

Presence of triploid cytotypes in the common fig (*Ficus carica* L.)

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Abstract: *Ficus carica* ($2n = 26$) is one of the oldest fruit trees of the Mediterranean basin. Recently there has been increasing interest in this species, in particular for questions related to germplasm such as genetic diversity and cultivar identification. This study was undertaken to gain more knowledge of *F. carica* cytogenetics and provide data useful for the characterization of its germplasm. Karyomorphological analysis and physical mapping of 18S–25S and 5S rRNA genes by the FISH technique contributed to defining the basic traits of the chromosome complement of *F. carica*. However, the most interesting result was the discovery of triploid ($2n = 39$) cytotypes of the cultivated common fig. This result demonstrates the importance of cytogenetic investigations in studies of fig germplasm and emphasizes the role of cross-fertilization as a source of variability not only in wild populations but also in cultivated forms. The results of pollen analysis suggest spontaneous sexual polyploidization as a possible origin of triploid cytotypes. Further studies are necessary to clarify the origin and effective spreading of polyploidy, the presence of other ploidy levels, and their distribution in wild and cultivated forms.

Key words: *Ficus carica*, fluorescence in situ hybridization, rRNA genes, triploidy, $2n$ gametes.

Résumé : Le *Ficus carica* ($2n = 26$) est l'un des arbres fruitiers les plus anciens du bassin méditerranéen. Au cours des dernières années, il suscite un intérêt croissant, particulièrement sur les questions liées aux ressources génétiques, comme la diversité génétique et l'identification variétale. Cette étude a été initiée pour acquérir une meilleure connaissance de la cytogénétique du *F. carica* et pour fournir des données utiles en vue de la caractérisation de ses ressources génétiques. Des analyses caryomorphologiques et la cartographie physique des gènes codant pour les ARNr 18S–25S et 5S par la technique FISH ont contribué à définir les caractéristiques de base du complément chromosomique chez le *F. carica*. Cependant, le résultat le plus intéressant est la découverte de cytotypes triploïdes ($2n = 39$) chez le figuier cultivé. Ce résultat démontre l'importance des études cytogénétiques pour la caractérisation des ressources génétiques chez le figuier et met en relief le rôle de la fécondation croisée comme source de variabilité non seulement au sein de populations sauvages mais également au sein des formes cultivées. Les résultats de l'analyse du pollen suggèrent que la polyploïdisation sexuelle spontanée serait possiblement à l'origine des cytotypes triploïdes. Des études additionnelles seront nécessaires pour élucider, en plus de son origine, l'efficacité de la dispersion de la polyploïdie, la présence d'autres niveaux de ploïdie et leur distribution au sein des formes sauvages et cultivées.

Mots-clés : *Ficus carica*, hybridation in situ en fluorescence, gènes d'ARNr, triploïdie, gamètes $2n$.

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Introduction

The common fig (*Ficus carica* L.) is a typical fruit tree of the Mediterranean basin which takes its scientific name from the region Caria, in ancient times the southwestern part of Turkey (Storey 1975). *Ficus carica* is one of the early domesticated (4000 B.C.) Mediterranean fruit trees, although the original distribution of this species before domestication, mainly with respect to its presence in the western Mediterranean regions, is still unclear (Dickson and Dickson 1996). According to the theory of Zohary and Hopf (2000), domestication of the common fig tree took place in the eastern Mediterranean and from there selected forms were brought

to other regions, especially the western Mediterranean areas. A recent study based on mtDNA RFLP (Khadari et al. 2005) offers evidence that fig populations were present in the western Mediterranean even before domestication, following the same pattern observed in *Olea europaea* (Besnard et al. 2002). Domestication produced substantial modifications in the primitive characteristics of the fig tree, for example by increasing the sugar content and the size of the fruit and determining a gradual shift toward vegetative propagation (Storey 1975). However, in the wild the spreading of the species is entirely dependent on seed (Zohary and Spiegel-Roy 1975). The reproductive biology of *F. carica* is regulated by a mechanism of extraordinary complexity characterized by the symbiosis with its wasp pollinator, the agaonid *Blastophaga psenes*, 3 functional floral forms, and 2 forms of tree. The symbiotic relationship between plant and insect is one of obligate mutualism. The fig tree and its pollinating wasp are completely dependent on one another for survival and reproduction, as the fig can be pollinated only by *B. psenes* and the wasp can reproduce only within the fig (Marussich and Machado 2007). The flowers, located on the

