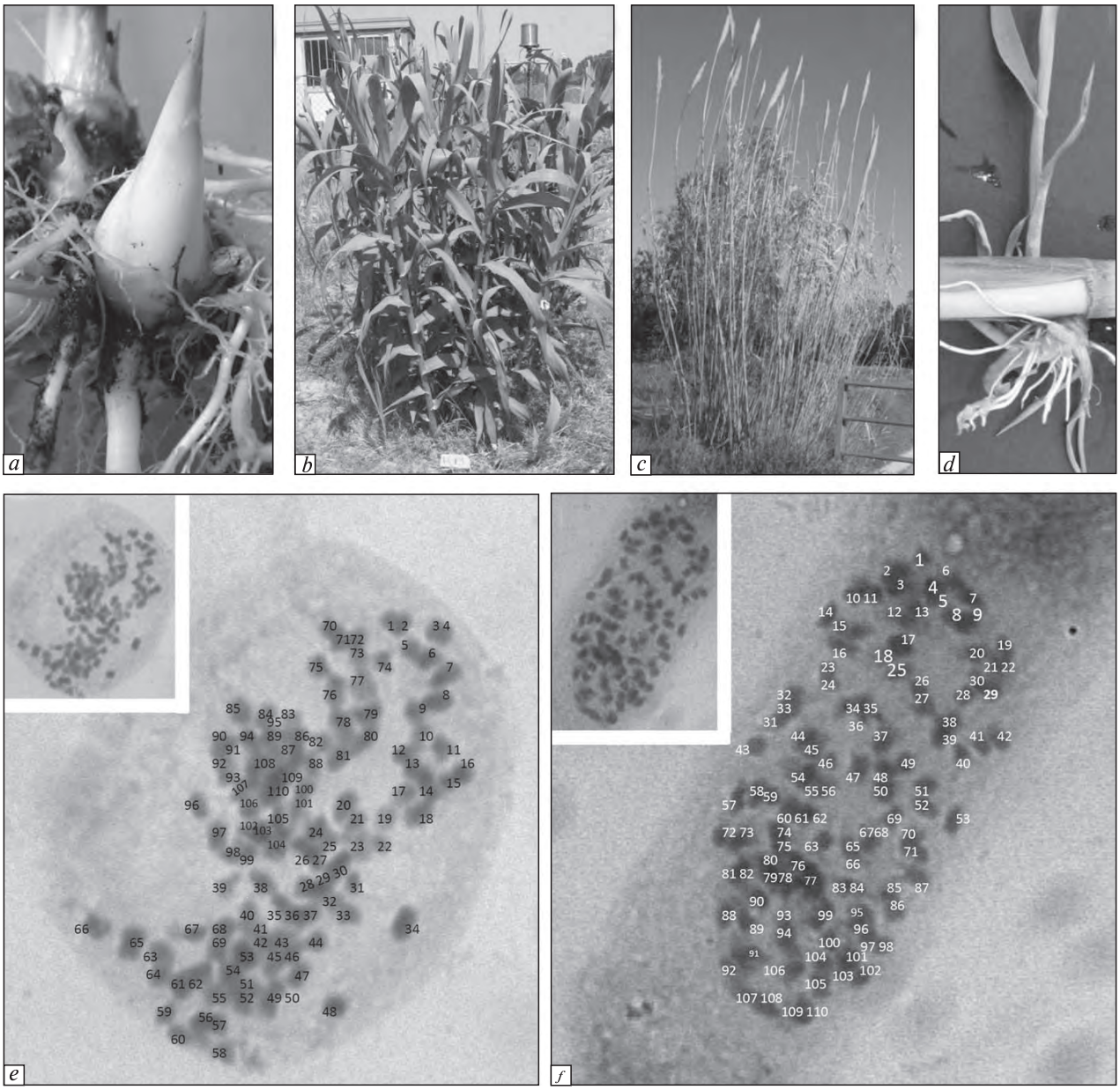

Fig. 1. *A. donax* life cycle and chromosome number: *a* – young rhizome; *b* – growing plants in spring; *c* – mature plants in winter; *d* – seedling obtained from cane node by hydroponic cultivation; *e, f* – metaphase plates

per 300 cm^3. After 2–3 weeks, each node generated one shoot which started to root after 2–3 weeks at 25 °C.

