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On the hybrid origin of *Dactylorhiza brennensis* and implications for the taxonomy of allotetraploid *Dactylorhiza*

Keywords
Orchidaceae; *Dactylorhiza brennensis, Dactylorhiza elata* subsp. *brennensis, Dactylorhiza majalis* subsp. *brennensis, Dactylorhiza majalis* subsp. *majalis, Dactylorhiza majalis* subsp. *integrata, Dactylorhiza majalis* subsp. *sesquipedalis*, hybridization, introgression, simple sequence repeats, plastid DNA haplotypes, species concept, systematics, taxonomy.

Summary
Hedrén, M. & D. Tyteca (2020): On the hybrid origin of *Dactylorhiza brennensis* and implications for the taxonomy of allotetraploid *Dactylorhiza*.- J. Eur. Orch. 52 (1): 33-64.
Dactylorhiza brennensis is an allotetraploid member of the *Dactylorhiza majalis* s.lat. complex, which comprises orchids that combine two genomes from the *D. incarnata* s.lat. lineage and two genomes from the *D. maculata* s.lat. lineage (incl. *D. maculata* subsp. *fuchsii*). The distribution of *D. brennensis* is restricted to central France. It was originally described as a subspecies of *D. elata*, which is distributed further to the south, but it has also been indicated that it resembles the northern *D. majalis* subsp. *integrata* (syn. *D. praetermissa*) and may be related to this taxon as well. On basis of detailed morphometric analysis, it has also been argued that it merits recognition as a separate species.

Here, we studied *Dactylorhiza brennensis* for variation at nuclear and plastid molecular markers and made a comparison with potentially related taxa. We show that *D. brennensis* combines genes from *D. majalis* subsp. *sesquipedalis* (syn. *D. elata* subsp. *sesquipedalis*) and *D. majalis* subsp. *integrata*, and that it originated by hybridization between already established allotetraploids, rather than by a separate allopolyploidization event from the parental *D. maculata* and *D. incarnata* lineages. We also show that *D. brennensis* integrates the

plastid genomes of both parental allotetraploids, which indicates that it has evolved by merger of populations, rather than by hybridization between single individuals. However, the hypothesis that it has hybridized with local population of *D. maculata* subsp. *fuchsii* could not be substantiated. Since *D. brennensis* and its parents belong to the same species according to the biological species concept, we provide the combination *D. majalis* subsp. *brennensis* (E.Nelson) D.Tyteca & Hedrén for those who use this concept as basis for taxonomic classification.

Zusammenfassung

Hedrén, M. & D. Tyteca (2020): Über die hybridogene Entstehung von *Dactylorhiza brennensis* und Implikationen für die Taxonomie allotetraploider *Dactylorhiza*.- J. Eur. Orch. 52 (1): 33-64.

Dactylorhiza brennensis ist eine allotetraploide Sippe des Komplexes von *Dactylorhiza majalis* s.lat. Bei den Orchideen dieses Komplexes liegen jeweils zwei Genome der Linie von *D. incarnata* s.lat. und zwei Genome der Linie von *D. maculata* s.lat. (inkl. *D. maculata* subsp. *fuchsii*) kombiniert vor. Das Verbreitungsareal von *D. brennensis* ist auf Zentral-Frankreich beschränkt. Die Art wurde ursprünglich als Unterart von *D. elata* beschrieben, deren Verbreitungsgebiet sich weiter südlich befindet. Dabei wurde auf gewisse Ähnlichkeiten mit und mögliche verwandtschaftliche Beziehungen zu der nördlich angrenzenden *D. majalis* subsp. *integrata* (syn. *D. praetermissa*) hingewiesen. Nach detaillierten morphometrischen Analysen wurde auch die Einstufung als eigene Art für gerechtfertigt gehalten.

In der vorliegenden Arbeit untersuchten wir *Dactylorhiza brennensis* hinsichtlich der Variation nuklearer und plastider molekularer Marker im Vergleich zu potentiell verwandten Taxa. Wir können zeigen, dass in *D. brennensis* Gene von *D. majalis* subsp. *sesquipedalis* (syn. *D. elata* subsp. *sesquipedalis*) und *D. majalis* subsp. *integrata* kombiniert sind und die Art durch Hybridisierung zwischen bereits bestehenden allotetraploiden Arten entstanden ist, weniger durch einen separaten Allopolyploidisierungsvorgang der Elternlinien von *D. maculata* and *D. incarnata*. Weiter zeigen wir, dass *D. brennensis* das Plastid-Genom beider allotetraploiden Eltern integriert und sich demnach durch eine Vermischung von Populationen, weniger durch Hybridisierung einzelner Individuen entwickelt hat. Demgegenüber kann die Hypothese einer Hybridisierung mit einer lokalen Population von *D. maculata* subsp. *fuchsii* nicht bestätigt werden. Demnach gehören *D. brennensis* und ihre Eltern entsprechend dem biologischen Artkonzept derselben Art an. Daher wird hier für die Anwender dieses Konzeptes zur taxonomischen Klassifizierung die dafür notwendige Kombination und Umstufung zu *D. majalis* subsp. *brennensis* (E.Nelson) D.Tyteca & Hedrén vorgenommen.

Résumé

Hedrén, M. & D. Tyteca (2020): Sur l'origine hybride de *Dactylorhiza brennensis* et implications pour la taxonomie des *Dactylorhiza* allotétraploïdes.- J. Eur. Orch. 52 (1): 33-64.

Dactylorhiza brennensis est un membre allotétraploïde du complexe de *Dactylorhiza majalis* s.lat., qui comprend des espèces combinant deux génomes de la lignée de *D. incarnata* s.lat. et deux génomes de la lignée de *D. maculata* s.lat. (qui inclut *D. maculata* subsp. *fuchsii*). La répartition de *D. brennensis* est limitée au Centre de la France. Au départ il a été décrit comme sous-espèce de *D. elata*, dont la répartition s'étend plus au sud, mais il présente aussi des ressemblances avec *D. majalis* subsp. *integrata* (syn. *D. praetermissa*), dont la répartition s'étend plus au nord, et peut être lié à ce taxon également. Sur base d'analyses morphométriques approfondies, il a aussi été suggéré que *D. brennensis* mérite d'être reconnue comme espèce distincte.

Dans cette recherche, nous avons étudié la variation de marqueurs moléculaires nucléaires et plastidiques de *Dactylorhiza brennensis*, en vue d'une comparaison avec les taxons potentiellement liés. Nous montrons que *D. brennensis* combine des gènes de *D. majalis* subsp. *sesquipedalis* (syn. *D. elata* subsp. *sesquipedalis*) et de *D. majalis* subsp. *integrata*, et qu'il trouve son origine dans une hybridation entre allotétraploïdes déjà établis, plutôt que dans un évènement distinct d'allopolyploïdisation à partir des lignées parentales de *D. maculata* et de *D. incarnata*. Nous montrons également que *D. brennensis* intègre les génomes plastidiques des deux allotétraploïdes parentaux, ce qui indique qu'il a évolué à partir d'une fusion entre populations, plutôt que par hybridation entre individus isolés. Par contre, l'hypothèse selon laquelle il se serait hybridé avec la population locale de *D. maculata* subsp. *fuchsii* n'a pas pu être corroborée. Les parents de *D. brennensis* sont souvent reconnus comme espèces distinctes dans la littérature récente, mais le constat qu'ils aient pu produire un intermédiaire totalement fertile et variable génétiquement, avec sa propre aire de répartition, montre que les parents font partie de la même espèce, si on se réfère au concept biologique de l'espèce. Pour cette raison, *D. brennensis* et ses parents devraient plutôt être reconnus comme sous-espèces de la même grande espèce, *D. majalis* s.lat. Pour se conformer à cette proposition, il est nécessaire de proposer la combinaison nouvelle *D. majalis* subsp. *brennensis* (E.Nelson) D.Tyteca & Hedrén.

* * *

1. Introduction

Dactylorhiza elata subsp. *brennensis* E.Nelson was described by NELSON (1976: 50) to accommodate populations of *Dactylorhiza elata* (Poir.) Soó from the northern margin of its distribution in the department of Indre in northwestern central France. It comprises fairly slender plants with narrow, erect, unspotted leaves and an inflorescence with a moderate number of large flowers, and it differs from more southern populations of *D. elata* in lower growth, a shorter inflorescence with flowers that are comparatively brighter than those of *D. elata* TYTECA & GATHOYE (1988a). The flowers have a shallowly lobed lip with a pattern of dots and dashed lines, and a large conical-cylindrical spur. *D. elata* subsp. *brennensis* grows in flat marshes on alkaline soils dominated by, e.g., reed, *Phragmites australis* and the sedges *Cladium mariscus* and *Schoenus nigricans*.

Dactylorhiza elata subsp. *brennensis* is a tetraploid with 80 chromosomes (GATHOYE & TYTECA 1989), and is a member of the *D. majalis* (Rchb.) P.F.Hunt & Summerh. s.lat. polyploid complex (HEDRÉN 1996, DEVOS et al. 2006, HEDRÉN et al. 2012). This complex includes a series of taxa formed by hybridization between members of the broadly defined *D. maculata* s.lat. (incl. *D. maculata* subsp. *fuchsii*) and *D. incarnata* s.lat. lineages, and subsequent stabilization by allotetraploidy (presence of two separate parental genomes in the same plant).