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inside surface of the syconium, a false fruit, are of 3 types: male flower, short-styled female flower, and long-styled female flower. Male flowers contain 3 to 5 anthers and produce dimorphic pollen which has been classified as triplicate spherical and diplicate ellipsoidal. Both types are produced within the same anther and can germinate on a receptive stigma (Beck and Lord 1988). Female flowers differ not only in stylus length but also in their functional role in the breeding system of the species. The short-styled type serves primarily as the oviposition site for the pollinating wasp, whereas the long-styled flowers function as seed producers. The male tree, called caprifig, produces syconia containing male and short-styled female flowers. The female tree produces only flowers of the long-styled type and it alone produces edible syconia. These characteristics led botanists to define *F. carica* as a gynodioecious species with a functional dioecious breeding system (Valdeyron and Lloyd 1979).

Among the fig cultivars, there are those that are totally dependent on pollination by *B. psenes* for fruit development. These cultivars produce fruit with fertile seeds when caprifigged, that is when the wasps bring pollen from the anthers of caprifig to the long-styled flowers of the female plant. Other cultivars are wholly or partly parthenocarpic and produce edible seedless fruits without pollination (Storey 1976).

From time immemorial the common fig has been cultivated in most areas around the Mediterranean basin, constituting a fruit crop of extraordinary importance. The fruit, much appreciated for its nutritive value, was a common component in the diet of Mediterranean populations, consumed fresh at the time of harvesting and dried to eat during the year. Nowadays the common fig is still an important crop for many regions around the world with warm temperate climates and it is surprising that, despite its long history of cultivation, genetic studies of this species are quite recent. DNA-based technologies have made it possible to attempt important aspects of germplasm characterization, such as assessment of genetic diversity and identification of cultivars (Khadari et al. 1995; Cabrita et al. 2001; Papadopoulou et al. 2002; Salhi-Hannachi et al. 2005). Despite advances in this field, cytogenetic studies in *F. carica* are almost nonexistent. It is known that the species is diploid ($2n = 26$) with very small chromosomes, but no other information is available (Oginuma and Tobe 1995).

The purpose of this study was to gain knowledge of *F. carica* cytogenetics and verify whether chromosome investigations can provide new information to characterize the germplasm of this species. To this end, chromosome counts, karyomorphological analysis, and physical mapping of rRNA genes by fluorescence in situ hybridization (FISH) were carried out on a large number of genotypes, which included wild and cultivated common figs. Since polyploid cytotypes with $2n = 39$ were discovered during this survey, chromosome investigations were followed by the analysis of pollen to verify, on the basis of the size of the pollen grains, the possible formation of $2n$ pollen. This is one of the most commonly used methods to ascertain the tendency of plants to form unreduced gametes, since, in general, the size of pollen grains is related to their ploidy level (Sala et al. 1989; Bretagnolle and Thompson 1995; Bretagnolle 2001).

Materials and methods

Plant materials

Forty-four genotypes collected during 2002–2005 were used for this study. They comprise wild plants (both caprifigs and female plants), cultivated figs of unknown identity, and cultivars. The accession numbers, identity, and origin of genotypes are listed in Table 1. All the cultivars were provided by a specialized nursery of central Italy. Cultivars Verdino, Verdolino, and Brogiotto are represented by more than one accession. Cuttings of each plant were placed in a warm bed with appropriate temperature and moisture for rooting and growing of leaves. All genotypes used for this investigation are conserved in the greenhouse of the Department of Applied Biology of the University of Perugia (Italy).

