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Phylogenetic relationships of *Deschampsia antarctica* (Poaceae): Insights from nuclear ribosomal ITS

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Abstract. *Deschampsia antarctica* E. Desv. is the only monocot in the Antarctic floristic zone. We evaluated the phylogenetic relationships of *Deschampsia antarctica* to other grasses using parsimony as the optimality criterion. Five different sets of gap, transversion and transitions costs were explored to analyze the effect of parameter choice on the phylogenetic results. Both internal transcribed spacers (ITS1 and ITS2) and the 5.8S subunit of nuclear ribosomal DNA were included in the analysis. A total of 43 species were analyzed including seven species of *Deschampsia*. *Deschampsia antarctica* forms a well supported group with five species of *Deschampsia*. *Deschampsia* does not appear monophyletic as *D. flexuosa* (L.) Trin. is not included in this clade. The clade to which *D. antarctica* belongs is sister to some Aveneae in all analyses. This study is the first contribution that evaluates the phylogenetic position of *D. antarctica* in relation to other species of *Deschampsia*.

Key words: *Deschampsia antarctica*, phylogenetics, 5.8S, ITS, sequence, Poaceae, Aveneae.

Deschampsia antarctica is one of the two native phanerogams known from within the Antarctic botanical zone. It occupies the tip of the American southern hemisphere, Antarctica and subantarctic islands, south of Argentina and Chile (Nicora 1978, Soreng 1997). The resistance of *D. antarctica* to the incredibly harsh environment of the Antarctic continent (air temperatures that average well below freezing all year round, strong winds that increases the effects of the cold, light which varies from months of total darkness to total sunlight) makes it a valuable resource for the identification of genes associated with freezing (Gidekel et al. 2003). It is also important in evaluation of the mechanisms that protect plants against elevated levels of solar UV-B radiation (Van de Staaij et al. 2002). Despite the importance of this only antarctic monocot, there are no previous studies on its phylogenetic position using molecular markers.

The Poaceae (grass family) occupies about one third of the earth's surface and has the fifth largest number of species of any flowering plant family (Clayton and Renvoize 1986). This family comprises nine major subfamilies belonging to the BEP (Bambusoideae,

Ehrhartoideae and Pooideae) and to the PACCAD clades (Panicoideae, Arundinoideae, Chloridoideae, Centothecoideae, Aristidoideae and Danthonioideae), respectively (GPWG 2001). Pooideae, has been divided in 13 tribes, but many conflicts exist between molecular and morphological data and relationships among some of the mayor lineages of the core Pooideae clade remain unresolved (GPWG 2001).

From a morphological point of view, *Deschampsia* has been traditionally placed within the tribe Aveneae (Nicora 1978, Clayton and Renvoize 1986, Tzvelev 1989, Watson and Dallwitz 1992, ProFlora 1996). However, there has been some controversy about the placement of the genus *Deschampsia* with molecular evidence. Results of some studies, using chloroplast DNA and nuclear (ITS) sequences, are in agreement with the traditional taxonomy (Hsiao et al. 1995, Soreng and Davis 2000). Results of other studies using chloroplast sequences suggested that *Deschampsia* should be placed among the genera most closely related to Poeae (Nadot et al. 1994, Catalan et al. 1997). Recently it has been proposed that the distinction between Aveneae and Poeae be abandoned in favor of the recognition of one tribe, Poeae, with a series of subtribes (Soreng and Davis 2000).

Nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) and the coding 5.8S sequences were chosen to analyze the phylogenetic position of *D. antarctica* within the Poaceae. The ITS1 and ITS2 spacers have been shown to constitute a valuable source of molecular characters to reconstruct plant phylogeny (Hsiao et al. 1994, 1999; Baldwin et al. 1995; Jobes and Thien 1997; Hershkovitz and Zimmer 1996; Aïnouche and Bayer 1999; Nishikawaa et al. 1999). The aim of this study is to provide sequence data to clarify the phylogenetic relationships of *Deschampsia antarctica* through ITS 1, 5.8S gene and ITS 2 sequences within the Poaceae family and determine the phylogenetic position of the genus *Deschampsia*.

