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# On the monophyly of *Dactylorhiza* Necker ex Nevski (Orchidaceae): is *Coeloglossum viride* (L.) Hartman a *Dactylorhiza*?

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Previous phylogenetic analyses of Orchidaceae subtribe Orchidinae resulted in the proposal to classify *Coeloglossum viride* (L.) Hartman within the genus *Dactylorhiza* in order to maintain its monophyly. In this paper, we report some results that contradict previous studies regarding the monophyly of the traditional *Dactylorhiza* and its phylogenetic relationship with *Coeloglossum*. Our results, which combine sequences of the internal and external transcribed spacers of the nuclear ribosomal DNA, support the monophyly of *Dactylorhiza*, with *Coeloglossum* being a sister clade. The position of *C. viride* in the phylogenetic tree, and the considerable morphological differences with respect to *Dactylorhiza*, incline us to retain both lineages as distinct genera. © 2006 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2006, **152**, 261–269.

ADDITIONAL KEYWORDS: external transcribed spacer – internal transcribed spacer nuclear ribosomal DNA – Wilcoxon signed-rank test.

## INTRODUCTION

The mainly Eurasian *Dactylorhiza* is one of the most taxonomically complex genera in the Orchidaceae. It has undergone several systematic treatments in recent decades (Heslop-Harrison, 1954; von Soó, 1980; Averyanov, 1990; Tyteca, 2001). Traditional classifications recognize four sections in the genus, namely *Aristatae*, *Sambucinae*, *Iberanthus*, and *Dactylorhiza s.s.* The section *Dactylorhiza s.s.* is by far the most species rich and taxonomically complex. It is composed of both diploid ($2n = 40$) and tetraploid ($2n = 80$) species; the latter evolved by polyploidization following hybridization between diploid species and between diploid and tetraploid species (Averyanov, 1990; Hedrén, 1996, 2002; Devos *et al*., 2005, 2006).

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The first comprehensive molecular phylogenetic studies of the subtribe Orchidinae were published by Pridgeon *et al*. (1997) and Bateman, Pridgeon & Chase (1997). These phylogenetic studies, which were based on the analysis of sequences of the internal transcribed spacers (ITSs) of the nuclear ribosomal DNA (nrDNA), yielded much valuable information regarding the classification of the Orchidinae. The phylogenetic tree published by Bateman *et al*. (1997) showed that the monotypic genus *Coeloglossum* was embedded between the *Dactylorhiza incarnata* group, on the one hand, and the remaining species of *Dactylorhiza*, including *D. aristata*, and the sections *Iberanthus* and *Sambucinae*, on the other. Thus, the genus *Dactylorhiza* in its traditional conception could no longer be considered monophyletic, and could only be resolved by incorporating *Coeloglossum* into *Dactylorhiza*. These results led Bateman *et al*. (1997) and Cribb & Chase (2001) to suggest the new combination *Dactylorhiza viridis* (L.) R.M. Bateman, Pridgeon & M.W. Chase. Several authors have used this new

classification in recently published monographs (e.g. Dusak & Pernot, 2002; Jacquet & Scappaticci, 2003; Foley & Clarke, 2005), whereas others, such as Rossi & Eldredge Maury (2002), Delforge (2005) and Baumann *et al*. (2005), have been reluctant to incorporate *Coeloglossum viride* within *Dactylorhiza*, arguing that current evidence is insufficient.