Chromosome preparations

Actively growing root tips were harvested from cuttings and pretreated in a saturated aqueous solution of α -bromonaphthalene for 6 h and then fixed in ethanol : acetic acid (3:1) overnight.

For determination of the chromosome number, slides prepared for FISH experiments and directly stained with DAPI were used.

Chromosome preparations for in situ hybridization experiments were set up as follows. The fixed root tips were rinsed in distilled water for 10 min and treated for 6 min in 0.25 N HCl at room temperature, rinsed again in distilled water, and immersed in enzyme buffer (10 mmol/L citric acid – sodium citrate, pH 4.6) for 20 min. Root tips were then excised and kept in the enzyme solution (4% cellulase Onozuka R10 and 1% pectolyase (Sigma) in distilled water) for 2 h at 37 °C. Slides were prepared according to the drop-ping method described by Leitch et al. (1994).

DNA probes and fluorescence in situ hybridization

The probes used to identify the loci of ribosomal RNA genes on mitotic chromosomes of *F. carica* were clone pTa71, containing the 18S–5.8S–25S rRNA genes and non-transcribed spacers of *Triticum aestivum* L. (Gerlach and Bedbrook 1979), and clone pXVI, containing the complete 5S rRNA gene and the spacer region of *Beta vulgaris* L. (Schmidt et al. 1994). The probes were labelled by nick translation, clone pTa71 with digoxigenin-11-dUTP (Roche) and clone pXVI with biotin-11-dUTP (Sigma).

FISH experiments were carried out according to the standard method (Falistocco 2000). Slides were pretreated with RNase A (100 μ g/mL) in $2\times$ SSC for 1 h at 37 °C, incubated with 80 units/mL of pepsin in 10 mmol/L HCl for 15 min at 37 °C, stabilized in freshly depolymerized 4% (w/v) paraformaldehyde in water, washed in $2\times$ SSC, and dehydrated in ethanol.

The hybridization mixture, consisting of 150 ng/ μ L of each labelled probe, 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS (sodium dodecyl sulfate), and 300 ng/ μ L sheared salmon sperm DNA, was incubated for 10 min at 70 °C and then applied to the chromosome preparation. The hybridization mixture and the chromosomes were denatured together at 70 °C in a modified thermocycler for 5 min and the temperature was gradually

Table 1. List of accessions of *F. carica* examined.

Accession No.	Genotype/cultivar name	Origin
1	Cultivated, unknown	Central Italy
2	Cultivated, unknown	Central Italy
3	Female plant	Central Italy
4	Female plant	Central Italy
5	Cultivated, unknown	Central Italy
6	Cultivated, unknown	South Italy
7	Caprifig	Central Italy
8	Cultivated, unknown	Sardinia
9	Female plant	Sardinia
10	Dottato	Commercial source
11	Cultivated, unknown	Central Italy
12	Cultivated, unknown	Sardinia
13	Cultivated, unknown	Central Italy
14	Cultivated, unknown	Sardinia
15	Cultivated, unknown	Central Italy
16	Female plant	Sardinia
17	Caprifig	Central Italy
18	Female plant	South Italy
19	Cultivated, unknown	Central Italy
20	Caprifig	Sardinia
21	Caprifig	Central Italy
22	Caprifig	Central Italy
23	Caprifig	Central Italy
24	Verdino	Commercial source
25	Verdino	Commercial source
26	Verdino	Commercial source
27	Caprifig	South Italy
28	Caprifig	South Italy
29	Caprifig	South Italy
30	Caprifig	South Italy
31	Verdolino	Central Italy
32	Verdolino	Central Italy
33	Cultivated, unknown	South Italy
34	Cultivated, unknown	South Italy
35	Cultivated, unknown	South Italy
36	Cultivated, unknown	Central Italy
37	San Pietro	Commercial source
38	Amelia Bianchelle	Commercial source
39	Nero Portogallo	Commercial source
40	Permaloso	Commercial source
41	Gentile Giallo	Commercial source
42	Brogiotto	Commercial source
43	Brogiotto	Commercial source
44	Female plant	Sardinia