Dactylorhiza elata in the broad sense has a wide distribution in southwest Europe and northwest Africa (NELSON 1976). The taxon comprises tall and robust plants with relatively narrow, unspotted (seldom spotted) leaves and elongate, many-flowered, lax to dense inflorescences. The flowers are pink to dark violet, large with a shallowly three-lobed, elongate lip and a prominent conical-cylindrical spur. *D. elata* grows in wet fens and meadows, often in seepage areas dominated by the sedge *Schoenus nigricans*. European and African populations are molecularly differentiated (PILLON et al. 2007) and the species has been subdivided into several taxa (NELSON 1976, DELFORGE 2001). European populations have been separated among others as *D. elata* subsp. *sesquipedalis* (Willd.) Soó and *D. elata* var. *ambigua* (Martrin-Donos) Soó. In the continuation, for reasons described further down, *D. elata* subsp. *sesquipedalis* (Willd.) Soó will be treated as *D. majalis* subsp. *sesquipedalis* (Willd.) H.A.Pedersen & Hedrén (HEDRÉN et al. 2012).

NELSON (1976: 49) also compared *Dactylorhiza elata* subsp. *brennensis* with the northwestern European *D. majalis* subsp. *integrata* (syn. *D. praetermissa*), which is similar to subsp. *brennensis* in the elongate, often unspotted

(in var. *integrata*) leaves, the fairly tall growth, and the pinkish flowers with a trilobed lip, the lobes being rounded, whole, and with an often indistict lip pattern of small dots (var. *integrata*). From the discussion given by Nelson, it appeared that he considered subsp. *brennensis* as potentially intermediate between *D. majalis* subsp. *sequipedalis* and subsp. *integrata*, Similar arguments also appeared in TYTECA & GATHOYE (1988a, b), who however, found the taxon to be morphologically homogeneous and distinct enough to be recognized as a separate species, *D. brennensis* (E.Nelson) D.Tyteca & Gathoye. It must be mentionned that the distribution areas of *D. majalis* subsp. *sesquipedalis* and subsp. *integrata* are slightly overlapping in northwestern France (TYTECA & GATHOYE 1999:160, Fig. 1), not far from the region where *D. elata* subsp. *brennensis* is found.

On basis of ITS and ETS sequence data from ribosomal DNA arrays, DEVOS et al. (2006), found that *D. brennensis* should be an allotetraploid with origins from the *D. maculata* s.str. and *D. incarnata* parental lineages. In addition, detailed analysis of cloned sequences also revealed events of recombination, suggesting that *D. brennensis* had been introgressed with *D. maculata* subsp. *fuchsii* (as *D. fuchsii*), which is also found within the distribution of *D. brennensis*. More precisely, they found that *D. brennensis* contains chimeric ITS sequence variants combining portions typical of *D. majalis* subsp. *sesquipedalis* (originally inherited from *D. maculata* subsp. *maculata* lineage) with portions typical of those of *D. maculata* subsp. *fuchsii*. Accordingly, the deviating morphology of *D. brennensis* compared to *D. elata* could then possibly be explained by introgression from *D. maculata* subsp. *fuchsii*.

However, a study of plastid haplotype variation (HEDRÉN et al. 2012) disclosed a somewhat modified scenario. It was found that *D. brennensis* contains a variety of plastid genomes, some of which are no longer found in present-day representatives of the parental lineages, but which are shared with other allotetraploid members of *Dactylorhiza*. One common haplotype, H156, is identical to a haplotype also present in populations of *D. majalis* subsp. *sequipedalis* in south central France, in populations in the Department of Aveyron. Another common haplotype is H87, which is otherwise confined to the *D. majalis* core complex (including subspp. *majalis*, *traunsteineri*, *alpestris*, etc.) and *D. majalis* subsp. *integrata*. From these data, it was concluded that *D. brennensis* has evolved by hybridization between two already established allotetraploids, one that must have been very similar or identical to *D. majalis* subsp. *sesquipedalis*, and another one that must have been close to either *D. majalis* subsp. *integrata* or a member of the *D. majalis* core complex.

In the present paper, we examine these hypotheses in further detail. We add data from nuclear simple sequence repeat regions (SSR, or nuclear microsatellites), and we investigate differentiation patterns given by individual accessions as well as population samples. We select relevant populations of *D. majalis* subsp. *sesquipedalis*, *D. majalis* subsp. *integrata* and *D. majalis* subsp. *majalis* from France as reference material and we also include accessions of *D. maculata* subsp. *fuchsii* from the same locality as *D. brennensis* to test for the possibility of local introgression from the latter. Combining our results with data from other studies, we try to find the scenario most likely to explain the origin of *D. brennensis*. Finally, we use our results for a general discussion on the subdivision and taxonomic treatment of allotetraploid members of the *D. majalis* polyploid complex.

2. Materials and methods

2.1 Plant material

The material analysed in this study includes 27 plants of *D. brennensis*[1] from one locality (two subsites) in Département Indre, 10 samples of *D. maculata* subsp. *fuchsii* from another subsite at the same locality, 55 samples of *D. majalis* subsp. *integrata* from two localities in northern France, 30 samples of *D. majalis* subsp. *sesquipedalis* from two localities in Département Aveyron in south central France, and 132 samples of *D. majalis* subsp. *majalis* from sites in the western Alps and the Pyrenées in France. Table 1 summarizes the information on the localities (site name, locality, latitude, longitude, orchid taxa, and number of plants) where the different population samples have been collected.

2.2 Molecular methods

Flowers with bracts were collected from plants in the field and desiccated in plastic bags with dry silica gel (CHASE & HILLS 1991). In the lab, DNA was extracted from one or two flowers per plant according to the 2× CTAB procedure (DOYLE & DOYLE 1990) modified to be carried out in 2 mL Eppendorf tubes.

Thirteen length-variable sites were studied in non-coding regions of the plastid genome (Table S1). The combined variation patterns at all marker sites were

[1] also proposed below as *D. majalis* subsp. *brennensis*, see sections 4 and 6

recognized as haplotypes and numbered according to the annotation system used in HEDRÉN et al. (2008). Haplotypes newly discovered here have been given additional numbers.

Table 1: Localities of population samples analysed in the present study.

Taxon	Locality	Latitude	Longitude	n
Dactylorhiza majalis subsp. *brennensis*	Région Centre, Département Indre, Saulnay, 1.3 km SE	46°51´N	01°17´E	13
Dactylorhiza majalis subsp. *brennensis*	Région Centre, Département Indre, Saulnay, 1.5 km SE	46°51´N	01°17´E	14
Dactylorhiza majalis subsp. *integrata*	Région Picardie, Département Aisne, Mauregny-en-Haye, Le Grand Marais	49°31´N	03°48´E	25
Dactylorhiza majalis subsp. *integrata*	Région Champagne-Ardenne, Département Marne, Prouilly, Marais de Neuf Ans	49°17´N	03°42´E	30
Dactylorhiza majalis subsp. *majalis*	Région Provence-Alpes-Côte d´Azur, Département Hautes-Alpes, Les Terasses	45°03´N	06°17´E	16
Dactylorhiza majalis subsp. *majalis*	Région Provence-Alpes-Côte d´Azur, Département Alpes-Maritimes, Bousiéyas	44°19´N	6°52´E	24
Dactylorhiza majalis subsp. *majalis*	Région Provence-Alpes-Côte d´Azur, Département Hautes Alpes, Col de Lautaret	45°02´N	06°24´E	24
Dactylorhiza majalis subsp. *majalis*	Région Roussillon, Département Pyrenées-Orientales, Col de Jau, 1.8 km W	42°42´N	02°14´E	31
Dactylorhiza majalis subsp. *majalis*	Région Midi-Pyrenées, Département Hautes-Pyrenées, Luz-Ardiden	42°53´N	00°04´W	37
Dactylorhiza majalis subsp. *sesquipedalis*	Région Midi-Pyrenées, Département Aveyron, Compregnac	44°05´N	02°58´E	7
Dactylorhiza majalis subsp. *sesquipedalis*	Région Midi-Pyrenées, Département Aveyron, Val de Trébans	44°14´N	03°06´E	23
Dactylorhiza maculata subsp. *fuchsii*	Région Centre, Département Indre, Saulnay, 1.5 km SE	46°51´N	01°17´E	10

Variation in the nuclear genome was studied by use of five SSR loci described in NORDSTRÖM & HEDRÉN (2007) and Table S2. Ms3 and ms8 are specific for the *incarnata* subgenome, whereas ms13, and ms14 are specific for the *maculata* s.lat. subgenome. Ms 11 amplifies well in each of the parental lineages, but in allotetraploid *Dactylorhiza,* fragments from the *maculata* subgenome amplify much more strongly than those of the *incarnata* subgenome, such that only alleles of *maculata* origins could be scored with certainty. Accordingly, none of the loci produced more than two fragments in any individual, and calculations could be performed as for diploids.

Identification of ITS alleles followed the technique developed by PILLON et al. (2007), in which alleles were identified by their combined fragment lengths at two size variable regions (Table S1). ITS allele frequencies were estimated from the relative amounts of PCR products detected on the automated sequencer used for separating the size variable fragments.

We refer to the above-mentioned publications for details on PCR primers and conditions. The PCR products from each reaction were mixed with 8.5 µl formamide containing appropriate size markers to enable exact size determination of the amplified fragments. They were then heated at 94° C for ca. 20 minutes before loading onto acrylamide gels and separated by size on an ALFexpress II automated sequencer (Amersham Pharmacia Biotech). Fragment sizes were determined by using ALFwin™ fragment analyser 1.03.01 software (Amersham Pharmacia Biotech).

2.3 Data analysis

The relationships between plastid haplotypes was estimated by median joining (MJ; BANDELT et al. 1999), in which the fragments identified at each size-variable plastid locus were treated as ordered characters, assuming a step-wise mutation model (SLATKIN 1985). The output of the analysis is a haplotype network composed of all trees of minimum length (Fig. 1). The MJ network was calculated in the computer program NETWORK 4.6.1.6 (FLUXUS TECHNOLOGY 2007).

