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Nuclear–Cytoplasmic male-sterility in diploid dandelions

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Male-sterility was found in diploid dandelions from two widely separated populations from France, and its inheritance was analysed by crossing a diploid male-sterile dandelion to diploid sexuals and triploid apomicts. Nuclear genetic variation, found in full-sib families, segregated for male-fertility, partial male-sterility, and full male-sterility, and also segregated for small-sized versus normally sized pollen. The crossing results are best explained by a cytoplasmic male-sterility factor in combination with two dominant restorer genes. Involvement of the cytoplasmic male-sterility factor was further investigated by chloroplast haplotyping. Male-sterility was exclusively associated with a rare chloroplast haplotype (designated 16b). This haplotype was found in seven male-sterile plants and one (apparently restored) male-fertile individual but does not occur in 110 co-existing male-fertile plants and not in several hundreds of individuals previously haplotyped. Apomicts with cytoplasmic male sterility were generated in some test crosses. This raises the question as to whether the male sterility found in natural dandelion apomicts, is of cytoplasmic or of nuclear genetic nature. As many breeding systems in *Taraxacum* are involved in shaping population structure, it will be difficult to predict the evolutionary consequences of nuclear–cytoplasmic male-sterility for this species complex.
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Introduction

The dandelion genus *Taraxacum* is well known for its various breeding systems, including outcrossing mediated by sporophytic self-incompatibility (Okabe, 1956), predominant selfing (Kirschner *et al*, 1994), fully apomictic reproduction or reproduction by one or more of its components involving diplospory, parthenogenesis and autonomous endosperm development (Van Dijk *et al*, 1999 and references therein; Tas and Van Dijk, 1999; PJ Van Dijk, personal communication). The presence of apomixis in the genus and its consequences for taxonomy have been the subject of many studies (see Kirschner and Stepanek, 1996; Wittzell, 1999).

Apart from its taxonomic consequences, apomixis and particularly its combination with male-sterility is important from an evolutionary point of view. One of the greatest questions in evolutionary biology is why so many species reproduce sexually and spend their energy in producing males and females? In the light of this problem, Maynard-Smith (1979) postulated that apomictic dandelions could become superior over sexuals by putting more resources into the production of seeds instead of into pollen production. An explanation for the fact that most apomictic dandelions produce pollen, which is not needed for reproduction, could be that these have not yet accumulated mutations that cause male-sterility (Maynard-Smith, 1979).

Our recent findings shed new light on this view. In this paper, we report the presence of male-sterility in diploid dandelions obtained from two widely separated localities in France. We investigated the inheritance of male-sterility and fertility restoration by analyzing crosses between a diploid male-sterile (MS) dandelion and diploid sexual or triploid apomictic pollen donors. Additionally, we used chloroplast haplotyping of MS and male-fertile (MF) dandelions sampled from populations to investigate the involvement of the cytoplasm in the inheritance of male-sterility. Our data suggest that this type of male-sterility shows nuclear–cytoplasmic inheritance.

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Materials and methods

Population samples

MS dandelions were found in two localities in France. The first site (in Central France) was a hay field at the hill slope 'Col de Croix' near Beaujeu, Rhône, from which dandelions were collected twice (nonsystematically) in April 1995 and April 1997 (referred to as samples TJX3 and TLX3, respectively). The second locality (in southern France) was situated on the limestone plateau 'Causse du Larzac' near Belvezet, Aveyron. The plants of this sample, designated TLX9 were taken (non-systematically with respect to male-sterility) by JCM Den Nijs in the summer of 1997. Plants were grown individually in plastic pots with compost and were individually labelled

(eg plant 20 of sample TJX3 is TJX3-20). All plants that could be successfully maintained were subjected to chloroplast haplotyping and were analysed for the ability to produce pollen (see below). A total of 60, 32, and 26 plants was analysed for TJX3, TLX3, and TLX9, respectively. Plants were determined to the level of section or microspecies by an expert taxonomist. All plants from TLX9 have red or brownish-red seeds (in fact fruits or achenes) and most, if not all, plants belong to the species *Taraxacum rubicundum* ssp *monspeliense* and all belong to the section *Erythrosperma* (P Oosterveld, personal communication). Virtually, all plants of TJX3 and TLX3 plants have been determined as specimens belonging to the section *Ruderalia*, but a few individuals seem to have some 'features' of the section *Celtica* (P Oosterveld, personal communication).