*Coeloglossum* includes only one widely recognized species, *C. viride* (L.) Hartman, and shows a circumboreal distribution that significantly overlaps with the distribution of *Dactylorhiza s.s.* (e.g. Luer, 1975; Pridgeon *et al*., 2001). *C. viride* is one of the most easily recognized species in the subfamily Orchidoidae. Its main characteristics include green or brownish flowers with a bifid lip and a globose spur, and sepals forming a protective hood over the gynostemium. The strikingly different floral morphological characters distinguishing *Coeloglossum* and *Dactylorhiza* (see Table 1) lend support to retaining *Coeloglossum* as a distinct genus. Moreover, only one nuclear region (i.e. ITS nrDNA) has been explored so far by Bateman *et al*. (1997, 2003). Amplified fragment length polymorphism data and sequence data from the chloroplast region *trn*L have failed to resolve the basal radiation of *D. incarnata*, *C. viride*, *D. sambucina* plus *D. romana*, and the remaining *Dactylorhiza* species (Bateman & Denholm, 2003). Although the ITS of 18S–26S nrDNA usually displays sufficient variation for the robust resolution of some generic and subgeneric relationships (Baldwin *et al*., 1995), it often presents too few variable nucleotide sites for robust resolution of rapidly evolving lineages. Combining the sequence data from different molecular markers is known to increase the phylogenetic accuracy (Wiens, 1998; Cognato & Vogler, 2001; Buckley *et al*., 2002; Teske, Cherry & Matthee, 2004) by recovering better resolved and better supported topologies than those derived from the analysis of sequence data from a single molecular marker. In this regard, the external transcribed spacer (ETS) of 18S–26S rDNA has recently been shown to be a good source of additional characters for increasing the resolution and phylogenetic accuracy of rDNA-based phylogenies (e.g. Baldwin & Markos, 1998; Bena *et al*., 1998; Linder *et al*., 2000; Markos & Baldwin, 2001).

In this paper, we investigate the monophyly of *Dactylorhiza* and its phylogenetic relationship with *Coeloglossum* by combining ETS and ITS sequence data. ITS sequence data have been used previously by Bateman *et al*. (1997, 2003) to infer the phylogenetic relationships between *Dactylorhiza* and *Coeloglossum*. The impact on the tree topology of the incorporation in the phylogenetic analysis of sequences from an additional region of the nuclear DNA is discussed.

## MATERIAL AND METHODS

Sequences of the ITSs and ETSs of nrDNA were obtained for two accessions of *C. viride*, one accession of *D. insularis*, one accession of *D. occitanica*, and one accession of *D. ochroleuca* (see Appendix). Moreover, 28 ITS and ETS sequences reported by Devos *et al*. (2005, 2006) in their phylogenetic analyses of the *Dactylorhiza* allotetraploid complex were also included in this phylogenetic study (Appendix). These sequences span 11 *Dactylorhiza* species that represent the main diploid and tetraploid lineages of this genus. ITS sequences for *D. markusii*, *D. romana*, *D. aristata*, and *D. euxina*, published by Pillon *et al*. (2006), were downloaded from GenBank and added to the data

**Table 1.** Comparison of the morphology of *Coeloglossum* and *Dactylorhiza*

| Character | *Coeloglossum* | *Dactylorhiza* |
|---|---|---|
| Flowers: general colour | Green, sometimes reddish or brownish | White, pink, purple, yellow |
| Perianth arrangement | Lateral sepals included in protective hood | Lateral sepals spread (exception: *D. iberica*) |
| Lip shape | Elongated, with bifid apex and small tooth in indentation | Generally three-lobed, with wide lateral lobes and variable median |
| | Oblong, with parallel lateral margins | lobe; seldom undivided |
| | | Elliptical to rhomboidal |
| Lip length vs. width | Much longer than wide | Wider than long or equal |
| Lip markings | None | Generally red to purple spots, lines and/or loops; seldom no markings |
| Spur shape and aspect | Roughly spherical, shiny | Cylindrical to conical, rarely sac-shaped, matte |
| Spur length | Much shorter than ovary | Much longer to roughly equal to ovary |
| Nectar production | Spur nectariferous | No nectar |
| Pollinia | Strongly divergent | Parallel, close to each other |
| Viscidia | Naked, in rudimentary bursicles | Joined together in a well-formed bursicle |

matrix in order to include as many *Dactylorhiza* species/lineages as possible.