Material and methods

Plant material. *Deschampsia antarctica* was collected from the Antarctic Peninsula (Jubany Argentine Navy Base 62°14'18''South 58°40'West) and complete specimens were frozen at –20°C previous to use. Specimens of *Bromus catharticus* Vahl., *Bromus* sp., *Poa annua* L. and *Stenotaphrum secundatum* (Walt) Kuntze, were collected in gardens of the Buenos Aires Metropolitan area (34°48'South, 58°West). DNA samples from each species were obtained from individual plants. *Triticum aestivum* L. was obtained from Banco de germoplasma del Instituto de Recursos Biológicos del INTA Castellar. In addition, ITS1, 5.8S gene and ITS2 sequences of 37 species from GenBank were included in the analysis resulting in a total of 43 sequences that includes seven *Deschampsia* species (approx. 30% of the genus), 11 more Aveneae and 17 Poeae (Table 1). Based on previous phylogenies (GPWG 2001) *Oryza sativa* was used as functional outgroup in order to root the trees. Names, voucher and GenBank accession numbers are listed in Table 1. Taxonomic determination of individuals sequenced in this study was kindly conducted by Dr. Fernando O. Zuloaga, specialist in Poaceae, with help of Dr. Gustavo Giberti. Voucher specimens were deposited at the Pharmacobotany Museum "Juan A. Domínguez" (Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina).

DNA extraction. Total genomic DNA was isolated from approximately 1 g of young leaves following a modified CTAB procedure (Glick and Thompson 1993) by adding 20 µl of proteinase K (60 U/ml) before the 55°C overnight incubation.

DNA amplification and sequencing. Two primers flanking the entire ITS1, 5.8S and ITS2 region were used to amplify this segment. These included a forward primer, ITS 5, annealing to the 18S gene (5′-GGAAGGAGAAGTCGTAA CAAGG-3′) (an adapted version of White et al. 1990 with modifications made in positions 6: T→G and 8: A→G), and a reverse primer, ITS 4, annealing to the 26S gene (5′-TCCTCCGC TTATTGATATGC-3′) (White et al. 1990). Amplification of the ITS1, 5.8S, and ITS2 region was conducted in a volume of 30 µL, reaction mix contained 19 µL sterile water, 3 µL of 10x reaction buffer, 0.6 µL of a mix of each 10 mM dNTP, 2.4 µL $MgCl_2$ (25 mM), using 0.15 µL (5 U/µl) of the *Taq* polymerase (Promega, Madison, USA),

Table 1. Species, Tribes, Sources, Pharmacobotany Museum and GenBank accession

Species	Tribe	Source	Pharmacobotany Museum Accession N°	GenBank Accession N°
Bromus catharticus Vahl.	Bromeae	Corach D. et al.	BAF 10284	AF 521898
Bromus sp.	Bromeae	Corach D. et al.	BAF 10276	AF 521899
Deschampsia antarctica E. Desv.	Aveneae	Corach D. et al.	BAF 10287	AF 521900
Poa annua L.	Poeae	Corach D. et al.	BAF 10281	AF 521901
Stenotaphrum secundatum (Walt) Kuntze	Paniceae	Corach D. et al.	BAF 10275	AF 521902
Triticum aestivum L.	Triticeae	Corach D. et al.	Banco de germoplasma[a]	AF 521903
Stipa ichu Kunth.	Stipeae	Hsiao C. et al.		AF019803
Deschampsia cespitosa (L.) P. Beauv.	Aveneae	Hsiao C. et al.		L36513
Avena longiglumis Dur.	Aveneae	Chatterton N. et al.		GI16001
Agrostis capillaris L.	Aveneae	Subbotin S. A. et al.		AF498395
Arrhenatherum elatius (L.) P. Beauv.	Aveneae	Hsiao C. et al.		AF019795
Brachypodium distachyon (L.) Beauv.	Brachypodieae	Torrecilla P. and Catalan P.		AF303399
Brachyelytrum erectum (Schreb.) P. Beauv.	Brachyelytreae	Hsiao C. et al.		AF019794
Diarrhena americana P. Beauv.	Diarrheneae	Hsiao C. et al.		AF019798
Helleria fragilis Luces.	Poeae	Catalan P. et al.		AF532960
Zizaniopsis miliacea (Michx.) Doell. & Asch.	Oryzeae	Kahn A. B. and Horne F.		AF169235
Nardus stricta L.	Nardeae	Hsiao C. et al.		AF019796
Arctagrostis latifolia (R. Br.) Griseb.	Poeae	Brysting A. K. et al.		AY237843
Arctophila fulva (Trin.) N.J. Andersson	Poeae	Brysting A. K. et al.		AY237832
Poa alpina var. alpina (L.)	Poeae	Brysting A. K. et al.		AY237837
Poa arctica R. Br.	Poeae	Brysting A. K. et al.		AY237842
Castellia tuberculosa (Moris) Bor.	Poeae	Catalan P. et al.		AF532954
Monerma cilindrica (Willd.) Cosson et Durieu	Poeae	Catalan P. et al.		AF532941
Sphenopus divaricatus (Gouan) Rchb.	Poeae	Catalan P. et al.		AF532939
Schedonorus arundinaceus (Schreb.) Dumort.	Poeae	Catalan P. et al.		AF532951
Micropyropsis tuberosa Romero-Zarco Cabezudo.	Poeae	Catalan P. et al.		AF532943
Dactylis glomerata L.	Poeae	Torrecilla P. and Catalan P.		AF393013
Deschampsia alpina (L.) Roem. & Schult.	Aveneae	Brysting A. K et al.		AY237845
Deschampsia flexuosa (L.) Trin.	Aveneae	Brysting A. K et al.		AY237846