decreased to 37 °C. The hybridization was carried out overnight at 37 °C. Stringent washing consisted of 10 min in 20% (v/v) formamide in 0.1× SSC at 42 °C and 10 min in 2× SSC at room temperature. Detection of the probes labelled with digoxigenin and biotin was done with anti-digoxigenin conjugated with FITC and streptavidin conjugated with Cy3, respectively. Finally, the slides were counterstained with 2 µg/mL DAPI and mounted in Vectashield antifade solution. Photographs were taken on Fujichrome 400 color slide film and digitized with a film scanner. Contrast optimization and superimposition of images were realized with the software Adobe Photoshop.

Pollen analysis

Pollen samples were collected from 5 caprifigs (accession Nos. 7, 17, 21, 22, and 23) and stained with a solution of acetocarmine and glycerol (1:1). The size of the pollen was determined by measuring, for each plant, the diameter of 1000 grains of both the spherical and ellipsoidal types. In the ellipsoidal grains only the major diameter was considered. Measurements were taken with an ocular micrometer.

Results

Chromosome number and morphology

DAPI-stained metaphases were used to determine the chromosome number of all the accessions. Most of the genotypes examined showed the expected number, $2n = 26$, typical of the species. But accessions 13, 26, and 32, corresponding, respectively, to a cultivated fig of unknown identity, one plant of Verdino, and one plant of Verdolino, exhibited the chromosome number $2n = 39$ (Fig. 1). Since *F. carica* is considered a diploid species ($2n = 2x = 26$), the $2n = 39$ genotypes were regarded as triploid variants. Karyomorphological analyses, carried out on several mitotic metaphases from the early to the late stage, showed that the chromosome complement of *F. carica* is constituted by metacentric chromosomes with length ranging from 0.6 to 2.0 µm. Only 2 large pairs (2.0 µm) were distinguishable from the others. The karyomorphological pattern obtained on $2n = 39$ cytotypes was similar to that of diploids. Six large chromosomes were observed in all metaphases examined and they constituted reliable evidence of the triploid constitution of these cytotypes. Other morphological traits useful for karyotyping, such as the position and number of secondary constrictions, could not be detected with certainty.

Chromosomal localization of 18S–25S and 5S rRNA loci

All genotypes used for this study were subjected to FISH treatment to investigate the chromosomal distribution of 18S–25S and 5S rRNA loci. The results of FISH mapping using two differentially labelled probes are summarized in Fig. 2. Two pairs of 18S–25S rRNA loci and one pair of 5S rRNA loci were consistently identified in all diploid genotypes. The green fluorescent signals indicated the 18S–25S rRNA loci were localized at the extremity of two pairs of small chromosomes, thus revealing the number and the position of the nucleolar organizer regions that could not be detected by DAPI staining (Fig. 2a). No substantial differences between the sizes of the mapped loci were observed. Red fluorescent signals, corresponding to 5S rRNA loci, appeared instead to be located in one of the larger chromosome pairs, close to the centromere (Fig. 2b). The number of hybrid signals produced by the two rRNA probes in accessions 13, 26, and 32 reflected the triploid nature of these cytotypes. The chromosomal positions of the rRNA loci were similar to those observed in diploids. Sites of 18S–25S rRNA were localized in the terminal region of 6 small chromosomes, while 5S rRNA sites were mapped to the proximal region of 3 large chromosomes (Figs. 2c–2d).

Pollen analysis

Pollen samples were collected from 5 caprifigs and exam-

Fig. 1. Mitotic metaphases of diploid ($2n = 26$) and triploid ($2n = 39$) genotypes of *F. carica* (common fig). Arrows indicate the larger chromosomes. Bar = 3 μm .