Total genomic DNA was isolated from leaf tissue using the cetyltrimethylammonium bromide procedure outlined by Doyle & Doyle (1987), but without RNAse treatment. The complete ITS region, and a fragment of approximately 1000 bp at the 3′ end of the ETS region, were amplified by polymerase chain reaction (PCR) according to the protocol described by Devos *et al*. (2005). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), and both strands of purified ITS and ETS PCR products were sequenced directly using BigDye terminators (Applied Biosystems). Complementary strands of both ITS and part of the ETS region were assembled using Sequencher 4.01 (Gene Codes Corporation, 1998). ITS and ETS sequences were deposited in GenBank (Appendix).

Using as a guide previously published molecular systematic studies of the subtribe Orchidinae (Bateman *et al*., 1997, 2003), *Gymnadenia conopsea*, *G. borealis*, *G. nigra*, and *G. austriaca* were selected as outgroup species because of their sister relationship with *Dactylorhiza* and *Coeloglossum*. Adding representatives of more distantly related genera as additional outgroup taxa did not improve the phylogenetic resolution of the ingroup. Adding distant outgroup taxa simply leads to the addition of long branches to the base of the tree increasing the possiblity of long-branch attraction (Maddison, Donoghue & Maddison, 1984; Sanderson & Shaffer, 2002). All sequences were aligned manually using MacClade 4.07 (Maddison & Maddison, 2005), with gaps inserted where necessary to preserve positional homology. The two data sets were merged into a single matrix prior to phylogenetic analysis, and gaps were coded as missing data. ETS sequences were unavailable for four ingroup species (i.e. *D. markusii*, *D. romana*, *D. aristata*, and *D. euxina*) and three species from the outgroup (*G. borealis*, *G. nigra*, and *G. austriaca*). The inclusion of taxa that are characterized by incomplete sequence data into a combined analysis is unlikely to be problematic as long as sufficient characters are present in one broadly sampled data set to allow the position of these taxa to be resolved (Wiens, 2003).

### Phylogenetic analyses

Phylogenetic relationships between taxa were investigated using both maximum parsimony (MP) and Bayesian inference (BI). Parsimony tree searches were performed in PAUP*4.0b10 (Swofford, 2002). Equally weighted heuristic tree searches were performed using 1000 random sequence additions and tree bisection–reconnection (TBR) branch swapping. Nodal support was evaluated in PAUP* with 500 non-parametric bootstrap pseudo-replicates, using a simple addition sequence of taxa and TBR branch swapping.

Bayesian phylogenetic inference was performed with MrBayes 3.0 (Huelsenbeck & Ronquist, 2001). A metropolis-coupled Markov chain Monte Carlo algorithm was employed for two million generations, sampling trees every 100 generations, with four independent chains running simultaneously. The general time-reversal model with a gamma distribution parameter of $\alpha = 0.5223$ was used to estimate the likelihood values. This model was determined by a hierarchical likelihood ratio test based on the Akaike Information Criterion, using the program MrModeltest version 2.2 (Nylander, 2002). All the resulting trees were imported into PAUP* and a 50% majority-rule consensus tree was generated after discarding the burn-in trees.

### Hypothesis testing

The phylogenetic hypothesis in which *C. viride* was placed between the *D. incarnata s.l.* group and the remaining *Dactylorhiza* species was tested against our most-parsimonious topology. To find the most-parsimonious topology compatible with the alternative hypothesis, a constraint tree was first constructed using MacClade 4.07 (Maddison & Maddison, 2005). This constraint tree was ((((remaining *Dactylorhiza* species),(*C. viride*)),(*D. incarnata s.l.*)),(*Gymnadenia* species)). Heuristic searches were then carried out in PAUP* with the constraint enforced. A two-tailed Wilcoxon signed-rank test (Templeton, 1983), as implemented in PAUP*, was used to determine whether our most-parsimonious tree was significantly shorter than the best alternative topology under the constraint, or whether the difference in length was statistically indistinguishable.

## RESULTS

The resulting aligned ITS plus ETS data matrix (including the outgroup) had a length of 1603 characters, 332 of which were variable and 186 were parsimony informative. The alignment of the two *C. viride* accessions with the *Dactylorhiza* species could only be achieved by the inclusion of two major gaps. One gap of 40 bp in length was inserted for the alignment of the ITS data set, and a gap of 110 bp was inserted in order to align the ETS sequences.