Table 1. (Continued)

Species	Tribe	Source	Pharmacobotany Museum Accession N°	GenBank Accesion N°
Deschampsia sukatschewii (Popl.) Roshev.	Aveneae	Brysting A. K et al.		AY237844
Deschampsia mejlandii C.E. Hubb.	Aveneae	Chiapella J. O.		AF486268
Deschampsia christophersenii C.E. Hubb.	Aveneae	Chiapella J. O.		AF486267
Secale montanum Guss.	Triticeae	Asay K. H. et al.		Z11760
Aegilops speltiodes Tausch.	Triticeae	Chatterton N. J.		Z11762
Calamagrostis epigejos (L.) Roth.	Aveneae	Jakob S. S. and Blattner F. R.		AJ306448
Zingeria trichopoda (Boiss) Smirnow.	Aveneae	Kotseruba V. et al.		AJ428835
Avena wiestii Steud.	Aveneae	Rodionov A.V. et al.		AY216261
Festuca rupícola Heuff.	Poeae	Penksza K. and Illyes Z.		AJ508379
Lolium temulentum L.	Poeae	Charmet G. et al.		AJ240145
Hordeum vulgare L.	Triticeae	Chatterton N. J. et al.		Z11759
Sclerochloa dura (L.) Beauv.	Poeae	Catalan P. et al.		AF532933
Dupontia fisheri R. Br.	Poeae	Brysting A. K et al.		AY237885
Oryza sativa L.	Oryzeae	Kahn A. B. and Horne F.		AF169230

[a] INTA Germplasm bank CIRN- CNIA INTA Castelar (Buenos Aires, Argentina)

1 μL of each primer (0.1 μg) and 1 μL of template. The PCR program consisted of a first denaturing step of 4 min at 94°C, 40 amplification cycles (94°C for 30 s, 50°C for 60 s, 72°C for 2 min) and a final extension of 5 min at 72°C in a Perkin-Elmer thermal cycler model TC1, USA. Amplicons were purified using Wizard PCR Prep (Promega, Madison, USA) or QIAquick (QIAGEN, Amsterdam, The Netherlands) following the manufacturers protocols. Cycle sequencing of the purified amplification products were conducted with Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, USA). Sequences were analyzed with an ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, USA).

Phylogenetic analysis. Sequence data were analyzed using the "direct optimization" method (Wheeler 1996; see also Wheeler and Hayashi 1998) performed with the computer software POY (Wheeler et al. 2002) with parsimony as the optimality criterion. This method, as opposed to the more classical two-step analyses (alignment + tree search), minimizes the weighted number of evolutionary changes over the entire tree, working in a one-step fashion (i.e. treats the indels as processes as opposed to the patterns implied by multiple sequence alignment). The results of this procedure are directly compatible with parsimony-based tree lengths but are much less computational demanding and appear to generate more efficient (simpler) explanations of sequence variation than multiple sequence alignment (Wheeler 1996).

Sequences where separated in three different files in areas of unambiguous alignment as suggested by Giribet (2003) as it increases computation efficiency.