For nuclear SSR loci, we used average numbers of pairwise differences (NEI & LI 1979) to describe differentiation between population samples. The calculations were performed in Arlequin 3.5.1.2 (EXCOFFIER et al. 2005). In order to summarize the results for visual inspection, we adopted a principal coordinate analysis (PCO) to rotate the resulting matrix of pairwise differences. The PCO was calculated in NTSYS-pc 2.2 (ROHLF 2005).

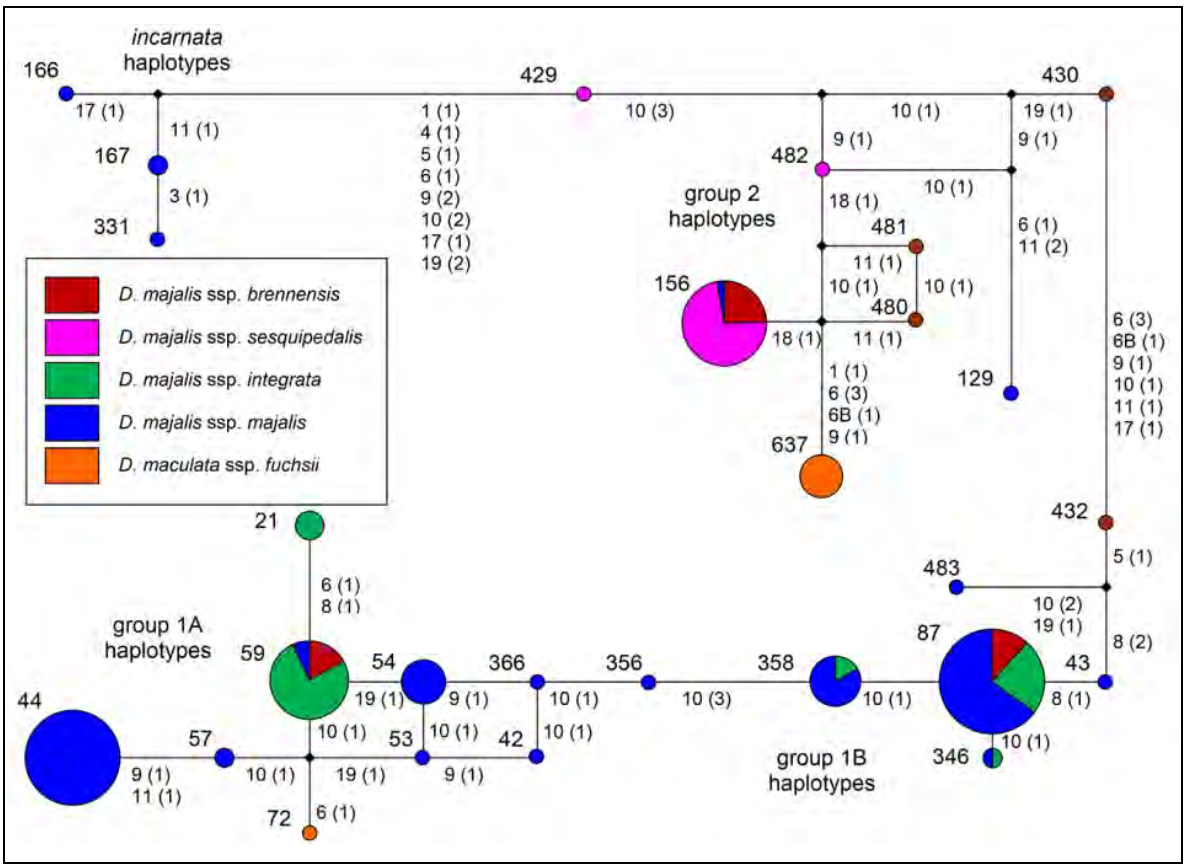

Fig. 1: Median-joining network of the plastid haplotypes encountered in the analyzed material of *Dactylorhiza majalis* subspp. *brennensis*, *majalis*, *integrata*, and *sesquipedalis*.
Haplotypes are represented by pie diagrams in which taxonomic origin is given by different colours. The sizes of the pie diagrams are proportional to the numbers of samples carrying the haplotypes. Marker sites separating haplotypes are given along branches and for each marker the number of mutations separating haplotypes have been indicated within brackets. Branch lengths are approximately proportional to the numbers of mutational steps. The haplotypes are identified by numbers to the upper left of each haplotype. Small filled diamonds denote median vectors.

An assignment test was adopted to explore the affiliation of individual samples of *D. brennensis* analysed from Saulnay with samples from the reference populations of *D. majalis* subspp. *majalis*, *integrata* and *sesquipedalis*. Assignment tests are useful to compare samples of potential hybrid origins with representative samples of the parental groups (WASER 1998). Based on allele frequencies within population samples, the assignment test calculates the posterior probability that any individual sample, on basis of its genotype, would belong to its original population, or to any of the other populations included in the test. Probability scores are given as the negative logarithm of

the probability that a sample would belong to any of the groups included in the analysis, so all probability scores are given as negative values. Since probability scores are calculated for all individual samples from all populations, individual samples would be located in a probability space with the same number of dimensions as the number of populations.

The assignment test conducted here included eleven groups, nine reference populations and two subsamples of *D. brennensis*. To obtain as much resolution as possible, the probability axes of the nine reference populations were rotated by means of a principal component analysis, PCA. The *D. brennensis* samples were inserted a posteriori in the resulting ordination plot, on basis of the probability scores obtained for each of the reference populations. They were accordingly not allowed to influence the divergence pattern formed by the reference populations. The PCA was calculated on basis of the variance-covariance matrix of the probability axes. The assignment test was conducted in the computer program Arlequin 3.5.1.2 (EXCOFFIER et al. 2005), and the PCA in the computer program NTSYS-pc 2.2 (ROHLF 2005).

3. Results

3.1 Population differentiation and population diversity patterns

Altogether 27 plastid haplotypes were identified by combining the size variants found at the 13 analysed plastid VNTR loci (Table S3, Fig. 1). The two haplotypes 87 and 156 were most common in *D. brennensis*, found in nine samples each. Haplotype 59 was found in five samples, and another four haplotypes were found in one plant each. Of the common haplotypes in *D. brennensis*, haplotype 156 was shared with *D. majalis* subsp. *sesquipedalis* only, and 87 and 59 were shared with both subsp. *majalis* and subsp. *integrata*, although haplotype 59 was only found at low frequency in subsp. *majalis*. The four uncommon haplotypes were restricted to *D. brennensis*. Three of those were related to haplotype 156 and one related to haplotype 87. To enable comparison, we have translated our annotation system to those of other studies in Table S4.

The plot of the first two axes from a PCO calculated on basis of average numbers of pairwise differences at nuclear SSR loci between populations is given as Fig. 2. The two populations of *D. majalis* subsp. *integrata* are found in the upper part of the plot, the five populations of subsp. *majalis* in the upper part to the right, and the two populations of subsp. *sesquipedalis* in the lower part to the right of the plot. The two subpopulations of *D. brennensis* are found

in the lower central part, at a position almost intermediate between the populations of *D. majalis* subsp. *sesquipedalis* and subsp. *integrata*.

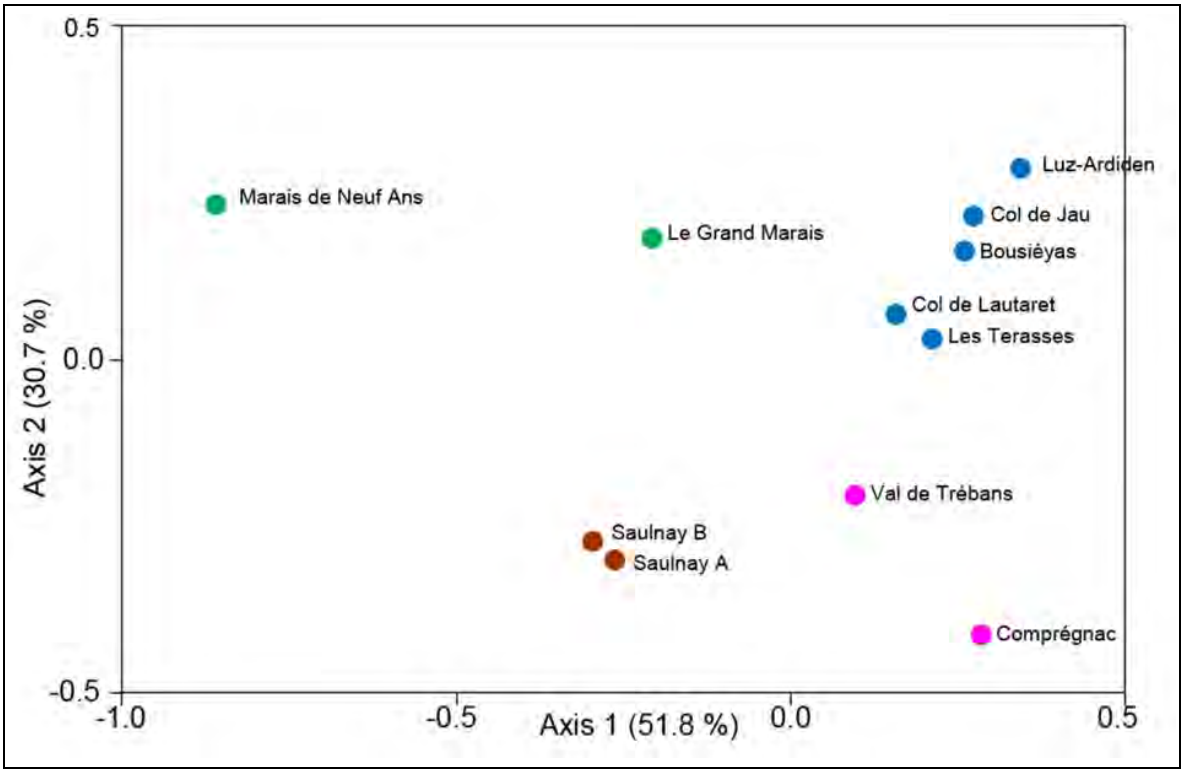

Fig. 2: Principal coordinate analysis (PCO) summarizing differentiation between analysed populations of *D. majalis* subspp. *brennensis* (brown spots), *majalis* (blue spots), *integrata* (green spots) and *sesquipedalis* (pink spots). The analysis was based on pairwise F_{ST} values between populations in SSR allele compositions.

