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Autopolyploidy in *Dactylis glomerata* L.: further evidence from studies of chloroplast DNA variation

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Summary. Chloroplast DNA variation has been used to examine some of the maternal lineages involved in the evolution of the intraspecific polyploid complex, *Dactylis glomerata* L. Diploid (2x) and tetraploid (4x) individuals were collected from natural populations of the subspecies *glomerata* (4x), *marina* (4x) and *lusitanica* (2x), as well as from sympatric 2x/4x populations of the Galician type. Digestion of their ctDNA with 11 restriction endonucleases revealed enough variation to characterise three ctDNA variants, designated MBMK, MBmK and mBMK. The distribution of these ctDNA variants reflects different stages in their spread among the populations. The MBMK ctDNA variant predominated at both ploidy levels in subspecies *glomerata, lusitanica* and *marina,* and in recent tetraploid Galician/*glomerata* hybrids. The MBmK variant was detected in a single tetraploid individual and probably results from a relatively recent mutation. Fixation of the mBMK minority variant in the diploid and tetraploid Galician populations adds to the evidence concerning the possible origin of the Galician tetraploids. It means that the Galician diploids were maternal ancestors of the tetraploids. This result complements evidence from earlier studies based on morphology or biochemical markers, and reduces the likelihood that the tetraploids arose by hybridisation between an ancient Galician diploid and an alien tetraploid. It is, however, consistent with a true autopolyploid origin of the tetraploids.

Key words: Autopolyploidy – Chloroplast DNA variation – *Dactylis glomerata* L. – Intraspecific evolution

Introduction

As stated by Levin (1983), "the role of polyploidy per se in the development of evolutionary novelty remains one of the outstanding questions in flowering plant evolution". The adaptive significance of polyploidy must be investigated mainly in natural autopolyploids, because induced polyploids have often been shown to differ appreciably from natural ones, especially with respect to their fertility or adaptive versatility (McCollum 1958; Borrill 1978). Unfortunately, natural autopolyploids are difficult to detect because, in most cases, individuals which have different ploidy levels have no obvious distinguishing characters. They are similar in overall size and morphology and differ from one another only in organ or cell size (Clausen et al. 1945; Jackson and Casey 1982; Soltis 1984; Soltis and Rieseberg 1986). Autopolyploidisation can be detected directly by chromosome counting in the progeny of diploids from species where gametic non-reduction occurs naturally (Denijs and Peloquin 1977). But, unfortunately, even if gamete non-reduction occurs more frequently than predicted (Love 1964; Harlan and De Wet 1975; De Wet 1980), this method is difficult and time-consuming, because the probability of polyploid detection is usually extremely low.

Consequently, evidence of autopolyploidy is usually obtained indirectly, but all indirect methods have their disadvantages. For example, autopolyploidy can be detected cytologically by multivalent formation during chromosome pairing (Carroll 1966), but multivalents are not always seen; even true autotetraploids may form only bivalents (e.g. Soltis and Rieseberg 1986). Genetic similarities between autopolyploids and their related diploids can also be detected biochemically using enzyme or flavonoid markers (e.g. Ardouin et al. 1987). However, in studies of some plant polyploid complexes, it has been found difficult to interpret such similarities, because divergence of the marker genes themselves is affected by polyploidy (e.g. Lumaret 1985, 1986; Soltis and Rieseberg 1986; Ardouin et al. 1987). Such problems can constitute major barriers to phylogenetic analysis.

A cytoplasmic genome such as chloroplast DNA (ctDNA), which has a clonal inheritance and a highly conservative mode of evolution (Curtis and Clegg 1984; Palmer 1987) is not expected to be modified by polyploidy. It is, therefore, particularly suitable as a marker for examining phylogenetic relationships in polyploid complexes. Restriction endonuclease analysis of ctDNA has already been invaluable in establishing the origin and specific parentage of many allopolyploid species (Vedel et al. 1981). The maternal parental genome has been identified in several interspecific complexes such as *Brassica* (Palmer et al. 1983; Erickson et al. 1983), *Triticum/Aegilops* (Bowman et al. 1983; Tsunewaki and Ogihara 1983; Terachi et al. 1984), *Nicotiana* (Kung et al. 1982; Salts et al. 1984), *Oryza* (A. Dally and G. Second, personal communication). It was, therefore, decided to use ctDNA variation to examine further the parental lineages involved in the evolution of a natural and well-documented intraspecific polyploid complex, that of *Dactylis glomerata* L.