Slide preparation. We used 5 root apices (0.5–1 cm length), obtained from thicker roots, to prepare each slide. The apices were incubated in hydroxyquinoline 0.002 M for 5 hours; then they were washed with a citrate solution at 4 °C for 15 min. Afterwards, the apices were treated at 37 °C for 7 h with cellulase (0.57 g/10 mL, C1184-5KU, «Sigma», USA) and pectinase (1.82 g/10 mL, 178389-10G, «Sigma», USA) dissolved in the citrate solution. The apices were washed with the citrate solution for 3 minutes at room temperature, then with distilled water for 15 min. Finally, the apices were crumbled in some drops of acetic acid

60 % using a Pasteur pipette and spread onto a slide pre-heated at 52 ºC. After drying, the slides were dipped for 15–20 min in Giemsa staining solution (40 mL 9.08 g/L of KH_2PO_4: 60 mL 11.88 g/L of NaH_2PO_4: 3 mL of Giemsa) and subsequently in distilled water for 10 min. After the complete drying of the slides they were mounted permanently using Euparal and a cover slip.

Metaphase plates observation. Slides were observed using a Zeiss Axiophot D1 microscope at 1000× magnification. Images were recorded with an Axiocam MRc5 camera («Zeiss», Germany) using the Axiovision program (version 4.1).

DNA extraction and SSR analysis. A piece of leaf of each clone was used for DNA extraction [12]. The clones used for the molecular analysis were: lane 1 ad18 clone (Scaldasole, PV, 45º 07' N, 08º 54' E); lane 2 ad19 clone (Brescia 45º 32' N, 10º 10' E); lane 3 ad20 clone (Leno, BS, 45º 20' N, 10º 16' E); lane 4 ad21 clone (Calcinato, BS, 45º28' N, 10º 24' E); lane 5 ad22 clone (Desenzano del Garda, BS, 45º 26' N, 10º 37' E); lane 6 ad23 clone (Nago Torbole, TN, 45º 52' N, 10º 52' E); lane 7 ad24 clone (Ala, TN, 45º 44' N, 10º 58' E). The 48 SSR markers were randomly chosen from MaizeGDB (http://www.maizegdb.org/ssr.php). The sequence of the SSR umc1221 was: forward primer GCAACAGCAACTGGCAACAG and reverse primer AAACAGGCACAAAGCATGGATAG. Polymerase chain reactions (PCR) and gel running conditions were performed as described in the SSR Methods Manual by MaizeGDB (http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php).

Results and discussion. So far the chromosome number of *A. donax* has not been accurately determined because of the small size and high number of chromosomes of this species, in fact several authors reported different chromosome number for *A. donax*. Christopher [13] reported 108 chromosomes, Pizzolongo [14] observed 110 chromosomes, earlier Hunter [15] encountered the same number whilst Avdulov [16] observed 100 chromosomes, Vittoria [17] reported three different counts on metaphase plates: 24, 48 and 96, and recently Mariani et al. [7] reported a number of 40 chromosomes (in this case these data were reported as unpublished observations). Thus, so far, the chromosome number, the origin and the cause of *A. donax* sterility are still open questions. In this work