The ITS allele composition of *D. brennensis* is compared to that of the reference material in Table S5. *D. majalis* subsp. *sesquipedalis* is dominated by ITS-I, subsp. *integrata* by a combination of ITS-III, ITS-V and ITS-X, and subsp. *majalis* by a combination of ITS-III, ITS-V and ITS-VI (with some variation among populations). The local *D. maculata* subsp. *fuchsii* combined ITS-III and ITS-V. *D. brennensis* included all these ITS alleles. In Table S6 the annotation system of ITS alleles used here could be compared to the enumeration of clades of ITS/ETS sequences used in DEVOS et al. (2006).

3.2 Differentiation patterns among individual samples

The resulting plot of the assignment test is given as Fig. 3, in which all material has been rotated in a PCA to show maximum degree of dispersion between the reference populations. Material belonging to *D. majalis* subsp.

majalis is located to the upper right, material of subsp. *integrata* to the upper center/left (depending on population), and material of subsp. *sesquipedalis* to the lower right. Material of *D. brennensis* is mostly positioned in the lower part of the plot, in between samples of *D. majalis* subsp. *sesquipedalis* and subsp. *integrata*. The material is somewhat spread out, but there is no obvious differentiation between material from the two subsites.

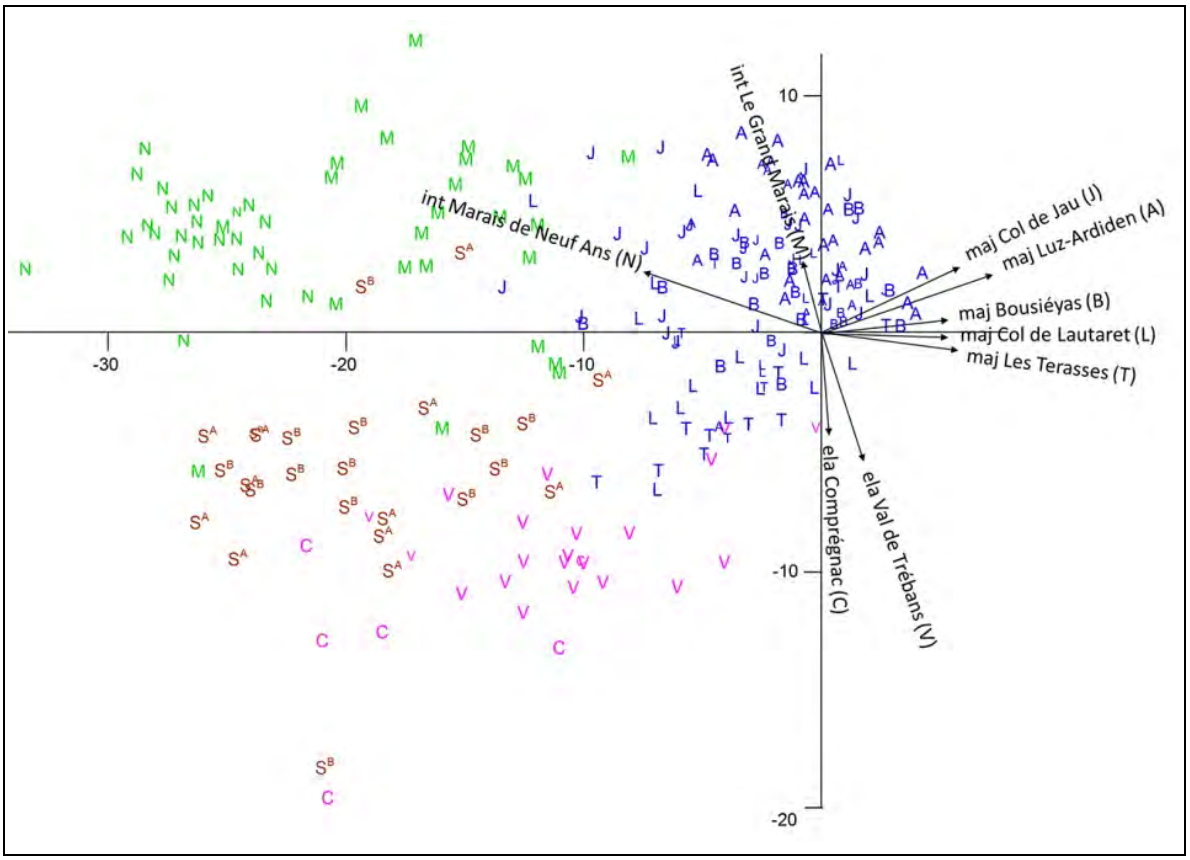

Fig. 3: Assignment test investigating the affiliation of individual samples of *D. majalis* subsp. *brennensis* (brown capitals) relative to reference samples of *D. majalis* subspp. *majalis* (blue capitals), *integrata* (green capitals), and *sesquipedalis* (pink capitals, sub "ela").
All samples were first analysed for the probability of belonging to any of the eleven populations included in the analysis. The variance-covariance matrix of the probability axes of the nine reference populations were then rotated by means of a principal component analysis, PCA, and finally the samples of *D. majalis* subsp. *brennensis* were inserted a posteriori. The positions of individual samples are given by coloured capital letters, indicating their population of origin, and the direction of the probability axes for the reference populations are given as vectors. The proportion of variation shown by the first two principal components were 61.6 % and 19.5 %, respectively, of the total variation.

The direction and strength of the probability axes for the various reference populations are shown by vectors originating at origo (the point of zero probability of belonging to any of the population included in the analysis). Obviously, all populations of *D. majalis* subsp. *majalis* tend to pull the samples in the same direction, towards the upper right, whereas the two populations of subsp. *integrata* pull the samples towards the upper left. Populations of subsp. *sesquipedalis* pull the samples towards the lower part of the plot.

4. Discussion

4.1 Origin of *Dactylorhiza brennensis*

Our data strongly suggest that an allotetraplod similar to the *D. majalis* subsp. *sesquipedalis* growing in south central France was one of the parents to *D. brennensis*. The other parent should have been *D. majalis* subsp. *integrata*, or a member of the *D. majalis* core complex. Following one line of arguments (see below), we treat *D. brennensis* as a subspecies of *D. majalis* s.lat. in the rest of this paper.

The most common plastid haplotypes encountered in *D. majalis* subsp. *brennensis* were haplotypes 156, 87, and 59 (Fig. 1, Table S3). These haplotypes are distinctly different from each other and are also found in other allotetraploid members of *Dactylorhiza*.

- Haplotype 156 is a group II haplotype in the classification of DEVOS et al. (2006) and given as haplotype B in PILLON et al. (2007). It is the dominant haplotype in populations of *D. majalis* subsp. *sesquipedalis* in southern France (HEDRÉN et al. 2012), but it is rare or absent from other allotetraploids and from present-day *D. maculata* s.lat.
- Haplotype 87 is a group I haplotype (haplotype C in PILLON et al. 2007) found in *D. majalis* subsp. *integrata* as well as members of the *D. majalis* core complex, but it is seemingly also absent from present-day *D. maculata* s.lat.
- In contrast, haplotype 59, belonging to group I, is common in present-day *D. maculata* subsp. *fuchsii* (haplotype A in PILLON et al. 2007) and is also widespread in other allotetraploids within *D. majalis* s.lat.

Nuclear SSR data suggest that *D. majalis* subsp. *integrata* rather than subsp. *majalis* contributed the 59 and 87 haplotypes to subsp. *brennensis*. The PCO based on SSR allele frequencies within populations (Fig. 2) and the multiple assignment test based on individual samples (Fig. 3) showed similar patterns.

D. majalis subsp. *brennensis* was located in between subsp. *integrata* and subsp. *sesquipedalis* in each case.

Considering the plastid haplotype diversity within *Dactylorhiza majalis* subsp. *brennensis*, the taxon must have originated on a minimum of three occasions: on one occasion with subsp. *sesquipedalis* as maternal parent, and on two occasions with subsp. *integrata* or subsp. *majalis* as maternal parent (or alternatively on one occasion with *D. maculata* subsp. *fuchsii* as maternal parent). However, since these are only minimum values, the origin of subsp. *brennensis* is probably better described as the merger of populations of parental taxa, rather than by hybridization between pairs of single individuals.

A few additional haplotypes were also identified in *D. majalis* subsp. *brennensis*; these may have been incorporated into subsp. *brennenis* by additional gene flow from its allotetraploid parents or from back-crossing with the *D. maculata* s.lat. lineage. However, on basis of plastid haplotype data, we did not find any evidence that the present-day, local population of *D. maculata* subsp. *fuchsii* was involved in such a process. In contrast, we found that the local subsp. *fuchsii* was dominated by a distinct group II haplotype (637; Fig. 1) that was not found in any of the subsp. *brennensis* individuals analyzed.

DEVOS et al. (2006) found that *D. majalis* subsp. *brennensis* contains ITS/ETS variants shared with *D. incarnata* (given as clade G), *D. maculata* subsp. maculata (clade C), and *D. maculata* subsp. *fuchsii* (clade A). In addition they found chimeric variants in subsp. *brennensis* that were composed by portions of clade A and clade C sequence variants. They interpreted their results such that subsp. *brennensis* should have originated by hybridization and allopolyploidization between members of the *D. maculata* subsp. *maculata* and *D. incarnata* lineages (similar to *D. majalis* subsp. *sesquipedalis*), and subsequent back-crossing with local *D. maculata* subsp. *fuchsii* (DEVOS et al. 2006).