Dactylis glomerata L. is a perennial and predominantly out-crossing species of grass, and the polyploid complex includes mainly diploid and cytologically autotetraploid subspecies. There are several morphologically distinct diploid subspecies, which are now generally allopatric (Lumaret 1985) but the most common, widely distributed forms of *Dactylis* are three main tetraploid subspecies: *D. glomerata* ssp. *glomerata,* ssp. *marina* and ssp. *hispanica.*

These three tetraploid subspecies combine the morphological, physiological and biochemical characteristics of a number of diploid subspecies (Lumaret 1988). To discover how the tetraploids arose, the phylogenetic relationships between these diploid and tetraploid subspecies have been extensively studied using morphological, cytological and biochemical markers (e.g. Lumaret 1988). The results of such analyses have already indicated autopolyploidy as the origin of a particular tetraploid *D. glomerata* type endemic to Galicia, in northwest Spain (Lumaret and Barrientos 1989).

In this paper, we have examined the ctDNA of individuals from several natural *D. glomerata* populations. Among these were diploid populations of *D. glomerata,* Galician type, that are thought to have contributed to the origin of the Galician tetraploids. The ctDNA variation detected among them has revealed different stages in the spread of the ctDNA variants and has provided decisive evidence of a direct relationship between the Galician diploids and tetraploids.

Materials and methods

Plant material

Analyses were carried out on individual plants from the natural *D. glomerata* populations from Galicia and Portugal, listed in Table 1. Most of the plants belong to the endemic Galician type. Sites 1, 2, 3, 12, and 13 provided morphologically indistinguishable Galician diploid and tetraploid individuals, plus hybrid tetraploids, that have arisen by recent hybridisation at the tetraploid level between the Galician type and ssp. *glomerata* introduced as cultivars in Galicia (Lumaret et al. 1987; Lumaret 1988). Sites 101, 151, 73 and 77 provided individuals from the ssp. *lusitanica* and *marina,* thought to be most closely related (both geographically and genetically) to the Galician type (Lumaret 1988).

Table 1. Origin and collection sites from which the 47 *Dactylis* plants from several diploid (2x) and tetraploid (4x) subspecies and hybrids were analysed. Site numbers refer to previous mapping (Lumaret 1986; Lumaret et al. 1987)

Subspecies	Origin	Collection site	Ploidy	Sampled plants
1. *lusitanica*	Central Portugal	101	2x	2
2. *lusitanica*	Central Portugal	151	2x	4
3. *marina*	Coastal Portugal	73	4x	3
4. *marina*	Coastal Portugal	77	4x	2
5. *marina*	Coastal Galicia	–	4x	2
6. *glomerata*	Cambridge (GB)	–	4x	2
7. *glomerata*	Galicia (Spain)	1	4x	4
8. *glomerata*	Galicia (Spain)	3	4x	2
9. *Galician*	Galicia	3	2x	4
10. *Galician*	Galicia	12	2x	2
11. *Galician*	Galicia	13	2x	4
12. *Galician*	Galicia	2	4x	3
13. *Galician*	Galicia	3	4x	2
14. *Galician*	Galicia	1	4x	2
15. hybrid (a) *Gal/glo*	Galicia	1	4x	5
16. hybrid (a) *Gal/glo*	Galicia	3	4x	4

(a) The frequency of hybridization at each Galician site was estimated previously using morphological (e.g. Lumaret et al. 1987) and enzyme markers (Barrientos 1985)

Preparation and restriction endonuclease analysis of ctDNA

Either ramets or, in a few cases, seeds from identified mother plants were collected in the natural sites and grown in a greenhouse in uniform conditions. The progeny of each mother plant were grown separately for 2 months before analysis. Adult plants or seedlings were placed in the dark for 36 h to destarch the leaves before the leaf tissue was harvested. The leaves were then ground in liquid nitrogen and freeze-dried.

Chloroplasts were isolated from samples of the freeze-dried leaf powder (0.5 g from 4x and 1.0 g from 2x individuals) using a nonaqueous procedure modified as described by Bowman et al. (1983), and total nucleic acid was extracted from the chloroplast pellets.