The unconstrained parsimony analysis of this data set resulted in 2803 equally most-parsimonious trees of 375 steps [retention index (RI) = 0.963; consistency index (CI) = 0.899]. Figure 1 presents the strict consensus of all the most-parsimonious trees recovered by the MP analysis. The Bayesian analysis of the

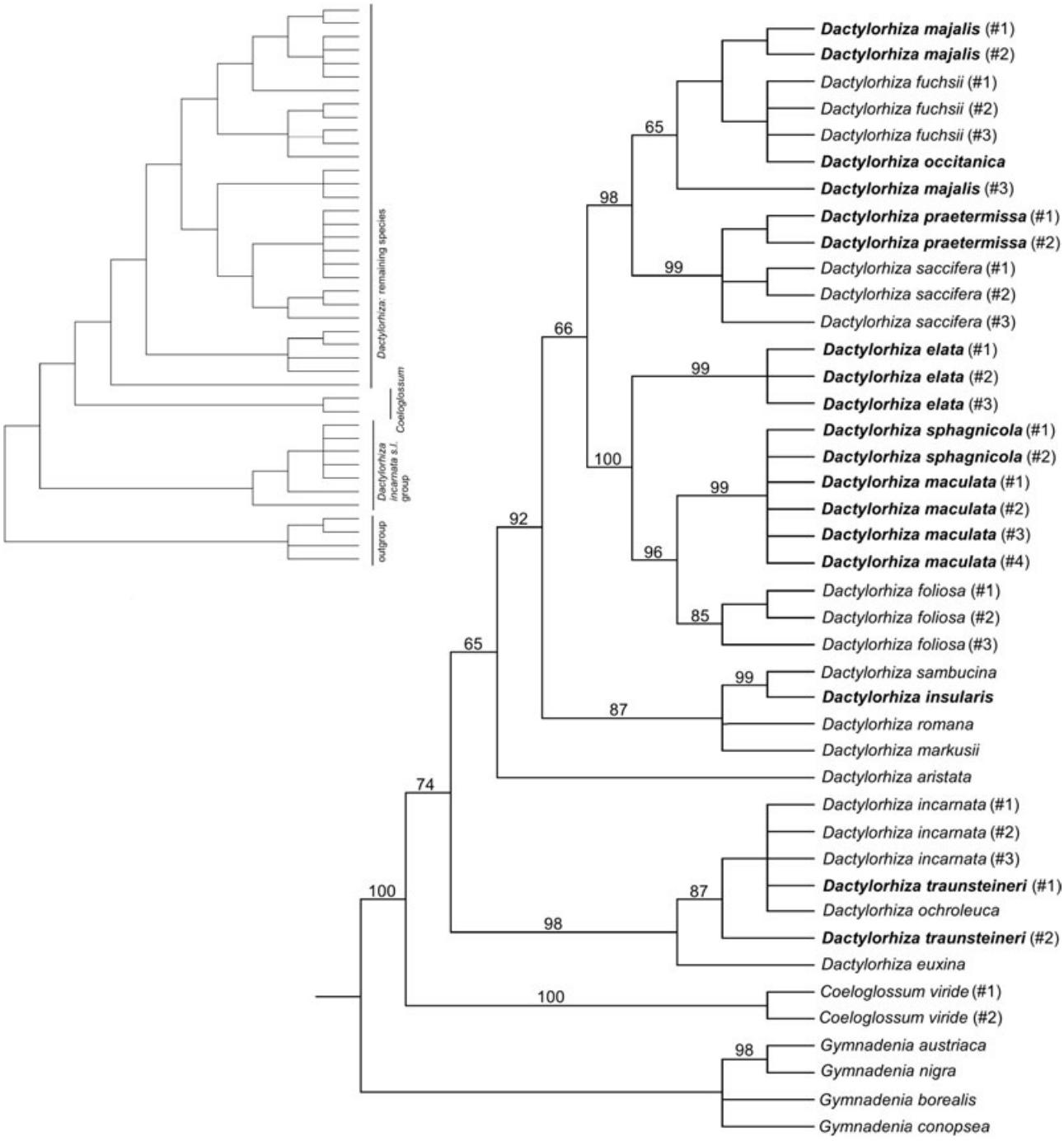


**Figure 1.** Strict consensus tree resulting from the parsimony analysis of the combined internal transcribed spacer (ITS) and external transcribed spacer (ETS) data set. Nodal support is indicated above the branches. Numbers after the species names indicate the accession number (Appendix). Terminals in bold are polyploid species ($2n = 80$, except *Dactylorhiza insularis* $2n = 60$). The inset represents the strict consensus of all the most-parsimonious trees that were constrained to reflect the alternative phylogenetic hypothesis in which *Coeloglossum* would be resolved within *Dactylorhiza*, following Bateman *et al*. (1997, 2003).

same data set, initiated from a random starting tree, converged on similar log-likelihood scores and reached stationarity after 22 000 generations. The initial 221 trees sampled were discarded and a 50% majority-rule consensus of the remaining 19 780 trees (Fig. 2) resulted in a phylogenetic hypothesis congruent with the phylogenetic tree derived from the MP analysis. Both the MP and BI analyses resolved the two *C. viride* accessions as sister clade of the *Dactylorhiza* genus. Nodal support for the branch separating *Coeloglossum* from *Dactylorhiza s.s.* was high (Figs 1, 2).

The MP search for the shortest tree that was constrained to include *Coeloglossum* within *Dactylorhiza*, as suggested by Bateman *et al*. (1997, 2003), yielded 2805 equally parsimonious trees of 386 steps (RI = 0.952, CI = 0.873, Fig. 1, inset). As determined by the Wilcoxon signed-rank test, the shortest constrained phylogenetic tree was significantly longer than the shortest unconstrained tree ($N = 17$, $Z = -2.6679$, $P = 0.0076$). The null hypothesis of *Coeloglossum* being part of *Dactylorhiza*, as suggested by Bateman *et al*. (1997, 2003), was therefore rejected.