In agreement with DEVOS et al. (2006), we could verify the presence of ITS variants corresponding to ITS/ETS clades G, C, and A, and we also found some variants corresponding to those of clade B, which are characteristic of subsp. *integrata*. However, because we used the simplified method for ITS variant identification of PILLON et al. (2007) and did also not perform any sequencing of cloned fragments, we had no possibility to identify chimeric sequence variants in the present study.

Based on plastid haplotype variation in *D. majalis* subsp. *brennensis*, we propose here a modified scenario, according to which subsp. *brennensis* originated from hybridization between two already established allotetraploids,

subsp. *sesquipedalis* and subsp. *integrata*. Our scenario is still fully compatible with the results presented by DEVOS et al. (2006). According to our scenario, the ITS/ETS variants of clade C could be contributed to subsp. *brennensis* by subsp. *sesquipedalis*, whereas ITS/ETS variants of clades G, A, and B could be contributed by subsp. *integrata* in which they are all fairly common. Once these variants had been brought together in subsp. *brennensis*, one could easily envision the formation of chimeric variants between clades A and C.

It could still be argued that some nuclear genes might have been brought to *D. majalis* subsp. *brennensis* by hybridization with the local *D. maculata* subsp. *fuchsii*. If the latter acted as pollen donor to the primary hybridization step, the plastid haplotype of subsp. *fuchsii* would not have ben transferred to subsp. *brennensis* (as the plastid genome is transmitted by the maternal (seed) parent in orchids (CORRIVEAU & COLEMAN 1988)). Hybrids between subsp. *brennensis* and subsp. *fuchsii* were identified and described in the study of TYTECA & GATHOYE (1988a).

Comparing *D. majalis* subsp. *integrata* and subsp. *majalis* for plastid haplotype and ITS variants, it appears most likely that the former was involved in the formation of subsp. *brennensis*. This parent must have contributed the major plastid genomes and ITS alleles not contributed by subsp. *sesquipedalis*, viz. haplotypes 59 and 87, and ITS alleles III and VI. Rare plastid haplotypes restricted subsp. *brennensis* might have been added by mutation from common haplotypes after the formation of subsp. *brennensis*, or they might just not have been detected in the restricted material of the putative parental taxa examined here. The chimeric ITS variants discovered by DEVOS et al. (2006), should also have been formed within *D. majalis* subsp. *brennensis* between ITS-III inherited from subsp. *integrata* and ITS-I inherited from subsp. *sesquipedalis*.

4.2 A scenario for the evolution of *Dactylorhiza majalis* subsp. *brennensis*

We interpret our data such that *D. majalis* subsp. *brennensis* evolved from hybridization between two already established allotetraploids in the *D. majalis* complex, *D. majalis* subsp. *sesquipedalis* and *D. majalis* subsp. *integrata*. Combining these results with data obtained from other studies of molecular variation in *Dactylorhiza* (e.g., DEVOS et al. 2003, 2006, PILLON et al. 2007, HEDRÉN 2003, HEDRÉN et al. 2008, 2011, 2012, NORDSTRÖM & HEDRÉN 2009, STÅHLBERG & HEDRÉN 2010), we here also try to describe the origin and evolution of the ancestral allotetraploids to *D. majalis* subsp. *brennensis* (Fig. 4). Such a reconstruction would help us to understand variation patterns and polyploidization dynamics within the *D. majalis* complex as a whole.

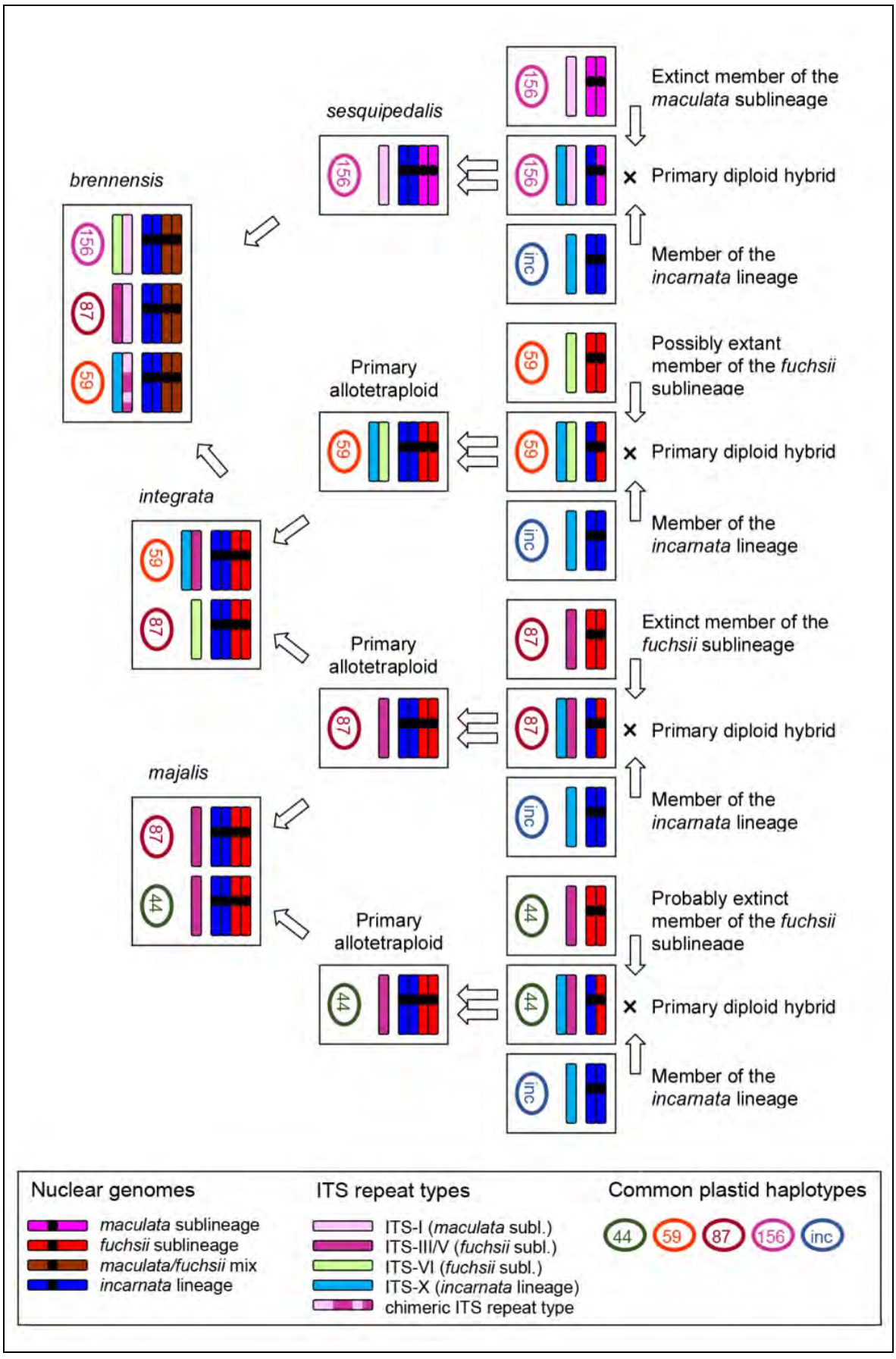

Fig. 4 (p. 48): A simplified scenario for the origin of *D. majalis* subsp. *brennensis* based on the distribution of molecular markers in its direct ancestors and in the parental lineages to the *D. majalis* polyploid complex. Note that the actual pathways that gave rise to present-day allotetraploids were probably much more complex than the scenario depicted here.
Primary allotetraploids arise occasionally from hybridization between the *D. maculata* s.l. and *D. incarnata* s.l. parental lineages and subsequent increase in ploidy level. These primary allotetraploids can include different subsets and different combinations of the genetic material present in the parental lineages; for instance, whereas some had maternal parents similar to present-day *D. maculata* subsp. *fuchsii*, others had maternal parents similar to present-day *D. maculata* subsp. *maculata*. Allotetraploids of wide distribution, such as, e.g., *D. majalis* subsp. *majalis*, may have formed by the merger of several of these primary allotetraploids. They may also preserve genetic material that has subsequently gone extinct in the parents, for instance the plastid haplotypes 87 and 156. Different allotetraploids share portions of their gene pools with each other as they have partly originated from the same primary allotetraploids or have exchanged genetic material by direct hybridization. Recombination within nuclear genomes of the same parental origins give rise additional diversity within the allotetraploids, whereas recombination between nuclear genomes of different parental origins is much more restricted. Plastid genomes are inherited from the maternal parents and reflect plastid haplotype diversity in the maternal parental lineage (usually *D. maculata* s.lat.). Fusion of population belonging to different allotetraploid taxa can give rise to new allotetraploids, such as *D. majalis* subsp. *brennensis*. Chimeric ITS repeat types can evolve as a result of recombination in regions of ribosomal DNA genes.

According to our hypothesis *D. majalis* subsp. *sesquipedalis* must have contributed the plastid haplotype 156 and ITS/ETS clade C (ITS-I) to subsp. *brennensis*. This haplotype is similar to haplotypes dominant in western European members of the *D. maculata* subsp. *maculata* sublineage, but there is no exact match with any of the haplotypes found in *D. maculata* today. This finding suggests that *D. majalis* subsp. *sequipedalis* is itself an old allotetraploid with origin from a now extinct member of the *D. maculata* subsp. *maculata* sublineage. Alternatively, the haplotype has evolved within *D. majalis* subsp. *sesquipedalis* after its origin, which also points to a high age. ITS variants belonging to clade C are still dominant in western European *D. maculata* subsp. *maculata* but are rare in *D. maculata* subsp. *fuchsii*.