Aliquots of 20 µg total chloroplast nucleic acid were incubated for 1 h at 37 °C with the following restriction endonucleases: EcoRI, HindIII, BamHI, DraI, ClaI, KpnI, SalI, XhoI, PstI or BglII in the presence of pancreatic A ribonuclease. Incubation was 1 h at 30 °C for SmaI. The digestion products were fractionated by electrophoresis on horizontal 0.85% agarose slab gels, as previously described (Bowman and Dyer 1982).

Results

The nature of the Dactylis glomerata ctDNA variation

When ctDNA from the 47 individuals listed in Table 1 was analysed by digestion with the 11 different restriction endonucleases, 13 different banding patterns were observed. These are illustrated in Fig. 1 a and b. Fragment patterns produced by nine of the enzymes revealed no useful ctDNA variation. However, digestion with BamHI or KpnI did reveal restriction fragment length polymorphism that could be used to identify three different ctDNA variants among the 47 individuals.

The majority BamHI pattern, MB (found in 31 individuals) is characterised by a 4.5 kbp fragment (Fig. 1, lane 3) which, in the minority pattern, mB (Fig. 1, lane 4), is replaced by a 4.3 kbp fragment. Similarly, the majority KpnI pattern, MK (found in 46 individuals), is characterised by a small Kpn fragment (Fig. 1, lane 8) which, in the minority KpnI pattern, mK (Fig. 1, lane 9), is replaced by a larger Kpn fragment. Both these length mutations could have been caused either by deletion/insertion or by point mutation at a restriction site.

The BamHI, HindIII, EcoRI and SalI patterns shown in Fig. 1 for fragments with sizes ranging from 1.4 to 23.1 kbp, are comparable to those which had been obtained previously by Lehvaslaiho et al. (1987) for *Dactylis,* using the same restriction enzymes. However, in the SalI pattern, only one large fragment (about 23 kbp) is described by these workers, whereas two distinct large fragments of about 23 and 22 kbp, respectively, were observed from each individual plant in our study. The fractionation conditions are too different to tell whether such a difference represents ctDNA variation or artifact.

The three intraspecific *Dactylis glomerata* ctDNA variants detected among the individuals in Table 1 were identified by their different combinations of the majority (M) or minority (m) DNA fragments in the BamHI and KpnI digests, and are named, accordingly, MBMK, MBmK and mBMK (Table 2).

The distribution of the Dactylis glomerata ctDNA variation

The distribution of the three different ctDNA variants (MBMK, MBmK and mBMK), among the sampled *Dactylis* subspecies, is shown in Table 2. No variation was found between ctDNA from individuals of the subspecies *lusitanica, marina* and *glomerata* (even those growing in Galicia); all had the MBMK ctDNA variant. As this variant was also observed in two other diploid subspecies, namely *himalayensis* (from India) and *sinensis* (from China) (R. Lumaret, unpublished results), it probably predominates in the species.

The MBMK ctDNA variant was also found in nearly all the hybrid Galician/*glomerata* tetraploids, and also in