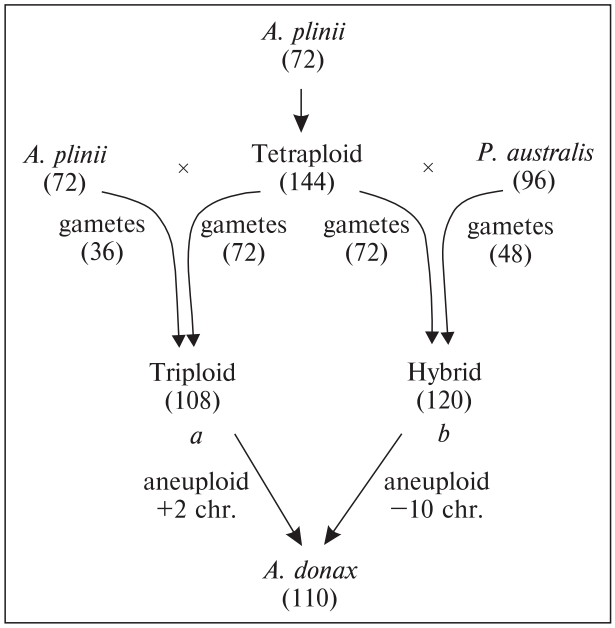

Fig. 2. Phylogenetic origin of *A. donax* assuming a chromosome number of 110. In the first hypothesis (*a*) *A. plinii* (72 chromosomes) underwent a chromosome doubling producing a fertile tetraploid (144 chromosomes) that crossed with a diploid *A. plinii* created the sterile triploid (108 chromosomes), the addition of two chromosomes resulted in the aneuploid *A. donax* (110 chromosomes). In the second hypothesis (*b*) the fertile *A. plinii* tetraploid crossed with *Phragmites australis* (96 chromosomes) to produce a sterile hybrid (120 chromosomes), the deletion of 10 chromosomes created the aneuploid *A. donax* (110 chromosomes). The numbers in brackets indicate the chromosome number

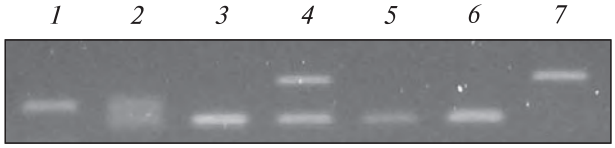

Fig. 3. Amplification pattern obtained by amplifying *A. donax* genomic DNA using maize *umc1221* SSR molecular marker: *1* – ad18 clone; *2* – ad19 clone; *3* – ad20 clone; *4* – ad21 clone; *5* – ad22 clone; *6* – ad23 clone; *7* – ad24 clone

light-microscopy analysis of metaphase plates was used to ascertain the correct chromosome numbers of *A. donax* diffused in Italy. We used hydroponic cultivation of cane nodes (Fig. 1, *d*) with the aim of obtaining root tips, rich in fresh meristem tissues, to be used to produce metaphase plates. In

the slides prepared, as described in Material and Methods, we found 17 metaphase plates where a chromosome count was achievable. In 14 plates we counted 110 chromosomes (Fig. 1 *e*, *f*) in one 118, in one 116 and in another 115. The average number was 111.11, the mode and median were 110:

Metaphase plate counts obtained from root tips

Metaphase plate	Chromosome number	Metaphase plate	Chromosome number
1	110	11	110
2	116	12	110
3	110	13	110
4	110	14	110
5	110	15	110
6	115	16	110
7	110	17	118
8	110	Average	111.11
9	110	Median	110
10	110	Mode	110