D. majalis subsp. *sesquipedalis* has often been regarded as part of *D. majalis* subsp. *elata* (as *D. elata* s.lat.), but the taxon is apparently heterogeneous. Populations from North Africa differ from European populations in having other plastid haplotypes and are also dominated by another ITS type (PILLON et al. 2007). Moreover, populations from Portugal differ from populations from southern France in having plastid haplotypes similar to those of present-day members of the *D. maculata* subsp. *maculata* sublineage (HEDRÉN et al. 2012). The dominance of haplotype 156 in *D. majalis* subsp. *sesquipedalis* in southern France suggests that populations from this region have served as one of the parents to subsp. *brennensis*. Interestingly, haplotype 156 was also identified in a single accession of *D. majalis* subsp. *majalis* from the Pyrenees, pointing to the possibility that subsp. *majalis* has also hybridized with subsp. *sesquipedalis*, or that the two taxa have some maternal parents in common.

The second parent to *D. majalis* subsp. *brennensis* was most probably *D. majalis* subsp. *integrata*, although an origin from subsp. *majalis* (or another member of the *D. majalis* core complex) cannot be ruled out completely. These allotetraploids have a more complex background than subsp. *sesquipedalis* and may themselves have formed by hybridization between independently formed primary allotetraploids. Furthermore, as suggested by the shared presence of haplotype 87, some of these primary allotetraploids may have contributed genetic material to both subsp. *integrata* and subsp. *majalis*. Alternatively, haplotype 87 could have been transferred between the two taxa by direct hybridization after their origins. Haplotype 87 has not been identified in present-day members of the *D. maculata* s.lat. lineage, but is common in both *D. majalis* subsp. *majalis* and subsp. *integrata*, suggesting that these subspecies include primary allotetraploids of relatively high age.

A common group I haplotype in *D. majalis* subsp. *integrata*, apart from 87, is haplotype 59. This haplotype is still common in *D. maculata* subsp. *fuchsii*, indicating that *D. majalis* subsp. *integrata* may also include primary allotetraploids of more recent origins. Furthermore, many populations of subsp. *integrata* contain ITS/ETS sequence variants of clade B (ITS-VI in PILLON et al 2007) in the nuclear genome. These variants are common in the southern European *D. maculata* subsp. *saccifera* (belonging to the subsp. *fuchsii* sublineage), leading DEVOS et al. (2006) and PILLON et al. (2007) to propose that subsp. *saccifera* served as the maternal parent to *D. majalis* subsp. *integrata*. The southern distribution of *D. maculata* subsp. *saccifera* indicates that *D. majalis* subsp. *integrata* may also have a southern origin, although its present-day distribution is restricted to NW Europe. However, ITS-VI is also found at low frequency in *D. maculata* subsp. *fuchsii* (PILLON et al. 2007, HEDRÉN et al. 2011), so this allele could have been inherited from the latter, which indicates that

D. majalis subsp. *integrata* might still have originated within its present area of distribution. Furthermore, the wide variation in *D. majalis* subsp. *integrata* suggests that this taxon has evolved by fusion of several independently derived allopolyploids, and/or that it has increased its gene pool by secondary hybridization and introgression from its parental species.

Dactylorhiza majalis subsp. *majalis* contains several plastid haplotypes in addition to 87 that are rare or absent in present-day representatives of the *D. maculata* lineage (including both group I and group II haplotypes; HEDRÉN et al. 2008, NORDSTRÖM & HEDRÉN 2009) and haplotype 59 has a low frequency in *D. majalis* subsp. *majalis*. These findings suggest that subsp. *majalis* has formed by merger of several primary allotetraploids of independent origins. Furthermore, presence of group II haplotypes in subsp. *majalis* suggests that the parents of these primary allotetraploids must also include members of the *D. maculata* subsp. *maculata* sublineage. However, the exact haplotypes have not been identified in present-day subsp. *maculata*, which indicates that some of the primary allotetraploids included in *D. majalis* subsp. *majalis* are of relatively high age.

At nuclear ITS, *D. majalis* subsp. *majalis* is dominated by ITS/ETS variants of clade A (ITS-III sensu PILLON et al. 2007), which is one of the most common variants in *D. maculata* subsp. *fuchsii*, but which is rare in subsp. *maculata* in western Europe. ITS/ETS variants of clade B (ITS-VI in PILLON et al. 2007) are rare in *D. majalis* subsp. *majalis*, but we nevertheless found this ITS type in the Bousiéyas population examined here.

The finding that *D. majalis* subsp. *brennensis* evolved by hybridization between existing allotetraploids − rather than de novo allopolyploidization from ancestral diploid species with divergent genomes − might indicate a general pattern in the evolution of allopolyploid *Dactylorhiza*. Hybridization between different allopolyploids could explain the high level of diversity found in widespread taxa such as subsp. *majalis*, subsp. *lapponica*, subsp. *integrata*, subsp. *cordigera*, and others. Alternatively, already existing allotetraploids could have been modified by secondary hybridization and introgression from variants of the diploid ancestors that gave rise to them, adding diversity to the original allotetraploid. However, these processes – multiple origins, hybridization between primary allotetraploids, and secondary hybridization with parental diploids – are very difficult to separate from each other, and their relative importance are difficult to estimate. The only thing we can establish at this stage is that good examples for all these processes have been described in the literature, and here we have given an obvious example of the second one.


Fig. 5-6: *D. majalis* subsp. *brennensis* (*D. brennensis*), Etang Neuf, Sainte-Gemme, Dépt. Indre, France, 6.6.1987, phot. DT.


Fig. 7-8: *D. majalis* subsp. *sesquipedalis,* Engayresque, Séverac d'Aveyron, Dépt. Aveyron, France, 31.5.2010, phot. DT.

Fig. 9-10: *D. majalis* subsp. *majalis*, Terre des Aujes, Beauraing, Prov. Namur, Belgium, 20.5.2007 (whole plant), 24.5.2010 (close up), phot. DT.

Fig. 11-12: *D. majalis* subsp. *integrata* (*D. praetermissa*), Marais du Temple, Pontavert, Dépt. Aisne, France, 18.6.2016, phot. DT.

4.3 Taxonomic implications

The two authors have different opinions on the taxonomic implications of the findings obtained in this study. Whereas the first author (MH) argues that *D. majalis* subsp. *brennensis* is best treated as part of *D. majalis*, with reference to the biological species concept, BSC, of MAYR (1942) and to the taxonomic species concept, TSC in its general sense (DU RIETZ 1930, HEDBERG 1957, JONSELL 2004), the second author (DT) argues that it is better recognized as a full species, with reference to the phylogenetic species concept, PSC, as formulated by WILEY (1981), and to the specific taxonomic species concept adopted by many authors studying the European orchid flora, following GÖLZ & REINHARD (1975).

According to the BSC (MAYR 1942), species are biological entities separated from other species by barriers to gene exchange, so called isolation mechanisms. Organisms connected by gene flow should then be recognized as members of the same species. Modern evolution biologists (COYNE & ORR 2004), still regard taxa as conforming to BSC even if restricted inter-species gene flow occurs, as long as species identity is not disrupted. Since the two allotetraploid parental taxa considered here, *D. majalis* subsp. *sesquipedalis* and *D. majalis* subsp. *integrata* have obviously produced the derivative and fully fertile subsp. *brennensis* by hybridization, it follows that these taxa are all parts of the same species if any of the above-mentioned forms of BSC is applied.

However, plant systematists often regard that the BSC often is too inclusive, especially when it is applied in the orchid family. Many orchid species are capable of producing hybrids under artificial or occasional conditions, but rarely hybridize in nature (GILL 1989). In spite of the fact that artificially produced hybrids could be fully or partly fertile, other factors such as pollinator specificity, geographic isolation, or phenology instead contribute to species separation.

In the set of taxa considered here, gene flow is largely impeded by the fact that the present-day population of *D. majalis* subsp. *brennensis* is geographically separated from its parental populations. Although subsp. *brennensis* must have once originated as a result of hybridization, this finding could just be regarded as part of its history and may not be relevant for present-day patterns of gene exchange.

In recent decades, phylogenetic systematists have largely abandoned the BSC in favour of lineage-based evolutionary or phylogenetic species concepts, PSC,

according to which species are defined as lineages evolving independently from other lineages through time (WILEY 1981). Variants of these concepts allow for minor gene exchange by occasional hybridization, as long as the lineages remain morphologically and genetically discrete. Further modifications of PSC also accept allopolyploids and other groups originating from hybridization as separate species (BATEMAN 2001), if the origin of such species could be traced back to discrete events in time and space, and if they could be assumed to evolve independently from other groups in the future.

Preferably, the identification and circumscription of species according to PSC should be supplemented by phylogenetic analyses of multiple individuals and populations and careful interpretation of trees resulting from such analyses. Since a species circumscribed according to the PSC constitutes a lineage evolving separately from other lineages, its component populations are expected to form a monophyletic cluster in the phylogenetic trees obtained from analyses where it is compared to populations of other species (GROSS & RIESEBERG 2003).

From a cladistic standpoint, the ability of two species to interbreed is regarded as an ancestral character state, or plesiomorphy (ROSEN 1978). Such character states are not relevant for classification, since lineages are recognized by possession of shared derived character states, synapomorphies in phylogenetic classification.