## DISCUSSION

As expected, the phylogenetic signal that emerged from the combined ITS and ETS data set was strong, as indicated by high bootstrap and posterior probability values. In the studies by Bateman *et al*. (1997, 2003), *C. viride* appeared inside the clade formed by *Dactylorhiza* species, whereas our analysis placed this species outside the *Dactylorhiza* clade. This difference can be attributed to the inclusion of the ETS sequences in our data set, which increased the total number of parsimony informative characters (48 for the ITS data set alone vs. 186 for the combined ITS and ETS data set). A bootstrap value of 74 and a posterior probability value of 98 for the node defining *Dactylorhiza* (Figs 1, 2) indicates potentially strong support for the monophyly of *Dactylorhiza*. A more definitive statistical criterion was to apply the Wilcoxon signed-rank test (Templeton, 1983; Felsenstein, 1985) to test whether the shortest tree violating the monophyly of *Dactylorhiza* (Fig. 1, inset) was significantly longer than the most-parsimonious tree. According to this test, our molecular data strongly support the monophyly of *Dactylorhiza s.s.* with *C. viride* being its sister taxon.

The most important morphological characters that differ between the two genera are floral traits (Table 1). There are no significant differences with regard to vegetative characters, because of the considerable variability of *Dactylorhiza* in this respect (e.g. Vermeulen, 1947; Nelson, 1976; Tyteca & Gathoye, 2000). Such variability in vegetative characters ensures that *Coeloglossum* characters fall within the *Dactylorhiza* ranges. Floral traits are highly significant with regard to pollination and insect attraction strategies, two important factors for plant speciation (especially in orchids). Of particular significance is the strongly different position of the pollinia. They are well separated and divergent in *Coeloglossum*, but are parallel and closely juxtaposed in *Dactylorhiza*. The viscidia are naked in *Coeloglossum* but held together in a bursicle in *Dactylorhiza* (Bournérias & Prat, 2005: fig. 3.14). One of the most striking differences between *Coeloglossum* and *Dactylorhiza* is related to their insect attraction strategy. *Coeloglossum* produces nectar, displaying a rewarding strategy. In contrast, *Dactylorhiza* species do not produce nectar and pursue a food-deceit strategy by attracting insects with their more showy flowers, which sometimes mimic flowers of other nectariferous species (e.g. Nilsson, 1981).