Thus our results indicated most probably a chromosome number of 110, in accord with Pizzolongo [14] and [15]. The three metaphase plates showing the higher chromosome numbers (respectively 115, 116 and 118) could be due to the hydroxyquinoline treatment of the root apices. Hence assuming that *A. donax* carries 110 chromosomes we can formulate a couple of hypotheses concerning the origin of this species. *A. plinii,* $2n = 72$ chromosomes with $x = 12$ [14] is a fertile species very similar to *A. donax*: we can conjecture that it underwent a chromosome doubling forming a fertile or partially fertile tetraploid individual (144 chromosomes) that crossed with the diploid ($2n = 72$ chromosomes) produced a sterile triploid (108 chromosomes) and by the acquisition of 2 chromosomes (aneuploid) formed the new species *A. donax* with 110 chromosomes (Fig. 2, *a*). Alternatively we can hypothesize that the tetraploid crossed with *Phragmites australis* (96 chromosomes), another species similar to *A. plinii*, producing a sterile hybrid (allopolyploid) with 120 chromosomes that lost 10 chromosomes (aneuploid) to create *A. donax* (Fig. 2, *b*). In both cases a sterile plant is obtained due to the uneven ploidy: in the first case the product is a triploid plant that acquired two chromosomes and in the second case an allopolyploid species that lost 10 chromosomes. These changes in chromosome number have been well demonstrated in *P. australis*, where both euploids and aneuploids have been found: in this species different euploid numbers, between $3x$ and $12x$, with $x = 12$, have been found: the predominant types in Europe are tetraploids while in Asia the octoploids with 96 chromosomes are more diffused [18] which agrees with the fact that the *Arundo* genus is supposed to have originated in East Asia [3, 7]. It is not surprising that this genetic plasticity in aquatic plants may occur, with a preponderantly vegetative reproducing system based on the fragmentation of parts of plants (rhizomes and canes): this reproductive system increases the probability of accumulating chromosomal mutations which are not filtered by the process of meiosis. To support the hypothesis of the involvement of a fertile tetraploid plant as one parent of *A. donax* we used a maize SSR molecular marker (*umc1221*), selected out of 48 SSRs tested (data not shown), that was able to point out the alleles carried by the different *A. donax* clones collected in the Italian territory. As shown in Fig. 3, analyzing 7 clones from northern Italy (the origin of the clones is described in Material and Methods) we observed the presence of 5 different polymorphisms (lanes *1*, *2*, *3*, *4* and *7*) where two bands present in lane *2* seem to be the same band present in lane 1 together with the band present in lane *3*, while two bands present in lane *4* seem to be the same bands as those present in lanes *3* and *7*. This pattern can be explained by supposing that the SSR sequence worked only on a single diploid genome (similar to maize) obtained from gametes of tetraploid plants: the clones *1*, *3*, *5* and 6 were homozygous and the clones *2* and *4* were heterozygous for the allele tested. In our hypothesis the probable ancestor could be *A. plinii*: the presence of multiple alleles could reflect a polyphyletic origin of *A. donax*. Concluding, in this work we suggest a chromosome number for *A. donax* present in Italian territory and we propose two hypotheses concerning its origin, supported by molecular markers that will help further work aimed to assess the relationship among *A. donax* and other similar species and to unravel the causes of its sterility.

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АНАЛИЗ ЧИСЛА ХРОМОСОМ И ПРЕДПОЛОЖЕНИЯ О ПРОИСХОЖДЕНИИ АРУНДО ТРОСТНИКОВОГО (*ARUNDO DONAX* L.).

Arundo donax (Арундо тростниковый) – многолетнее корневищное растение, происходящее из Азии, которое в наши дни распространено по всему миру. Интерес к этому виду растет благодаря значительной продукции биомассы и высокой приспособляемости к условиям малоплодородных земель. *A. donax* может использоваться как важная и перспективная энергетическая культура для получения топлива и биоэтанола второго поколения. *A. donax* размножается агамно с помощью фрагментации ризома и узлов побегов, что сильно затрудняет возможности генетического улучшения путем селекции. Стерильность может быть вызвана тем фактом, что *A. donax* является гибридом с нечетной плоидностью или триплоидным видом. Трудно предложить объяснение такой стерильности, так как число хромосом у *A. donax* до сих пор является предметом дискуссий из-за их большого количества и маленького размера. В библиографиях сообщается о размахе признака от 40 до 110 хромосом. Для определения хромосомного числа *A. donax* мы изучили 17 метафазных пластинок, полученных из кончиков корешков при гидропонном выращивании междоузлий. Наши подсчеты показали, что вероятнее всего *A. donax* имеет 110 хромосом. Эти данные, а также результаты изучения SSR молекулярных маркеров позволили нам выдвинуть две возможные гипотезы об эволюционных механизмах возникновения *A. donax*.

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