Diploid fathers crossed to MS plant TJX3-20

The diploid MS individual, TJX3-20 (section *Ruderalia*, P Oosterveld, personal communication), was crossed to two different diploid, fully MF, pollen donors, TG23-2, and TLX9-30. The resulting progeny of first- and second-generation crosses was analysed for segregation of MF and MS plants. Pollen donor TG23-2 (section *Ruderalia*) originated from a seed sampled in 1992 by JCM Den Nijs in a hayfield near Cluny, France and was used because of its recessive morphological marker and its ability to self-fertilize (see Results). Pollen donor TLX9-30 came from population TLX9.

Second-generation progeny

BC_1 progeny was obtained from the cross MS TJX3-20 $\times$ (MS TJX3-20 $\times$ MF TLX9-30) designated as $BC_{1'B'}$ (see Table 1). F_2 progeny were obtained by crossing two half-sibs (MS TJX3-20 $\times$ MF TG23-2) $\times$ (MS TJX3-20 $\times$ MF TLX9-30) designated as $F_{2'A'}$ (see Table 1).

Triploid apomicts crossed to MS TJX3-20

The MS plant TJX3-20 was also crossed individually to four triploid apomicts (TJX4-79, TLX11-02, TLX11-38, and T68). These triploid apomicts were sampled as roots from meadows. TJX4-79 was sampled near Langres, France in April 1995; TLX11-02 and TLX11-38 were sampled in Grassberg, near Bremen, Germany in April 1997. T68 was sampled from a meadow near Heteren, The Netherlands by P Van Dijk. Triploid pollen donors were used for three reasons. The first and most important reason for making crosses with apomicts was to infer whether hybrid MS apomicts could be obtained. From a population genetic perspective, preference should be given to MF members of the same population as MS TJX3-20. At the time these crosses were made, TJX3-20 was the only MS diploid known and it was crossed to the triploid apomicts listed, simply because flowers were available from these individuals. Although we are aware of complex inheritance in crosses between polyploids and diploids, our second aim was to use the data available from these crosses to analyse the inheritance of male-sterility. The third objective was to evaluate TJX3-20 as a test plant for investigating the inheritance of apomixis (here defined as spontaneous seed set).

Table 1 Segregation ratios and proposed genotypes for F1's and second-generation crosses made between MS tester TJX3-20 and individual father plants

Type of cross	*Cross*	*MS*	*PMS*	*MF*	*Suggested ratio MS:(PMS+F)*	*Model 1*	*Model 2*
$F_{1''A''}$	TJX3-20 (2x,MS) $\times$ TG23-2 (2x,MF)	17	42	13	1:3 ($P=0.79$) or 1:1****	(S)++, ++ $\times$ R+, R+	Ffhh $\times$ ffHh
$S_{1(P2''A'')}$	TG23-2 (2x,MF)	0	0	50	0:1 or 1:15*	selfing (N) R+, R+	Selfing ffHh
$F_{1''B''}$	TJX3-20 (2x,MS) $\times$ TLX9-30 (2x,MF)	0	9	28	0:1 or 1:3***	(S)++, ++ $\times$ RR, R+	Ffhh $\times$ (F:HH or ffHH but not ffHh)
$BC_{1''B''}$	TJX3-20 MS $\times$ $F_{1''B''}$	16	0	39	1:3 ($P=0.48$) or 1:1 ****	(S) ++, ++ $\times$ (S) R+, R+	Ffhh $\times$ ffHh
$F_{2\ (A''\times B'')}$	$F_{1''A''}$(MS) $\times$ $F_{1''B''}$	14	5	10	1:1 ($P=0.85$) 3:5 ($P=0.23$) or 1:3***	(S) ++, ++ $\times$ (S) R+, ++	Ffhh $\times$ FFHh or Ffhh $\times$ FfHh

MS, PMS, and MF refer to male sterile, partially male-sterile and male-fertile, respectively. In model 1, '+' refers to a wild-type allele, 'R' refers to a dominant restorer. (S) refers to MS cytoplasm and (N) refers to male-fertile cytoplasm. In model 2, a dominant gene 'F' causes male sterility where a dominant gene 'H' suppresses the action of F. $*P<0.05$, $**P<0.01$, $***P<0.005$, $****P<0.001$.