In the case of the *Platanthera s.l.* + *Galearis* + *Pseudorchis* + *Gymnadenia* + *Coeloglossum* + *Dactylorhiza s.s.* clade (e.g. Bateman *et al*., 2003), the relationship between the traditional *Dactylorhiza* and *C. viride* is of particular importance in order to reconstruct the evolutionary history of pollination strategies onto the phylogenetic tree. If the ancestor of this clade was characterized by a rewarding pollination strategy (most of these genera are well known for producing nectar, with the notable exception of *Dactylorhiza*), the phylogenetic hypothesis in which *C. viride* (nectariferous) has a basal position relative to *Dactylorhiza* (non-nectariferous) would imply the acquisition of the food-deceit pollination strategy along the branch leading to the ancestor of the *Dactylorhiza* species. In the alternative phylogenetic hypothesis, in which *C. viride* is embedded inside the lineage that represents the genus *Dactylorhiza*, two steps would be required to explain the evolution of the pollination strategies. The food-deceit pollination strategy would have evolved along the branch leading to the *Dactylorhiza* (including *C. viride*) clade and would have reverted later to a rewarding strategy along the branch leading to *C. viride*. Thus, the phylogenetic hypothesis in which the *C. viride* lineage has a basal relationship with the *Dactylorhiza s.s.* lineage is most parsimonious, at least for this particular character.

The topology of our molecular phylogenetic tree, and the extreme morphological divergence between *Coeloglossum* and *Dactylorhiza*, are in favour of maintaining *Coeloglossum* as a separate genus. Maintaining *Coeloglossum* would be compatible with the monophyly of *Dactylorhiza s.s.* and with the distinct floral morphology and insect attraction strategy that both genera display.