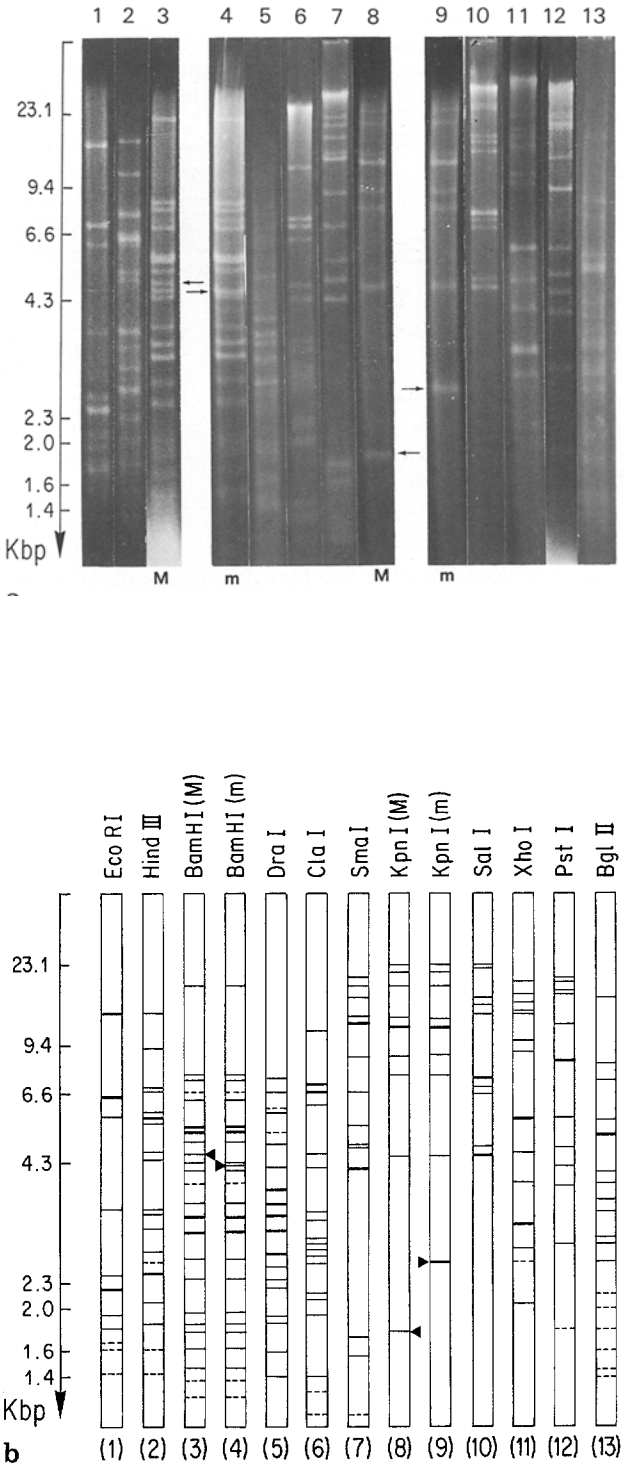

Fig. 1 a and b. Restriction fragment patterns obtained by digestion of *Dactylis glomerata* ctDNA with *1* – EcoRI; *2* – HindIII; *3 and 4* – BamHI; *5* – DraI; *6* – ClaI; *7* – SmaI; *8 and 9* – KpnI; *10* – SalI; *11* – XhoI; *12* – PstI; or *13* – BgIII. The minority fragment patterns for the BamHI and KpnI digests are shown in *lanes 4 and 9* respectively. **a** Digests fractionated by agarose gel electrophoresis and stained with ethidium bromide. **b** Diagrammatic representation of the restriction fragment patterns shown in **a**

Table 2. Distribution of ctDNA variants in plants from the several *Dactylis glomerata* subspecies and in hybrids. M and m correspond to the majority and minority patterns, respectively, B and K refer to the BamHI and KpnI endonucleases, respectively

Subspecies or type	Ploidy	ctDNA variant		
		MBMK	MBmK	mBMK
lusitanica	2x	6	–	–
marina	4x	7	–	–
glomerata	4x	8	–	–
Galician	2x	–	–	10
Galician (site 2)	4x	–	–	3
Galician (site 3)	4x	–	–	2
Galician (site 1)	4x	1	–	1
hybrid *Gal/glo* (site 1)	4x	4	1	–
hybrid *Gal/glo* (site 2)	4x	4	–	–

one tetraploid showing the Galician morphotype sampled from site no. 1. The single exception among the hybrid Galician/*glomerata* tetraploids was an individual from site no. 1 with an MBmK ctDNA variant. This was the only individual found to have the MBmK variant (with the minority KpnI fragment).

The mBMK variant (with the minority BamHI fragment) was restricted to the diploids and tetraploids belonging morphologically to the Galician type.

No individuals were found with an mBmK ctDNA variant. Chloroplast DNA was also analysed in several individual progeny from a reciprocal cross between two Galician tetraploids which differed for their ctDNA BamHI restriction fragment pattern. Allozyme markers were used to check the identification of progeny obtained from selfing and/or by cross-fertilization. The maternal ctDNA was always recovered in the progeny from cross-fertilization (results not shown), indicating that there is essentially maternal inheritance of ctDNA in *Dactylis*.

Discussion

The level and spread of Dactylis glomerata ctDNA variation

Restriction endonuclease analysis revealed a low level of ctDNA variation between the *D. glomerata* subspecies, particularly in comparison with the amount of allozyme variation that has been found in the same plant material (Lumaret 1985; Ardouin et al. 1987; Lumaret 1988). Similarly, low levels of intraspecific ctDNA variation have been found in several other plant species (e.g. Palmer and Zamir 1982; Clegg et al. 1984a, b; Banks and Birky 1985).