Scoring male-fertility

Male-fertility was determined in two different experimental set-ups, including either variable (ambient greenhouse) or 'constant' (20–25°C max.). temperatures. Basically, there are two kinds of phenotyping: (1) absence/presence of pollen and (2) pollen size. The absence/presence of pollen on the pollen-bearing stigmatic branches was determined by eye and checked using a light microscope. To this end, pollen samples were taken by dipping the stigmatic branches of flower heads in a drop of water on an object glass followed by inspection at 10×10 or 10×40 magnification. Plants were scored in three phenotypic classes: 'MS' male-sterile, 'PMS' facultatively male-sterile/partially male-sterile, and 'MF' male-fertile. The phenotypic class PMS represents all plants capable of producing pollen but only under favourable conditions (see below). From our seasonal scoring records (not shown) and greenhouse temperature records, it became apparent that virtually all plants capable of producing pollen will do so, at least in early spring. When greenhouse temperature rises (late spring–summer) some of the plants (accordingly designated 'PMS') lose the ability to produce pollen. This occurred about 2 weeks after the temperature reached a critical maximum temperature (average day temperature >17°C, max. probably $\gg 25$°C). Pollen production of PMS plants is usually regained 2 weeks after the plants have been exposed to temperatures below a certain critical value (average day temperature <15°C, max. probably $\ll 25$°C) caused by cool cloudy weather. This meant, that in practice, PMS can only be discriminated from either MS or MF by exposure of plants to variable temperatures. Therefore, we kept seasonal records for all F_1 plants by scoring these every 2 days (when flowering permitted this) for a period of 3 years.

A second way to phenotype male-fertility is by scoring the size of pollen of samples. Pollen was mixed and compared to haploid pollen that lacks pigment obtained from a special mutant plant (described below) under a light microscope. In this way, three classes were determined: (1) 'MS': no pollen, (2) 'small': containing pollen smaller than haploid and usually half the size of haploid pollen, and (3) 'MF-2': showing at least a fraction of pollen equal to haploid pollen size. Alternatively, MS and 'small' may be taken together in a class of plants not capable of producing normal-sized pollen designated 'MS-2' (see Results).

For the F_1 plants, grown at variable temperatures, the pollen of nearly all PMS plants could be classified as 'small', indicating that the two types of phenotypic classes were fully overlapping. The two classes were, however, not associated in the BC_1 and F_2 families kept at temperatures never exceeding 25°C. This was very likely caused by the lack of a variable critical temperature, which would reveal PMS plants. In this study, we will not postulate a comprehensive genetic model but will provide individual models to explain the segregation of both individual ways of phenotyping (absence/presence of pollen and the pollen size or quality). There is probably a wide spectrum of phenotypes associated with climate (a phenomenon found in *Petunia* by Van Marrewijk, 1969) that would require extensive climate studies using replications and several cuttings per individual to interpret the total pollen-phenotype, which is beyond the scope of this study. Genetic models were tested with standard χ^2 analyses.

Chloroplast haplotyping

To find out whether male-sterility is associated with a certain cytoplasm, chloroplast types were determined for individual plants from TJX3 and TLX3 and TLX9. DNA was isolated using the protocol of Van Der Hulst *et al* (2000) and used as templates for amplification of chloroplast regions by PCR, which were subsequently analysed for length and/or restriction site polymorphism. The regions were: the intron *trnL*, the *trnL–trnF* intergenic spacer (Taberlet *et al*, 1991), the *psbB–psbF* intergenic spacer (Hamilton, 1999), and the *psbA–trnH* intergenic spacer (Sang *et al*, 1997). The enzymes used to uncover restriction site polymorphism were *Apa*II, cutting the *trnL–trnF* intergenic spacer, *Hpa*II, cutting the *psbB–psbF* intergenic spacer, and *Dra*I, cutting the *psbA–trnH* intergenic spacer. After it was observed that male-sterility was associated with a particular chloroplast haplotype (see Results), all MS individuals were subjected to sequence analysis of the *trnL–trnF* intergenic spacer. Sequencing was performed to increase resolution, to ascertain whether these plants had an identical haplotype. Fragments were PCR amplified using Super-Taq (HT Biotechnology), cloned in the PGEM-T-easy vector (Promega) or directly sequenced on an LI-COR fluorescent automated sequencer (LI-COR Inc., Lincoln, Nebraska). For samples TLX9-25 and TLX3-22, the *trnL–trnF* PCR product was directly sequenced by BaseClear (Leiden, The Netherlands) and the Amsterdam Medical Centre, respectively.

DNA content measurements

DNA content of individual dandelions from samples TJX3 and TLX3 and DNA content of the progeny of TJX3-20 crossed to triploid apomicts was measured on a Partec flow-cytometer using methods described by Tas and Van Dijk (1999). The interpretation of DNA content with respect to chromosome numbers follows Tas and Van Dijk (1999) and Van Dijk *et al* (1999).