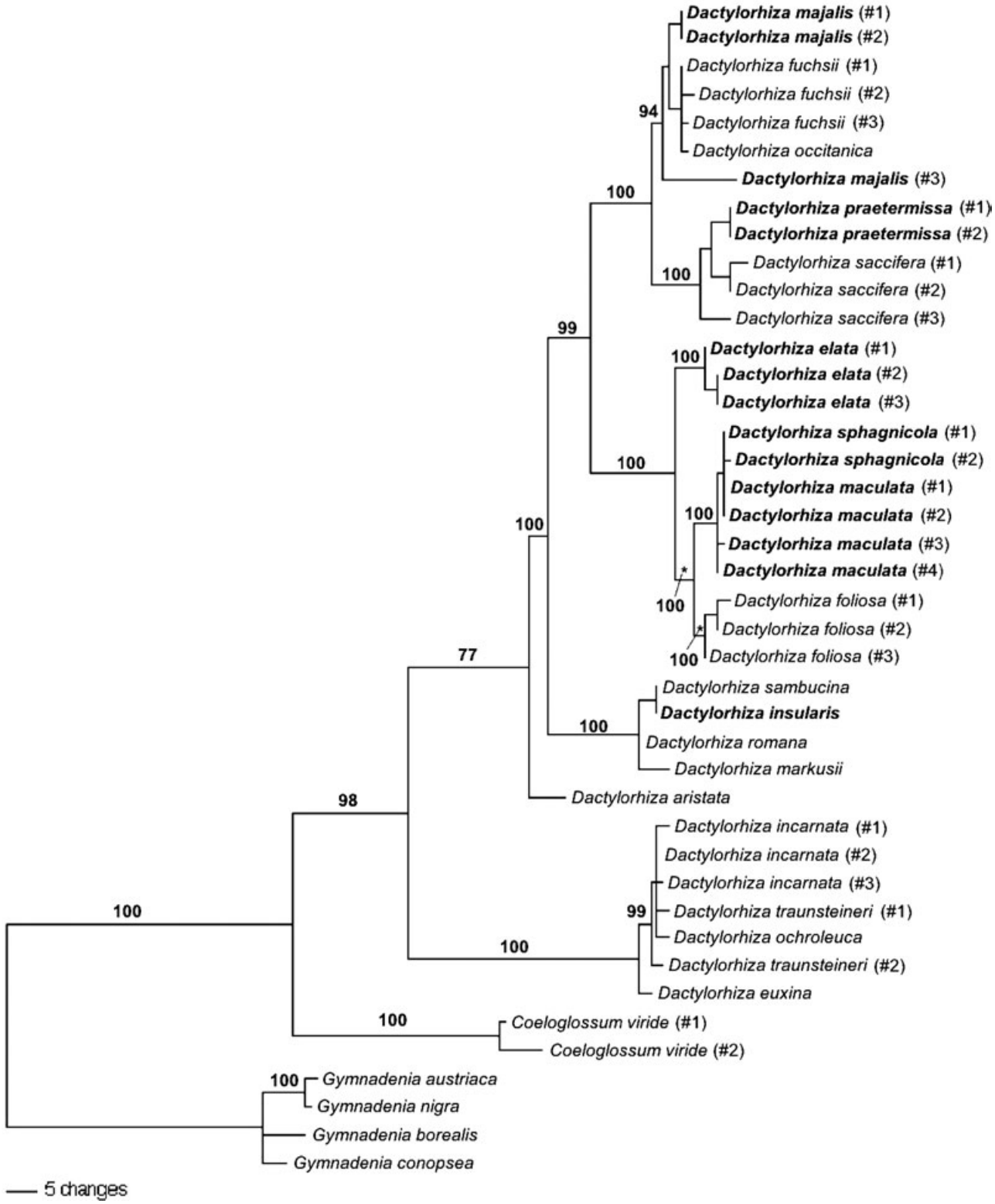


**Figure 2.** Fifty per cent majority-rule consensus of the trees recovered by Bayesian analysis. Bayesian posterior probabilities are given above the branches. Terminals in bold are polyploid species ($2n = 80$, except *Dactylorhiza insularis* $2n = 60$). Number after the species name refers to the accession number (Appendix).

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## APPENDIX

List of taxa analysed in this study with GenBank accession numbers for internal transcribed spacer (ITS) and external transcribed spacer (ETS) regions (–, missing sequences, not sequenced)

| Species | Geographical location | Accession number | ITS | ETS | Published |
|---|---|---|---|---|---|
| *Coeloglossum viride* (L.) Hartman | Bure, Belgium | 1 | DQ303385 | DQ303390 | This study |
| | Fondamente, France | 2 | DQ303386 | DQ303391 | This study |
| *Dactylorhiza aristata* (Fischer ex Lindley) Soó | | | DQ022872 | – | Pillon *et al*. (2006) |
| *D. elata* (Poiret) Soó | Col de l'homme mort, France | 1 | DQ074230 | DQ074305 | Devos *et al*. (2006) |
| | Col de l'homme mort, France | 2 | DQ074231 | DQ074307 | Devos *et al*. (2006) |
| | Col de l'homme mort, France | 3 | DQ074233 | DQ074308 | Devos *et al*. (2006) |
| *D. euxina* (Nevski) Czerepanov | | | DQ022886 | – | Pillon *et al*. (2006) |
| *D. foliosa* (Solander ex Lowe) Soó | Portela, Madeira | 1 | AY699480 | AY699553 | Devos *et al*. (2005) |
| | Encumeada, Madeira | 2 | AY699484 | AY699557 | Devos *et al*. (2005) |
| | Queimadas, Madeira | 3 | AY699486 | AY699559 | Devos *et al*. (2005) |