There was, however, enough variation to characterise the three distinct *D. glomerata* ctDNA variants, MBMK, MBmK and mBMK, whose distribution is represented in Table 2. The MBMK variant predominates, occurring in most subspecies at the diploid and polyploid level. However, at one site (no. 1), this majority variant coexists with the two minority variants in the same tetraploid population. Moreover, one of these minority variants, mBMK, has become fixed in the endemic Galician type, perhaps because of genetic drift. This distribution of the *D. glomerata* ctDNA variants presumably reflects different stages in their spread. The number and distribution of these *D. glomerata* ctDNA variants are extremely similar to data reported in a study of ctDNA diversity in 100 individuals from 20 natural populations of *Lupinus texensis* (Banks and Birky 1985). Four *L. texensis* ctDNA variants were detected. One variant predominated, two were found in single individuals from different populations and the fourth was fixed in one of the populations. Therefore, although there have been few studies of this kind, these data may well represent a typical spread of ctDNA variants in natural, higher plant populations.

The phylogenetic relationship between diploids and tetraploids native from central Galicia

The Galician diploid and tetraploid plants are morphologically indistinguishable except for cell size, and grow sympatrically, although they differ in habitat preference (Lumaret et al. 1987). The diploids, which are mainly confined to the forest-floor habitat in woodlands of mostly ancient origin (remains of the Galician primitive forest), are considered to be more ancient than the tetraploids, which become predominant in open disturbed areas and are clearly favoured by human activity (Lumaret et al. 1987). The occurrence of flavonoid compounds, being considered as "more primitive" in the Galician diploids and "more evolved" in the Galician tetraploids, supports the more ancient origin of the diploids (Ardouin et al. 1987).

Fixation of the *D. glomerata* mBMK ctDNA variant in these native diploid and tetraploid populations implies direct and very close phylogenetic relationships between them. As *Dactylis* ctDNA is maternally inherited (results not shown), these data indicate that the tetraploids derived their cytoplasmic organelle genomes, and at least one nuclear genome complement, from the Galician diploids.

The phylogenetic relationships between these diploid and tetraploid populations have already been studied extensively using morphological, cytological and biochemical markers, particularly allozyme and flavonoid analyses (e.g. Ardouin et al. 1987; Lumaret and Barrientos 1989). Despite all these studies, the origin of the Galician tetraploids is still unclear. They could have arisen from the diploids either by autopolyploidisation or perhaps by hybridisation. That hybridisation could have been with another diploid subspecies (when the

diploids were more widespread, Stebbins and Zohary 1959) or with a distinct tetraploid subspecies. The ctDNA analyses described in this paper complement these earlier studies and add to the evidence concerning the possible origin of the Galician tetraploids.

To be considered first is the possibility that the Galician tetraploids did have a hybrid origin. If so, their inheritance of the diploid (mBMK) ctDNA means that a Galician diploid must have been the maternal parent in the ancestral cross. The possibility of hybridisation between two diploid subspecies was investigated by including, in the analysis, ctDNA from several accessions of the subspecies *lusitanica* (Table 1). This is the closest relative of the Galician diploids thought to have been involved in such a cross (Ardouin et al. 1987; Lumaret 1988). The ctDNA analysis eliminates *D. g. lusitanica* as a possible maternal ancestor, but cannot eliminate it as an ancestral pollen parent. However, the limited morphological, biochemical and physiological similarity of ssp. *lusitanica* to the Galician tetraploids (Lumaret 1988) does argue against ssp. *lusitanica* as a pollen parent.

The maternal inheritance of the diploid ctDNA by the Galician tetraploids has other important implications. It reduces the probability that the tetraploids arose through ancestral hybridisation between diploid and tetraploid. It has already been shown that, due to differences in habitat preference and flowering period, direct hybridisation between diploids and tetraploids is rare in natural *Dactylis* populations (e.g. Zohary and Nur 1959; Borrill and Lindner 1971; Lumaret 1988). In addition, diploid/tetraploid hybridisations that do occur are known to be considerably less successful when the diploid is the seed parent (Borrill 1978; Lumaret 1988). Our knowledge that a Galician diploid was the maternal parent of the tetraploids, therefore, reduces the probability that the tetraploids arose through a successful ancestral diploid/tetraploid hybridisation, with the diploid as seed parent. It must be added that because of the geographical and environmental isolation of Galicia in the past, the likelihood of extensive contact between an ancient Galician diploid and an alien tetraploid is also thought to have been remote.