Results

Segregation of MS:PMS + MF in diploids

Evidence for a two-locus model: Nuclear genetic variation (segregation of MS, PMS, and MF plants) was found in progeny obtained by crossing several pollen donors to MS plant TJX3-20. The results for F_1 crosses and second-generation crosses obtained by using diploid pollen donors are listed in Table 1. Segregation ratios are presented for MS:PMS:MF. For modelling, however, the partial MS and fully MF phenotypic classes are combined into single class; MS (no pollen):PMS + MF (cf. Van Damme, 1983). The majority of F_1 individuals of the cross TJX 3-20 (MS) $\times$ TG23-2 (MF), designated $F_{1''A''}$, appeared male-fertile. Monogenic inheritance of male-fertility was rejected for $F_{1''A''}$ because of the significant deviation from a 1:1 ratio ($P<0.0001$). Results obtained for $F_{1''A''}$ were consistent with a 1:3 ratio and suggested that male-fertility is controlled by two genes. Our first model (model 1) assumed nuclear–cytoplasmic inheritance, where dominant genes restore male-fertility in plants with sterilizing cytoplasm (S). In 'model-1',

presented in Table 1, 'R' represents a dominant restorer and '+' indicates a wild-type recessive allele that is unable to restore male-fertility. An alternative model ('model-2') assumes strict nuclear genetic or 'genic' inheritance as postulated by Lewis and Crowe (1956). In this model, a dominant gene 'F' (female) causes male-sterility and a dominant gene 'H' (hermaphrodite) suppresses the action of F. Both models 1 and 2 could explain all segregation ratios for all crosses listed in Table 1.

Evidence for involvement of the cytoplasm: Involvement of the cytoplasm controlling male-sterility (needed for model 1) was deduced from the data obtained from pollen donor TG23-2 ($F_{1''A''}$, Table 1) that was also used as a seed parent ($S_{1(p2''A'')}$, Table 1). For MF individual TG23-2, progeny was obtained from self-fertilisation. The ability of TG23-2 to self-fertilize, which is exceptional for self-incompatible *Ruderalia* species (Richards, 1973, but see Morita *et al*, 1990), was detected by paternity tests using microsatellite MSTA 64 (obtained from Falque *et al*, 1998, data not shown) in several test crosses using TG23-2 as a seed parent. These test crosses were performed to study an apparently recessively inherited mutation that causes the lack of pigment in both viable pollen and fruits, designated '*albino*', of TG23-2 (RvdH, unpublished results). For TG23-2, we obtained 50 S_1 individuals from spontaneous selfing in one flower head. All of these S_1 individuals were albino but, more importantly, were fully MF. If it is hypothesized that male-sterility is not caused by the cytoplasm, but results from recessive genes, representing a loss of normal fertility, this could also explain the results obtained for cross $F_{1''A''}$. This assumption, however, implies that a 1:15 ratio (MS:PMS + MF) should be observed in the cross $S_{1('p2'A')}$, which is rejected ($P < 0.05$, see Table 1). Consequently, the data are best explained by 'model 1' in which it is assumed that TG23-2 carries nonsterilizing cytoplasm and all other parent plants a MS cytoplasm, designated N and S, respectively.

Segregation of MS-2:MF-2

If instead of absence or presence of pollen grains, pollen grain size was scored and a class lacking normal sized or containing abortive (small) pollen was defined as MS-2 (*vs* plants capable of producing normal pollen MF-2), the segregation ratios differed from the initial way of phenotyping (data in Tables 1 and 2). These segregation ratios could, as for the previous phenotyping, also be explained by nuclear–cytoplasmic inheritance. However, a different model, in this case, based on epistatic interaction of two genes was needed. This model, designated 'model 3', assumed that restored fertility in sterilising S cytoplasm requires the combined action of dominant fertility restorer genes Rf_1 and Rf_2, at two different loci. Consistent with our model 1 (explaining ratios MS: PMS + F), model 3 also assumed that TJX3-20 has S cytoplasm and that TG23-2 has N cytoplasm. The genic model of Lewis and Crowe (1956) failed to explain the ratios observed for the $F_{1''A''}$ (the highest fraction of MS is obtained for the cross Ffhh × ffHh, MS-2:MF-2 = 1:1 $P < 0.001$) and consequently was rejected.