APPENDIX *Continued*

| Species | Geographical location | Accession number | ITS | ETS | Published |
|---|---|---|---|---|---|
| *D. fuchsii* (Druce) Soó | Bievres, France | 1 | AY699435 | AY699523 | Devos *et al*. (2005) |
| | Baronville, Belgium | 2 | AY699424 | AY699513 | Devos *et al*. (2005) |
| | Kerseguenou, France | 3 | AY699427 | AY699516 | Devos *et al*. (2005) |
| *D. incarnata* (L.) Soó | Platte de sous les Monts, France | 1 | DQ074218 | DQ074293 | Devos *et al*. (2006) |
| | Kerseguenou, France | 2 | DQ074221 | DQ074296 | Devos *et al*. (2006) |
| | Col de Perjuret, France | 3 | DQ074222 | DQ074297 | Devos *et al*. (2006) |
| *D. insularis* (Sommier) Landwehr | Serra de Montejunto, Portugal | | DQ303383 | DQ303388 | This study |
| *D. maculata* (L.) Soó | Wesomont, Belgium | 1 | AY699447 | AY699534 | Devos *et al*. (2005) |
| | St-Hubert, Belgium | 2 | AY699448 | AY699535 | Devos *et al*. (2005) |
| | Guisseny, France | 3 | AY699459 | AY699545 | Devos *et al*. (2005) |
| | Dourbies, France | 4 | AY699460 | AY699546 | Devos *et al*. (2005) |
| *D. majalis* | Herock, Belgium | 1 | DQ074206 | DQ074281 | Devos *et al*. (2006) |
| (Reichenbach) P.F. | Herock, Belgium | 2 | DQ074208 | DQ074283 | Devos *et al*. (2006) |
| Hunt & Summerhayes | Lanuejols, France | 3 | DQ074217 | DQ074292 | Devos *et al*. (2006) |
| *D. markusii* (Tineo) H. Baumann & Künkele (unpublished) | | | AY369085 | – | S. Bernardos, J.L. Revuela, M.D.L.A. Santos & F. Amich (unpubl. data) |
| *D. occitanica* Geniez, Melki, Pain & Soca | Mas-de-Londres, France | | DQ303384 | DQ303389 | This study |
| *D. ochroleuca* (Wüstnei ex Boll) J. Holub | Ettal marsh, Germany | | DQ303382 | DQ303387 | This study |
| *D. praetermissa* | Guisseny marsh, France | 1 | DQ074224 | DQ074299 | Devos *et al*. (2006) |
| (Druce) Soó | Keremma, France | 2 | DQ074225 | DQ074300 | Devos *et al*. (2006) |
| *D. romana* (Sebastiani) Soó | | | DQ022872 | – | Pillon *et al*. (2006) |
| *D. saccifera* | Venaco, Corsica | 1 | AY699488 | AY699561 | Devos *et al*. (2005) |
| (Brongniart) Soó | Vizzavona, Corsica | 2 | AY699490 | AY699563 | Devos *et al*. (2005) |
| | Val di Vari, Italy | 3 | AY699498 | AY699573 | Devos *et al*. (2005) |
| *D. sambucina* (L.) Soó | Seoane, Spain | | DQ073238 | DQ074313 | Devos *et al*. (2006) |
| *D. sphagnicola* | Plaine-Haie, Belgium | 1 | DQ074240 | DQ074315 | Devos *et al*. (2006) |
| (Höppner) Soó | Plaine-Haie, Belgium | 2 | DQ074241 | DQ074316 | Devos *et al*. (2006) |
| *D. traunsteineri* | Praubert, France | 1 | DQ074227 | DQ074302 | Devos *et al*. (2006) |
| (Sauter ex Reichenbach) Soó | Praubert, France | 2 | DQ074228 | DQ074303 | Devos *et al*. (2006) |
| Outgroups | | | | | |
| *Gymnadenia austriaca* (Teppner & E. Klein) Delforge | | | DQ022893 | – | Pillon *et al*. (2006) |
| *G. borealis* (Druce) R.M. Bateman, Pridgeon & M.W. Chase | | | DQ022889 | – | Pillon *et al*. (2006) |
| *G. conopsea* (L.) R. Brown | Lavaux St. Anne, Belgium | | AY699508 | AY699574 | Devos *et al*. (2005) |
| *G. nigra s.s.* (L.) Reichenbach f. | | | DQ022892 | — | Pillon *et al*. (2006) |