A critique sometimes raised against lineage-based classifications in which individuals or populations are the fundamental units of analysis, is the difficulty to decide which branches of a tree represent species and which branches either represent clades within a species or clades composed of multiple species (MISHLER 2010). A commonly used principle to solve this problem is to use the hierarchic level at which a phylogenetic tree collapses into polytomy as indicator of species level. This level should be the level at which different characters largely disagree with each other for grouping, presumably due recombination and lateral gene flow between the objects under investigation, a pattern that would also agree with species delimitation according to BSC. However, a polytomy may also arise due to lack of data, such that species recognized by criteria other than those used in the analysis can be artificially grouped together. On the other hand, insufficient data, or use of too few objects for classification, may result in arbitrary branching patterns and inflated numbers of lineages accepted as separate species.

In the absence of molecular or experimental data, a morphological, or taxonomic species concept, TSC, may be applied. According to TSC, groups of

individuals separated in taxonomically important characters could be considered as different species. However, a major problem with a purely morphological species concept is the divergent opinions on criteria for species recognition vs. species lumping (STACE 1980). A widely used principle in general plant taxonomy is the requirement that all accessions accepted as members of a particular species should differ consistently in at least two presumably independent morphological characters from any other species (DU RIETZ 1930, HEDBERG 1957). It should be observed that taxonomic species circumscribed in this way often agree with species circumscribed according to the BSC (FUTUYMA 1986). Such concordance follows from the absence of gene flow between biological species. Groups isolated from each other will slowly accumulate unique mutations, and given enough time, these mutations may become fixed by selection or random processes and finally manifested as separating characters expressed in morphology. Exceptions could be young species, in which clear separating characters have not yet developed, or cryptic species, which are well separated according to molecular or cytological criteria invisible to the human eye.

Crosses between biological species are hybrids, not species, and they could often be recognized by reduced fertility. Hybrids could be intermediate between their parents in morphology but could sometimes transgress both parents in certain characters (e.g. size) due to hybrid vigour, or develop poorly as result of genetic incongruence or imbalance.

In contrast, authors specialising in the systematics of European orchids have often used a different form of taxonomic species concept based on the phenetic distance measure designed by GÖLZ & REINHARD (1975). This measure summarizes differences in mean character values between population samples and weigh the studied characters by their average standard variations. Populations separated by difference values above 35 are considered as belonging to different species, while difference values between 20 and 35 correspond to closely related species or subspecies.

TYTECA & GATHOYE (1988a, b) collected morphometric data for *D. majalis* subsp. *brennensis* and compared it to other Western European members of *Dactylorhiza* by using the distance measure designed by Gölz and Reinhard (TYTECA & GATHOYE 1988b, 1989). They found that subsp. *brennensis* was most closely similar to *D. delphinensis*, a member of the *D. majalis* complex described from the western Alps (TYTECA & GATHOYE 1988c; syn. *D. angustata*, TYTECA & GATHOYE 2000), but that it differed enough from other members of the *D. majalis* complex to motivate segregation as full species using the threshold levels proposed by GÖLZ & REINHARD (1975).

From the studies of Tyteca and Gathoye, it appears that the phenetic concept of GÖLZ & REINHARD (1975) corresponds to species circumscribed according to PSC. However, there are several reasons why great care must be taken when translating the Gölz and Reinhard distances into taxonomy (PEDERSEN 2010).

- First, the threshold levels given by Gölz and Reinhard are arbitrary and were originally established to correlate with already recognized species of *Ophrys*. By convenience, the same threshold levels have been adopted in studies of other European genera, which has been motivated by using data from similar sets of characters.
- Second, the Gölz and Reinhard distance was never meant as anything else but a purely phenetic distance and the degree of differentiation between any two entities may, or may not, correspond to differences observed in independent sources of data, for instance distribution, breeding system, karyotype, secondary compounds, or DNA.
- Third, the Gölz and Reinhard distance is based on population mean values but does not consider patterns of differentiation between individuals. Individuals from two predefined groups might turn out to remain in well-defined clusters, indicating a clear separation, but might alternatively show extensive overlap, indicating hybridization and exchange of genetic material.

As observed by PEDERSEN (2010), many studies in *Dactylorhiza* using the Gölz and Reinhard distance have often resulted in narrow species circumscriptions, and studies supported by molecular data have often failed to verify the high number of species segregated on basis of morphological criteria (e.g., DEVOS et al. 2006, PILLON et al. 2007, PEDERSEN 2010, BRANDRUD et al. 2019).

Lastly, we observe that many allotetraploid members of *Dactylorhiza* segregated as species according to the PSC, often fulfil the criteria for recognition at subspecies level according to standard taxonomic principles outlined in, e.g. JONSELL (2004). According to JONSELL (2004: 163-164), subspecies are regional variants of a species that may differ in distribution, ecology or a combination thereof from other subspecies. Subspecies should differ from each other in at least two presumable independent characters in which they can still show some degree of overlap.

Crosses between subspecies are not hybrids as they still belong to a species, and they are best classified as intermediates. They are typically as fertile as their parental subspecies, and they less often than hybrids transgress their

parents in size or suffer from genetic imbalances. Intermediates between subspecies often appear in transitional zones separating different subspecies in the geography (FUTUYMA 1986). Subspecies can be recognized in situations where a majority of studied individuals could be determined to correct subspecies using morphological criteria. In situations where variation is large, but no clear groupings could be identified, the situation is best left as a single variable species. According to SIMPSON (1961) it would be reasonable to segregate a species into subspecies if at least 75% of all studied samples could be correctly assigned to subspecies without any prior knowledge of their geographic origin.

Since subspecies do not need to be as rigidly circumscribed as full species, use of the subspecies category allows for a wider morphological variation within a recognized taxon. Instead, more emphasis could be put on geographic distribution, habitat preference, phylogeny, or other factors that may be more relevant from a biological perspective. The subspecies concept also allows for a higher degree of flexibility when the decision is made on how many, and which, subsets of a species merit formal recognition. In the case studied here, one might consider whether it is necessary to recognize *D. majalis* subsp. *brennensis* as a separate taxon, or whether it should just be seen as a transitional form between *D. majalis* subspp. *sesquipedalis* and *integrata* confined to a small area in between the distribution areas of the parental subspecies.

Although it might be preferable to segregate allotetraploid members of the *D. majalis* complex as subspecies rather than species from a biological perspective, such a treatment may not be useful in communication with ecologists, conservationists and the general audience. One problem is that subspecies are often not recognized by conservation authorities or by the legislative systems in many countries. Although subspecies may contain as many unique genotypes and as much genetic diversity as a full species, such diversity may accordingly be overlooked when the conservation value of an area occupied by a particular subspecies is assessed. On the other hand, it is the duty of taxonomists to work for a better understanding of the taxonomic system, such that infraspecific categories could also be accepted for conservation. Explaining these concepts may convey a better understanding of the evolutionary dynamics in *Dactylorhiza* and an insight that we need to consider evolutionary processes as much as we consider static taxa.

5. Conclusion

The allotetraploid *Dactylorhiza majalis* subsp. *brennensis* is a fertile intermediate that has originated by fusion of populations of *D. majalis* subsp. *sesquipedalis* and subsp. *integrata*. The three taxa are members of the *D. majalis* polyploid complex in which evolutionary patterns are highly reticulate due to repeated origins of allotetraploids from hybridization between the *D. maculata* s.lat. and *D. incarnata* s.lat. parental lineages, backcrossing and introgression from the parents, and hybridization between allotetraploids of independent origins.

From the standpoints of general systematics and evolutionary biology, the allotetraploids in the *D. majalis* complex are best treated as subsets of a broadly circumscribed *D. majalis* s.lat. rather than separate species. However, from a phylogenetic standpoint, *D. brennensis* can be accepted as a full species as it is regarded to have a single unique origin. Moreover, since it is restricted to a geographically separated area, one could predict that it will evolve independently of other allotetraploid taxa also in the future. Recognition of allotetraploid taxa at species level could also be motivated from ease of communication (short names), a large diversity in terms of morphology and habitat among allotetraploids, allopatric distributions, ignorance about infraspecific categories among ecologists and the general audience, and the inability of the legislative system in many countries to consider other than full species in conservation planning.

6. Nomenclatural note

For those who prefer to treat *Dactylorhiza elata* subsp. *brennensis* as a subspecies of *D. majalis*, we provide the following new combination:

Dactylorhiza majalis (Rchb.) P.F. Hunt & Summerh. **subsp. *brennensis*** (Nelson) D. Tyteca & Hedrén, comb. nov.

Basionym: *Dactylorhiza elata* (Poir.) Soó subsp. *brennensis* E. Nelson in Monographie und Ikonographie der Orchidaceen-Gattung *Dactylorhiza*: 50. 1976. Zürich.

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References

BANDELT, H.-J., FORSTER, P. & A. RÖHL (1999): Median-joining networks for inferring intraspecific phylogenies.- Mol. Biol. Evol. 16(1): 37-48.

BATEMAN, R.M. (2001): Evolution and classification of European orchids: insights from molecular and morphological characters.- J. Eur. Orch. 33(1): 33-119.

BRANDRUD, M.K., BAAR, J., LORENZO, M.T., BATEMAN, R.M., CHASE, M.W., HEDRÉN, M. & O. PAUN (2019): Phylogenomic relationships of diploids and the origins of allotetraploids in *Dactylorhiza* (*Orchidaceae*): RADseq data track reticulate evolution.- Systematic Biology 69(1): 91-109. DOI:10.1093/sysbio/syz035.

CHASE, M.W. & H.G. HILLS (1991): Silica gel: an ideal material for field preservation of leaf samples for DNA studies.- Taxon 40(2): 215-220.

CORRIVEAU, J.L. & A.W. COLEMAN (1988). Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species.- Am. J. Bot. 75(10): 1443-1458.

COYNE, J.A & H.A. ORR . (2001): Speciation.- Sinauer, Sunderland, MA.