Segregation of male-fertility using triploid pollen donors

The segregation of male-fertility in crosses between MS individual TJX3-20 and four different triploid apomictic pollen donors is shown in Table 3. Segregation of male-fertility in these crosses could all be explained by the action of independent dominant restorer genes, as in model 1. However, as the result of heterozygosity, polyploidy, and small family sizes, the number of possible genotypes becomes unwieldy, and therefore they are not shown. The dominant nature of fertility restoration followed from the data obtained for two families, designated $F_{1''C''}$ and $F_{1''E''}$. In these two families, MS plants were found among diploid progeny, but not in tetraploid progeny (Table 3). Segregation in the diploids could be explained by assuming that the pollen parent contributed reduced (haploid) gametes with either dominant restorers or recessive maintainer alleles. The tetraploid plants very likely resulted from unreduced pollen gametes, and would be expected to inherit all dominant restorer genes from their pollen parent. As MS plants were lacking in the tetraploids ($F_{1''C''}$ and $F_{1''E''}$, Table 3), fertility restoration is unlikely to be recessive. The results for $F_{1''F''}$ (Table 3), that was completely MS (at all ploidy levels) could be explained by the lack of dominant restorers in both parents. Alternatively, the results obtained for $F_{1''D''}$ that was fully MF (Table 1) could be explained by assuming that the paternal plant is fixed for dominant restorers. Model 2, the model of Lewis and Crowe (1956) is rejected for the polyploid crosses because it fails to explain the ratios observed for the $F_{1''F''}$ family. In this family, all plants are MS. It was assumed that the diploid mother of this family has the

Table 2 Segregation ratios for plants producing no pollen or abortive pollen MS-2 and plants producing normal-sized pollen MF-2 and a model explaining the ratios

Type of cross	*Cross*	*MS-2*	*F-2*	*Suggested ratio MS-2:MF-2*	*Model 3 (see text)*
$F_{1''A''}$	TJX3-20 (2x,MS) × TG23-2 (2x,F)	47	11	3:1 ($P = 0.29$) 1:1****	(S) $Rf_1rf_1rf_2rf_2$ × (N) $rf_1rf_1Rf_2rf_2$
$S_{1(P2''A'')}$	TG23-2 (2x,F)	0	50	0:1	(N) $rf_1rf_1Rf_2rf_2$ (×)
$F_{1''B''}$	TJX3-20 (2x,MS) × TLX9-30 (2x,F)	9	28	1:3 ($P = 0.92$) 1:1***	(S) $Rf_1rf_1rf_2rf_2$ × $Rf_1rf_1Rf_2Rf_2$
$BC_{1''B''}$	TJX3-20 MS × $F_{1''B''}$	24	32	1:1 ($P = 0.29$)	(S) $Rf_1rf_1rf_2rf_2$ × (S) Rf_1Rf_1 Rf_2rf_2
$F_{2\ (A''\ \times\ B'')}$	$F_{1''A''}$(MS) × $F_{1''B''}$	18	10	3:1 $P = 0.19$ 1:1 $P = 0.13$	(S) $rf_1rf_1rf_2rf_2$ × (S) Rf_1*Rf_2rf_2

The models assume that combined gene action of two loci, for which least one dominant restorer allele 'Rf' per locus is needed, restores male fertility. (S) refers to MS cytoplasm and (N) refers to MF cytoplasm (MF-2). ***$P < 0.005$, ****$P < 0.0001$.

Table 3 Segregation for male-sterility (MS), partial male-sterility (PMS) and male-fertility (MF) in F_1 progenies of diploid MS plant TJX3-20, crossed individually to four triploid MF apomicts

Cross no.	*Triploids*	*Ploidy level*	*MS*	*PMS*	*MF*
$F_{1''C''}$	TJX3-20 (2x,MS) × TJX 4-79 (3x,MF)	2x	9	6	0
		±3x	0	2	0
		4x	0	6	9
$F_{1''D''}$	TJX3-20 (2x,MS) × TLX11-02 (3x,MF)	2x	0	0	2
		±3x	0	0	10
		4x	0	0	16
$F_{1''E''}$	TJX3-20 (2x,MS) × TLX11-38 (3x,MF)	2x	3	2	1
		±3x	0	2	0
		4x	0	0	7
$F_{1''F''}$	TJX3-20 (2x,MS) × T68 (3x,MF)				
		2x	16	0	0
		±3x	14	0	0
		4x	1	0	0

2x, ±3x, and 4x refer to a diploid, (nearly)triploid, and tetraploid levels, respectively. Note that Families $F_{1''D''}$ and $F_{1''F''}$ are families, fixed for male-fertility and male-sterility, respectively. The families $F_{1''C''}$ and $F_{1''E''}$ segregated (mainly) at the diploid level, pointing to the dominant nature of fertility restoration.