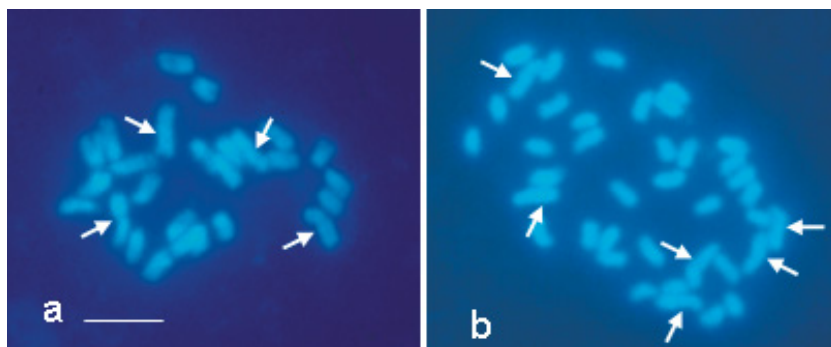
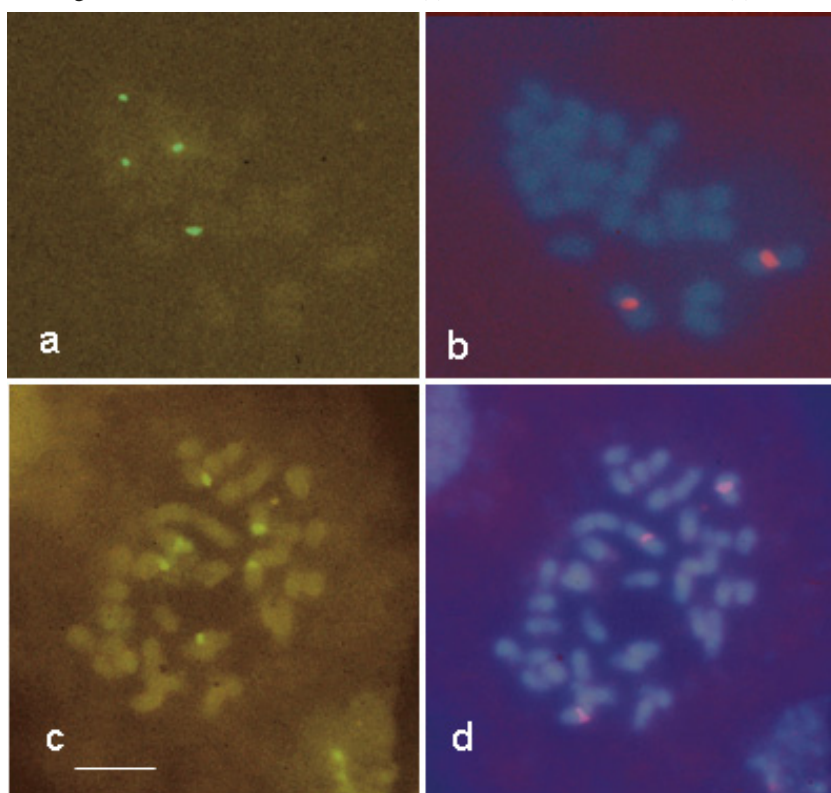


Fig. 2. Double-target in situ hybridization with 18S–25S rDNA (green) and 5S rDNA (red) probes to mitotic chromosomes of *F. carica*. Metaphase of a diploid ($2n = 26$) cytotype showing four loci of 18S–5.8S–25S rDNA (a) and two loci of 5S rDNA (b). Metaphase of a triploid ($2n = 39$) cytotype showing six loci of 18S–5.8S–25S rDNA (c) and three loci of 5S rDNA (d). Bar = 3 μm .