DELFORGE, P. (2001): Guide des Orchidées d´Europe, d´Afrique du Nord et du Proche-Orient, 2nd ed.- Delachaux et Niestlé, Lausanne.

DEVOS, N., TYTECA, D., RASPÉ, O., WESSELINGH, R.A. & A.-L. JACQUEMART (2003): Patterns of chloroplast diversity among western European *Dactylorhiza* species (*Orchidaceae*).- Pl. Syst. Evol. 243(1-2): 85-97.

DEVOS, N., RASPÉ, O., OH, S.-H., TYTECA, D. & A.-L. JACQUEMART (2006): The evolution of *Dactylorhiza* (*Orchidaceae*) allotetraploid complex: Insights from nrDNA sequences and cpDNA PCR-RFLP data.- Mol. Phyl. Evol. 38(3): 767-778.

DOYLE, J.J. & J.L. DOYLE (1990): Isolation of plant DNA from fresh tissue.- Focus 12(1): 13-15.

DU RIETZ, G.E. (1930): The fundamental units of biological taxonomy.- Svensk Bot. Tidskr. 24(3): 333-428.

EXCOFFIER, L., LAVAL, G. & S. SCHNEIDER (2005): Arlequin (version 3.0): An integrated software package for population genetics data analysis.- Evol.

Bioinform. Online 1: 47-50.
FLUXUS TECHNOLOGY (2007): Network 4.2.0.1. User Guide.- http://www.fluxus-technology.com (accessed 29.08.2007).
FUTUYMA, D.J. (1986): Evolutionary biology, 2nd ed.- Sinauer, Sunderland, MA.
GATHOYE, J.-L. & D. TYTECA (1989): Contribution à l´étude cytotaxonomique des *Dactylorhiza* d´Europe occidentale.- Mém. Soc. Roy. Bot. Belg. 11: 30-42.
GILL, D.E. (1989): Fruiting failure, pollinator inefficiency, and speciation in orchids.- In: OTTE, D. & J.A. ENDLER (eds.): Speciation and its consequences.- Sinauer, Sunderland, MA, pp. 458-481.
GÖLZ, P. & H.R. REINHARD (1975): Biostatistische Untersuchungen über *Ophrys bertoloniiformis* O. et E. Danesch.- Ber. Schweiz. Bot. Ges. 85(1): 31-56.
GROSS, B.L, SCHWARZBACH, A.E. & L.H. RIESEBERG (2003): Origin(s) of the diploid hybrid species *Helianthus deserticola* (Asteraceae).- Amer. J. Bot. 90(12): 1708-1719.
HEDBERG, O. (1957): Afro-alpine vascular plants. A taxonomic revision.- Symb. Bot. Upsal. 15(1).
HEDRÉN, M. (1996): Genetic differentiation, polyploidization and hybridization in northern European *Dactylorhiza* (*Orchidaceae*): evidence from allozyme markers.- Pl. Syst. Evol. 201(1-4): 31-55.
HEDRÉN, M. (2003): Plastid DNA variation in the *Dactylorhiza incarnata/maculata* polyploid complex and the origin of allotetraploid *D. sphagnicola*.- Mol. Ecol. 12(10): 2669-2680.
HEDRÉN, M., NORDSTRÖM, S. & D. STÅHLBERG (2008): Polyploid evolution and plastid DNA variation in the *Dactylorhiza incarnata/maculata* complex (*Orchidaceae*) in Scandinavia.- Mol. Ecol. 17(23): 5075-5091.
HEDRÉN, M., NORDSTRÖM, S. & R.M. BATEMAN (2011): Plastid and nuclear DNA marker data support the recognition of four tetraploid marsh-orchids (*Dactylorhiza majalis* s.l., *Orchidaceae*) in Britain and Ireland but require their recircumscription.- Biol. J. Linn. Soc. 104(1): 107-128.
HEDRÉN, M., NORDSTRÖM, S. & D. STÅHLBERG (2012): Geographical variation and systematics of the tetraploid marsh orchid *Dactylorhiza majalis* subsp. *sphagnicola* (*Orchidaceae*) and closely related taxa.- Bot. J. Linn. Soc. 168(2): 174-193.
JONSELL, B. (ed.) (2004): Flora Nordica, General volume.- Stockholm.
MAYR, E. (1942): Systematics and the origin of species.- Columbia University Press, New York.
MICHLER, B.D. (2010): Species are not uniquely real biological entities.- In: AYALA, F.J. & R. ARP (eds.): Contemporary Debates in Philosophy of Biology.- Wiley-Blackwell, Chicester, UK, pp. 110-122.

NEI, M., & W.H. LI (1979): Mathematical model for studying genetic variation in terms of restriction endonucleases.- Proc. Nat. Acad. Sci. USA 76(10): 5269-5273.

NELSON, E. (1976): Monographie und Ikonographie der Orchidaceen-Gattung *Dactylorhiza*.- Speich, Zürich.

NORDSTRÖM, S. & M. HEDRÉN (2007): Development of polymorphic nuclear microsatellite markers for polyploid and diploid members of the orchid genus *Dactylorhiza*.- Mol. Ecol. Notes 7(4): 644-647.

NORDSTRÖM, S. & M. HEDRÉN (2009): Genetic diversity and differentiation of allopolyploid *Dactylorhiza* (*Orchidaceae*) with particular focus on the *D. majalis* ssp. *traunsteineri/lapponica* complex.- Biol. J. Linn. Soc. 97(1): 52-67.

PEDERSEN, H.Æ. (2010): Inadequate morphometric analyses have contributed to oversplitting in European orchids: a case study in *Dactylorhiza* (*Orchidaceae*).- In: SEEBERG, O., PETERSEN, G., BARFOD, A.S. & J.I. DAVIS, Diversity, phylogeny and evolution in the Monocotyledons.- Aarhus University Press, Aarhus, pp. 193-212.

PILLON, Y., FAY, M. F., HEDRÉN, M., BATEMAN, R. M., DEVEY, D. S., SHIPUNOV, A. B., VAN DER BANK, M. & M.W. CHASE (2007): Evolution and temporal diversification of western European polyploid species complexes in *Dactylohiza* (*Orchidaceae*).- Taxon 56(4): 1185-1208.

ROHLF, F.J. (2005): NTSYSpc: Numerical Taxonomy System, ver. 2.20.- Exeter Publishing, Setauket, NY.

ROSEN, D.E. (1978): Vicariant pattern and historical explanation in biogeography.- Systematic Zoology 27(2): 159–188.

SIMPSON, G.G. (1961): Principles of animal taxonomy.- Columbia University Press, NY.

SLATKIN, M. (1995): A measure of population subdivision based on microsatellite allele frequencies.- Genetics 139(1): 457-462.

SOÓ, R. von. (1980): *Dactylorhiza* Necker ex Nevski.- In: TUTIN T.G., HEYWOOD V.H., BURGES N.A., MOORE D.M., VALENTINE D.H., WALTERS S.M. & D.A. WEBB (eds.) Flora Europaea, vol. 5, pp. 337-342. Cambridge University Press, Cambridge.

STACE, C.A. (1980): Plant taxonomy and biosystematics.- Edward Arnold, London.

STÅHLBERG D. & M. HEDRÉN (2010): Evolutionary history of the *Dactylorhiza maculata* polyploid complex (*Orchidaceae*).- Biol. J. Linn. Soc. 101(3): 503–525.

TYTECA, D. & J.-L. GATHOYE (1988a): Discussion du statut de *Dactylorhiza brennensis* et description de trois hybrids de cette espece.- L´Orchidophile 81: 55-61.

TYTECA, D. & J.-L. GATHOYE (1988b): Les *Dactylorhiza* d´Europe occidentale:

approche biostatistique.- Natural. belges 69, 2 (spécial Orchid. 2): 65-97.
TYTECA, D. & J.-L. GATHOYE (1988c): Une orchidée nouvelle des Hautes-Alpes: *Dactylorhiza delphinensis*.- Natural. belges 69, 2 (spécial Orchid. 2): 47-54.
TYTECA, D. & J.-L. GATHOYE (1989): Contribution à l´étude biostatistique des *Dactylorhiza* d´Europe occidentale.- Mém. Soc. Roy. Bot. Belg. 11: 43-64.
TYTECA, D. & J.-L. GATHOYE (1999): Morphometric analysis of *Dactylorhiza occitanica* and related populations from eastern France (*Orchidaceae*).- Belg. J. Bot. 132(2): 158-174.
WASER, P.M. (1998): Genetic signatures of interpopulation dispersal.- Trends Ecol. Evol.13(2): 43-44.
WILEY, E.O. (1981): Phylogenetics. The theory and practice of phylogenetic systematics.- Wiley, NY.

Supporting information available online:

https://www.orchids.de/jeo/supplements.html

Table S1: Description of plastid DNA and ITS markers used in the study of *Dactylorhiza majalis* subsp. *brennensis* and reference taxa.

Table S2: Description of nuclear microsatellite markers used in the study of *Dactylorhiza majalis* subsp. *brennensis* and reference taxa.

Table S3: Summary of plastid haplotype variation in populations of *D. majalis* subsp. *brennensis* and reference taxa.

Table S4: Annotation of ITS/ETS clades identified in Devos et al. (2006) compared to annotation of ITS alleles identified in Pillon et al. (2007).

Table S5: ITS allele frequencies estimated according to Pillon et al. (2007) in populations of *D. majalis* subsp. *brennensis* and reference taxa. Allele frequencies are reported in per cent ± 1 standard deviation. Range is given within parentheses).

Table S6: Annotation of common plastid haplotypes identified in the present study, compared to annotations used in some previous studies.

Appendix S1: Base data used in the study of *Dactylorhiza majalis* subsp. *brennensis* and reference taxa.

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