Ffhh genotype (to explain the results in the diploid crosses; Table 1). Segregation of MFs in diploid progeny of $F_{1''F''}$ is then expected unless the paternal plant carries a high number of F alleles. However, because the pollen donor is MF it should have at least one H allele. This H allele should restore the fertility in the tetraploid plant in the $F_{1''F''}$ that inherits an unreduced gamete from its father. This plant is, however, MS, which therefore rejects model 2.

Polyploid crosses were scored for male-sterility, partial male-sterility, and male-fertility (MS, PMS, and MF), but not for pollen size (MS-2:MF-2). This was because the pollen of most of the plants resulting from polyploid crosses varies either in chromosome content and/or pollen grain size-related to male sterility, which could not be separated in these siblings. Therefore model 3 cannot be tested here.

MS and cpDNA haplotyping

The results shown above favoured model 1 or possibly model 3, suggesting that the cytoplasm is involved in male-sterility. This result was strongly supported by cpDNA haplotyping of natural populations. In our two populations ($n = 115$), seven plants were identified that were at least partly MS. Two MS plants (TJX3-20 and TLX3-22) and three PMS producing abortive pollen (TLX3-11, 25, and 109) were found in samples from Beaujeu. In Belvezet, one MS and one PMS plant producing abortive pollen were found (TLX9-41 and 34 respectively). Table 4 lists the results of chloroplast DNA haplotyping in the two male-sterility source populations, together with results obtained from extensive haplotyping in other populations, that were used to describe the geographic distribution of haplotypes (Meirmans *et al*, in prepration). The haplotyping in our current study involved an 'exclusion procedure' aimed at investigating the possible similarity of cytoplasm among male-sterile plants and dissimilarity of cytoplasm between MS and MF plants. All MS and PMS plants belonged to the same haplotype, designated 16b (see Table 4, nomenclature explained in Discussion). One MF plant from Belvezet (TLX9-25) also had haplotype 16b and should consequently contain restorer genes. The remaining MF plants ($n = 107$) all differed from haplotype 16b. Our results indicate that sterility (either MS or PMS with abortive grains) and the rare haplotype 16b are not independent ($P < 0.0001$, by exact permutation test; Zaykin *et al*, 1995). In addition to the diagnostic markers, listed in Table 4, the identity of 16b haplotypes was further confirmed by PCR amplification of the *pbsA–trnH* chloroplast fragment. Upon digestion using *Dra*I, seven MSs and (MF) TLX9-25 were found to have an extra site in the fragment compared to all 87 hermaphrodite individuals from Beaujeu that only have one site. As a final test for identity, the (441 bp) *trnL–trnF* fragment was sequenced. This revealed full sequence identity of five (partial) MSs, TJX3-20, TLX3-109, TLX9-34, and TLX9-41, and TLX3-11 and the MF TLX9-25. The *trnL–trnF* fragment of two remaining PMS plants differed by one or two single nucleotide polymorphism, but this very likely resulted from the fact that only single cloned fragments (including possible sequence errors introduced by the Taq polymerase) were sequenced. TLX3-22 differed at position 130 (T→C) and TLX3-25 differed at positions 44 and 281 (both T→C) from 16b. The putative base substitution (281;T→C) in the *trnL–trnF* fragment of TLX3-25 should create an additional *Ava*I, *Hpa*II, and *Sma*I restriction site compared to16b and could thus be verified by restriction analysis of the amplified fragment using plant DNA. It appeared that all the *trnL–trnF* fragments of (partially) MS plants, including TLX3-25, were only cut by these enzymes at a common restriction site at position 193 (not at position 281). Resequencing (without prior cloning) the *trnL–trnF* fragment of TLX3-22 proved that the fragment is also identical to the 16b type. This supports our explanation that formerly observed nucleotide differences had resulted from PCR artefacts. Thus, we were finally able to conclude that the haplotypes found in all (P)MS plants are fully identical and belong to the 16b haplotype.

Table 4 Summary of chloroplast haplotyping showing that male-sterility is associated with haplotype 16b

	trnL-F *length (bp)*	*Number of ApaII sites in* trnL-F	*Number of* HapII *sites in* PsbB–pbsF	*Number of plants from Beaujeu*		*Number of plants from Belvezet*		*Additional number of plants from Meirmans (unpublished)*
				MS or PMS	*MF*	*MS or PMS*	*MF*	
Haplotype 16b	441	2	0	5		2	1	
	441	2	1				7	
	441	1	nt					2 (1/1)
	364	1	1				8	
Haplotype 'non-16b'	364	1	0				6	
	364	1	nt		87 (48/22)			268 (231/54)
	364	0	nt					11 (9/0)