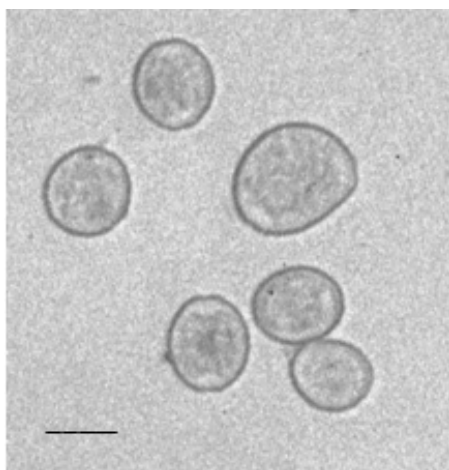


ined to assess the tendency of the plants to produce unreduced ($2n$) gametes. Both spherical and ellipsoidal grains showed a very uniform size; however, in genotypes 17, 21, and 23, grains of notably larger size were observed (Fig. 3). Diameter measurements confirmed the results of the visual analysis. In all caprifigs examined, the diameters of normal spherical and ellipsoidal grains ranged from 44.0 to 45.0 μm and from 53.0 to 54.0 μm , respectively. In contrast, large spherical and ellipsoidal grains measured 50.0–55.0 μm and 60.0–65.0 μm , respectively. Pollen grains of intermediate size were not found. The highest frequency of large pollen, 2.1%, was found in genotype 17, whereas values of 1.0% and 0.8% were obtained in genotypes 23 and 21, respectively.

Discussion

This paper is the first extensive study of *F. carica* cytogenetics. Owing to the uniform morphology of many chromosomes, karyological analyses could provide only a generic description of the chromosome complement of this species based on centromere position and chromosome length. On the other hand, the FISH technique turned out to be a very reliable approach for obtaining insight into the chromosome structure of this species, also supplying molecular markers for chromosome identification. The physical position of the 18S–25S rRNA loci led to the identification of two small chromosome pairs, whereas the two largest chromosome pairs could be distinguished one from the other by the presence or absence of the 5S rRNA loci.

Fig. 3. Pollen sample collected from caprifig No. 21 showing normal and large pollen grains. Bar = 40 μ m.



The discovery of triploid cytotypes is of particular interest with respect to the purpose of this investigation because it demonstrates the importance of cytogenetic studies for acquiring information to help characterize fig germplasm. Although further investigations are necessary to assess the real diffusion of polyploidy, the high incidence of triploids (6.8%) detected in the sample examined indicates that polyploidy in *F. carica* is a rather widespread phenomenon and not a sporadic event. Therefore, the ploidy level could be an important additional trait to be considered in studies of fig germplasm. In addition, the presence of polyploid variants focuses attention on the reproductive system of the species and emphasizes the role of cross-fertilization as a source of genetic variability not only in wild populations but also in cultivated varieties.

A fig cultivar is defined as a collection of individuals obtained by vegetative propagation from a wild genotype that was chosen for its agronomic features and introduced into cultivation (Khadari et al. 1995). Because of the widespread practice of vegetative propagation, intra-cultivar genetic variability is regarded principally as the result of natural mutations. Keeping in mind that wild populations reproduce entirely from seed, it is reasonable to suppose that cultivars have a multiple origin. Several related genotypes, including polyploid variants, may have contributed to the development of a single cultivar. Events of sexual reproduction may be expected to occur also within already established cultivars. It is known that some of the fig cultivars are totally or partially dependent on pollination for fruit production. After pollination these plants develop their fruit, which contains numerous fertile seeds. Plants derived from seed exhibiting desirable characteristics may have been selected by fig breeders and growers and propagated as new clones. Both situations could have occurred during the long history of fig cultivation and must be taken into account in explaining the existence of triploid genotypes in the common fig.