A third possibility is that the tetraploids arose by autopolyploidisation of the diploids, i.e., by intrasubspecific autopolyploidy. The observed inheritance of the diploid mBMK ctDNA by the tetraploids is completely consistent with an autopolyploid origin, but it clearly cannot eliminate the possibility that the second nuclear genome complement was derived from a different diploid subspecies by hybridisation. Autopolyploidisation is, however, considered to be the most probable origin of the tetraploids. Very recent studies have revealed that, in *Dactylis,* a small number of diploids, including plants from Galicia, show substantial gametic non-reduction in pollen, in ovules or in both (Huguessen 1986; Casler and Huguessen 1988; Lumaret 1988). Tetraploid plants can be produced from crosses between diploids growing at the same site, either directly from two individuals or (more frequently) via triploid formation.

Hybridisation and ctDNA flow between tetraploid subspecies

As a consequence of the recent introduction of commercial tetraploid *D. glomerata* cultivars into Galicia, hybridisations have occurred between the two tetraploid entities, Galician and *glomerata.* The resulting hybrid Galician/*glomerata* tetraploids are characterised by a morphotype intermediate between the Galician and *glomerata* morphotypes (Lumaret et al. 1987; Lumaret 1988) and by their isozyme patterns (Barrientos 1985; Lumaret and Barrientos 1989).

Chloroplast DNA was analysed from several hybrid Galician/*glomerata* tetraploid individuals from two Galician sites, sites 1 and 3 (Table 2). The ctDNA variant MBMK was observed in all the tetraploid hybrids that were studied, with the exception of one individual (Table 2). This result was unexpected, as crosses ought to occur both ways between these tetraploid subspecies, as they flower at the same time (Lumaret et al. 1987). However, examination of environmental conditions in which numerous hybridisations between tetraploid plants occur in Galicia provides a plausible explanation for such a bias. It has been shown that the *"glomerata"* tetraploids that become established in pastures, and are mixed with the native tetraploids, possess tougher leaves than native Galician plants. The *glomerata* tetraploids are grazed less so that, although they are less numerous, they make more effective seed parents. The native plants, which are more numerous, are mainly pollen producers (Lumaret 1989). Consequently, gene flow is found to be more important from the *"glomerata"* to the "Galician" types when measured with a cytoplasmic marker such as ctDNA, and to be predominantly in the reverse direction when mesured using nuclear markers such as enzyme polymorphism (Lumaret 1988, 1989).

The exceptional individual was the only hybrid tetraploid to have the MBmK ctDNA variant rather than the MBMK variant (Table 2). In fact, it was the only one of the 47 individuals studied to have the MBmK variant. The apparent low frequency of this variant and the fact that it occurs in the tetraploids means that the KpnI fragment length mutation could be relatively recent.

Lastly, there was a single tetraploid individual which, despite having the Galician tetraploid morphotype, showed the MBMK ctDNA variant, typical of the hybrid tetraploids (Table 2). This was collected at site no. 1, where the rate of hybridisation between tetraploids is known to be particularly high (Lumaret and Barrientos 1989). Evidence that this plant contains a few *"glome-*

rata" alleles (Barrientos 1985) implies that, genotypically, it arose as a hybrid Galician/*glomerata* tetraploid, and that the Galician morphotype has been conferred by successive hybridisation and backcrossing with the native Galician type.

In summary, there was enough ctDNA variation among the populations in this study to reveal some of the maternal lineages in the evolution of subspecies belonging to the *D. glomerata* complex. Evidence that the Galician diploids were maternal ancestors of the Galician tetraploids complements evidence from earlier studies of these populations and gives further insight into the possible origin of the tetraploids. It reduces the probability that the tetraploids arose by hybridisation between an ancient diploid and an alien tetraploid. It is, however, consistent with the idea that they arose through autopolyploidisation of the Galician diploids. Chloroplast DNA analysis of recent hybrids between the Galician and *glomerata* entities has also given some insight into the current evolution of the complex. It appears that, while the hybrids usually inherit their ctDNA from ssp. *glomerata* and initially have an intermediate morphotype, subsequent hybridisation and backcrossing can confer the "Galician" morphotype.

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