MS, PMS, and MF refer to male-sterility, partial male-sterility and male-fertility, respectively. *trnL-F* refers to a PCR-amplified chloroplast region either 441 or 364 base pairs in size digested with *Apa*II to detect 0, 1, or 2 restriction sites. *PbsB–psbF* is a PCR-amplified chloroplast region digested with *Hap*II to detect 0 or 1 restriction sites. 'nt' means: not tested. Frequencies in the table refer to number of individuals found for a particular haplotype. Some haplotypes were found among both diploid and triploid individuals. In these cases, the minimal number of genotypes can be inferred from the numbers shown in parentheses; # diploids/# distinct AFLP-fingerprinted triploids. For example: 87(48/22) refers to 87 individuals of which 48 were diploid, whereas the rest were triploid of which at least 22 had different AFLP fingerpint types (RvhH unpublished results).

Spontaneous seed set

Apomixis, here defined as spontaneous seed set, was exclusively associated with polyploidy, but polyploidy itself was not strictly associated with apomixis. In the cross with TJX4-79 ($F_{1''D''}$), all polyploids were capable of spontaneous seed set and all plants tested (nine plants) set seed after emasculation, but in the cross with TLX11-38 ($F_{1''E''}$) all but one of the nine polyploids had spontaneous seed set. For siblings obtained using parent TLX11-02 ($F_{1''D''}$), all polyploids (triploid or higher) except two set seed spontaneously. The $F_{1''F''}$ family also segregated polyploid apomicts and sexual diploids and is still being investigated in more detail and for more individuals (P Van Dijk *et al*, in prepration). In all crosses all diploids failed to set seed spontaneously.

Discussion

The first aim of this study was to reveal the mode of inheritance of male sterility in *Taraxacum* by interpreting the segregation patterns in the progenies derived from a diploid MS tester crossed to diploid and triploid pollen donors. Crossing studies revealed genetic variation for fertility restoration among pollen donors resulting in different segregation ratios. Phenotyping male-sterility/fertility was performed in two different ways and each was explained by a different model. The absence/presence of all types of pollen grains (MS:PMS:MF) could be explained as the result of a cytoplasmic factor interacting with independent dominant restorer genes (our model 1). The absence/presence of normal-sized pollen, could be explained as the result of the combined (epistatic) action of two dominant restorer genes Rf_1 and Rf_2 (our model 3). Model 2 (Lewis and Crowe, 1956) failed to explain all of the data.

For either model (1 and 3) for fertility restoration, examples are found in the literature. Cytoplasmic male-sterility with independent as well as combined action of restorer genes has been found in *Plantago coronopus* by Koelewijn and Van Damme (1995). Combined action of dominant restorers explains the results found in maize (Laughnan and Gabay-Laughnan, 1983) and several other species (see Koelewijn and Van Damme, 1995 for references). For our data, it appeared difficult to fit the results of both phenotyping approaches into a single genetic model. In some families, the small-sized phenotype (MS-2) and PMS appeared highly correlated but in other families, grown at different temperatures, the two phenotypes behaved differently (compare Tables 1 and 2). In order to obtain a comprehensive genetic model, plants should be cloned and grown in different climates with several replicates per treatment followed by detailed phenotyping using multiple classes of phenotypes and marker (QTL) analysis (see eg, Miedaner *et al*, 2000). QTL analysis has been successful in unravelling the number of restorers in many nuclear–cytoplasmic male sterility systems (eg Yao *et al*, 1997; Taan *et al*, 1998; He *et al*, 1999; Miedaner *et al*, 2000) but this was beyond the scope of this study.

Although models 1 and 3 differ in one respect (independent versus epistatic interaction), they were similar in that both assume that fertility restoration is dominant and that the cytoplasm is involved. The assumption of dominance fits with the literature where

both recessive and dominant action of restorer have been reported for gynodioecious (Van Damme, 1983, Koelewijn and Van Damme, 1995) as well as for crop systems (Saumitou-Laprade *et al*, 1994; Vedel *et al*, 1994; Schnable and Wise, 1998).

The evidence of a MS S cytoplasm from our crossing studies encouraged us to test for the presence of S cytoplasm by screening natural populations, the second important aspect of this study. We confirmed that a cpDNA haplotype, 16b, is associated with male-sterility, and thus represents S cytoplasm. All seven (P)MS plants have the same haplotype, 16b, that is found in only one of 109 co-occurring MF plants. The one exception is an apparently restored individual with S cytoplasm.