The discovery of the $3x$ genotypes proves that individuals belonging to the same cultivar may derive from genetically different lineages. Among natural polyploids, triploids represent a minority, although they are not rare among herbaceous (Zohary and Nur 1959; Lord and Richards 1977; Bretagnolle 2001) and woody species (Ananthawat-Jónsson

and Thórsson 2003; Dzialuk et al. 2007) as well as some important fruit species (Einsett 1952; Nsabimana and van Staden 2006). Investigations into the origin of $3x$ plants in wild populations led to the identification of 2 different pathways: sexual polyploidization by means of the formation of unreduced $2n$ gametes (Rhoades 1936), and hybridization between diploids and tetraploids (Müntzing 1937). At present a large consensus exists on the role that $2n$ gametes play in the genesis of polyploids, both auto- and allo-polyploids (Bretagnolle and Thompson 1995). These gametes, characterized by the somatic chromosome number of the species instead of by the normal reduced number, are the results of meiosis affected by alterations of chromosome pairing, spindle formation, or cytokinesis (Ramanna 1983; Veilleux 1985). One of the most used methods to test the tendency of a diploid plant to form $2n$ gametes consists of screening for variability in the size of pollen grains. Since the size of the grains increases with increasing DNA content, the presence of large pollen is generally considered a reliable indication of the production of $2n$ pollen (Ramanna 1974; Orjeda et al. 1990; Bretagnolle and Thompson 1995; Becerra Lopez-Lavalle and Orjeda 2002). Following the $2n$ gametes pathway, a triploid plant is generated by the fusion between a reduced and a non-reduced gamete. Therefore, spontaneous sexual polyploidization must be taken into account as the most likely source of neotriploids in wild diploid populations. Unreduced gametes have an important function in the formation of polyploid fruit crops such as sweet bananas (*Musa acuminata*). Many of the domesticated banana varieties have proved to be triploid ($2n = 3x = 33$) with a genome constitution of AAA. Raboin et al. (2005), using RFLP markers, demonstrated that sweet banana cultivars belonging to the subgroups Cavendish and Gros Michel have a common diploid ancestor that was incorporated in the triploid cultivars by means of $2n$ gametes, whereas two genetically distinct donors provided the third genome by means of a normal haploid gamete.

The alternative pathway leading to the production of triploids, i.e., hybridization between $2x$ and $4x$ genotypes, implies the existence of tetraploids and the establishment of hybrid zones involving diploids and the related tetraploids (Husband and Schemske 1998).

Data obtained from this study led to some consideration of the presence of polyploid forms in *F. carica*. The frequency of triploid cytotypes is clear evidence that the triploid block does not operate in this species or, at least, has minimal consequences. The occurrence of triploid events in cultivated figs argues that the $3x$ condition may confer attractive characteristics accounting for their selective advantage. If this is the case, the spreading of $3x$ plants would be favoured by human intervention by means of vegetative propagation, regardless of their fertility. The recurrent appearance of triploids in species under cultivation sustains a plausible connection between the selective advantage and their triploid constitutions. In the apple, for example, it has been observed that triploid cultivars are more vigorous and produce larger fruit than diploids (Brown 1975). Attempts to verify this hypothesis in *F. carica* will be done with the continuation of this study.

Large pollen produced by some caprifigs indicates the tendency of *F. carica* to form unreduced pollen and supports

spontaneous sexual polyploidization as the possible origin of 3x cytotypes. On the other hand, this result cannot exclude the hypothesis of hybridization between 2x and 4x genotypes, since hypothetical tetraploid figs could derive from a bilateral sexual polyploidization process, demonstrating in this way also the formation of 2n eggs.

The study in *F. carica* highlights several aspects that must be clarified for understanding the importance of polyploidy in the evolution of the species and in the development of the crop. Investigation is continuing to assess the effective spreading of polyploidy and to verify the existence of other ploidy levels and their distribution within wild populations and cultivars. Further analyses will be carried out to estimate the production of 2n pollen. Because it is known that the formation of 2n gametes may be highly variable, being largely genotype and environment dependent (Bretagnolle and Thompson 1995), a larger number of caprifigs, growing under different climatic conditions, will be examined.

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