Wittzell (1999) has grouped 46 identified *trnL–trnF* sequence haplotypes into 20 groups on the basis of a 31 bp deletion, the number of pseudo-gene copies and two informative sites, at positions 138 and 148. In this group-system, the male-sterility-associated haplotype would be a member of 'group 16'. In accordance with the system of Wittzell (1999), the P(MS)-associated haplotype is designated as '16b'. It would have been interesting to exploit the available chloroplast sequence data to estimate the age of our CMS haplotype 16b, but unfortunately it is impossible to construct an unambiguous phylogeny, as indicated by Wittzell (1999).

The haplotype 16b is extremely rare. It was not found in an additional set of 281 MF plants obtained from other European dandelion populations (Table 4; Meirmans *et al*, in prepration), nor in the 237 dandelion microspecies haplotyped by Wittzell (1999). The rarity of 16b and its local appearance suggest a recent origin of this type, and that 16b has not had the time to spread since it emerged.

Cytoplasmic male-sterility was first postulated to occur in *Taraxacum* by Malecka (1971a, b). She suggested that the typical pattern of tapetal layer degeneration is characteristic for cytoplasmic male-sterility because it has been reported to occur in many other species with this sexual system (Malecka 1971a, b and references therein). Remarkably, premature abortion (apoptosis) of tapetal cells indeed is taking place in our MS plants as was determined by Nomarski microscopy (P Van Baarlen, unpublished results), which differs from the normal development described by Van Baarlen *et al* (2000). The MS plants Malecka (1971a, b) described were polyploid *T. balticum* (haplotype 11a: Wittzell, 1999), and *T. sileciacum* ('non-16b', T Mes, personal communication), which both have cpDNA differing from the 16b type. So if Malecka (1971a, b) indeed found cytoplasmic male sterility, although no genetic data were provided to support this, it must have a different origin compared to haplotype 16b.

It has been speculated that cytoplasmic male-sterility may sometimes account for patterns of differential cytoplasmic versus nuclear introgression (reviewed in Rieseberg and Wendel, 1993). Wittzell (1999) found that so called 'advanced sections' of *Taraxacum* exhibit several cpDNA lineages and hence are probably the result of repeated hybridization. Our detection of cpDNA 16b, associated with male-sterility in both *Ruderalia* species and *T. rubicundum* ssp. *monspeliense* (*Erythrosperma*), suggest that CMS may facilitate hybridization between *Taraxacum* sections.

Evolutionary considerations

As already stated in the Introduction, male-sterility and particularly its association with apomixis is an interesting phenomenon from an evolutionary point of view. Apomicts that have strict female reproduction may benefit from the lack of a male function. By not producing pollen, dandelion apomicts may allocate resources to further increase female fitness (Maynard-Smith, 1979). In several populations, apomicts and sexual diploids co-occur (see Menken *et al*, 1995) suggesting that the two are in some sort of (dynamic) equilibrium. In extreme scenarios, the increase of female fitness of apomicts by the presence of cytoplasmic male sterility may cause them to outcompete sexuals and perhaps to dominate these populations absolutely. From crosses between our diploid tester and apomicts, MS apomicts could be obtained, which indicates that apomicts indeed can acquire cytoplasmic male-sterility. Male-sterility does occur in apomicts (Fünfkranz 1960, Dudman and Richards 1997), but their genetic status is unknown.

Also MF apomicts were obtained from crosses, implying the presence of restorer genes in the parents. This is remarkable because two apomicts used for crossing were collected near Bremen: in this area no diploid sexuals are present (see Roetman *et al*, 1988; Menken *et al*, 1995), nor are there any reports of 16b haplotypes, and it is far away from the two 16b-CMS source populations in Southern France. The presence of restorer genes outside 16b-source populations either suggests that 16b haplotype/S cytoplasm may have had a wider (or has an unnoticed) distribution resulting in positive selection for restorer genes. Another possibility is that fertility restoration occurs as a pleiotropic effect of genes that naturally occur in dandelion populations. Whatever the explanation is, the presence of restorer genes in the gene pool of dandelions will complicate the evolutionary dynamics of this sexual system (Gouyon *et al*, 1991). In particular, restorer genes will limit the possibility of apomicts to acquire male sterility and outcompete sexuals. Our crossing results indicate that restorer genes are dominant and because apomicts are polyploid, a low frequency of restorers may be sufficient to restore fertility in apomicts, which will reduce their female advantage. Ironically, the fact that the genus *Taraxacum* has so many breeding systems (self-incompatibility, breakdown of self-incompatibility by triploid pollination, apomixis, strict selfing), to which nuclear–cytoplasmic male-sterility can now be added, making it both fascinating and complicated to study their joint effects on the evolutionary dynamics of *Taraxacum*.

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