Nodal support was estimated by Bremer support (Bremer 1988). Since this approach generally overestimates nodal support, the support of certain nodes was also tested by more exhaustive constraint

Table 2. Tree length for the molecular data sets at different parameter values, and number of optimal trees for each parameter set

TS:TV:gap	trees Length	Number of optimal trees
1:1:1	1608	2
2:1:1	1904	3
4:2:1	2985	1
5:1:1	2592	2
16:4:1	6859	2

searches. Constraints were also used to force monophyly of some clades that do not appear in most parsimoniovs trees. Nodal support was estimated in each of the parameter set analysis.

Searching strategy consisted of 400 Wagner trees generated through random addition sequences followed by TBR holding two trees per replication (Complete command line used for one step matrix (511): poy.exe 1ter43.txt 2ter43.txt 3ter43.txt -molecularmatrix mat5.txt -nooneasis -replicates 400 -seed -1 -nospr -buildsperreplicate 2 -stopat 4 -minstop 50 -holdmaxtrees 600 -poy-strictconsensuscharfile constrain.txt -maxtrees 2 -tbr > 16_4_1.out). Since parameter choice is arbitrary, a sensitivity analysis (sensu Wheeler) is considered a way to explore the data and to discern between robust relationships (those supported throughout a wide range of parameters) and unstable relationships (those that appear only under particular parameter sets). In total, five combinations of parameters were employed in the analysis (Transition, transversion and gap costs: 1:1:1, 1:1:2, 1:1:5, 1:2:4 and 1:4:16).

Results and discussion

The length and number of optimal trees under each parameter set is shown in Table 2. The genus *Deschampsia* does not appear as monophyletic in the optimal trees under any parameter set (Figs. 1, 2). While six species of the genus, *D. alpina*, *D. sukatschewii*, *D. christophersenii*, *D. cespitosa* P. Beauv., *D. mejlandii* and *D. antarctica*, form a well-supported clade under all conditions (Bremer support : 8 to 10 depending on the parameter set), *D. flexuosa* is not included in this group. The constraint for monophyly of the genus resulted in trees with 2 to 18 weighted steps more than the optimal trees depending on the alignment condition. The optimal trees obtained in each analysis differ only in the position of *D. antarctica*. This species appeared in three positions: as sister to all other *Deschampsia* (excluding *D. flexuosa*), as a sister group of *D. cespitosa- D. alpina-D. sukatschewii*, and as sister to *D. mejlandii–D. christophersenii*. Moreover, *D. cespitosa-D. alpina- D. sukatschewii* and *D. mejlandii–D. christophersenii* form groups that are not sensitive to parameter set variation, with Bremer support of 4 to 13 and 4 to 12, respectively. In conclusion the phylogenetic position of *D. antarctica* could not be satisfactorily answered. However, from a biogeographic point of view, it is important to notice that *D. antarctica* never appear as sister group of a clade formed exclusively for Northern hemisphere species (Fig. 1).

One point of controversy is the position of *Deschampsia* respect to Aveneae/ Poeae. In these analyses, the *Deschampsia* clade (except *D. flexuosa*) appears as a sister group to an Aveneae clade in every tree irrespective of the condition considered, although with a weak support (Bremer value 1 to 5). This is in agreement with the results of both Soreng and Davis (2000) and Hsiao et al. (1995) in which *Deschampsia* forms a clade with species of Aveneae and not with species of Poeae as suggested by Nadot et al. (1994) and Catalan et al. (1997). Our results also agree with those of Soreng and Davis (2000), since in the present work Poeae and Aveneae did not appear as monophyletic in any tree under any condition. Different authors have indicated that high nodal stability is not necessarily linked to high nodal support (Goloboff et al. 2003, Giribet 2003).

In our analysis we found cases in which this correlation did exist, but other cases in which it is clearly contradicted. For example, the node *Deschampsia* (excluding *D. flexuosa*) + Aveneae appeared as a monophyletic group under every condition, but with low support under each condition (1 to 5). In

contrast, the clade formed by all the ingroup except *Zizaniopsis miliacea* and *Stenotaphrum secundatum* generated a Bremer value of 18 in the condition 4:2:1, but this clade is not present in the optimal trees under the conditions 1:1:1, and 2: 1:1. This study is the first that evaluate the taxonomic position of *D. antarctica* at a molecular level and also is the first time that many species of the genus are analyzed together. However a more comprehensive molecular analysis incorporating more species of *Deschampsia* and close rela-

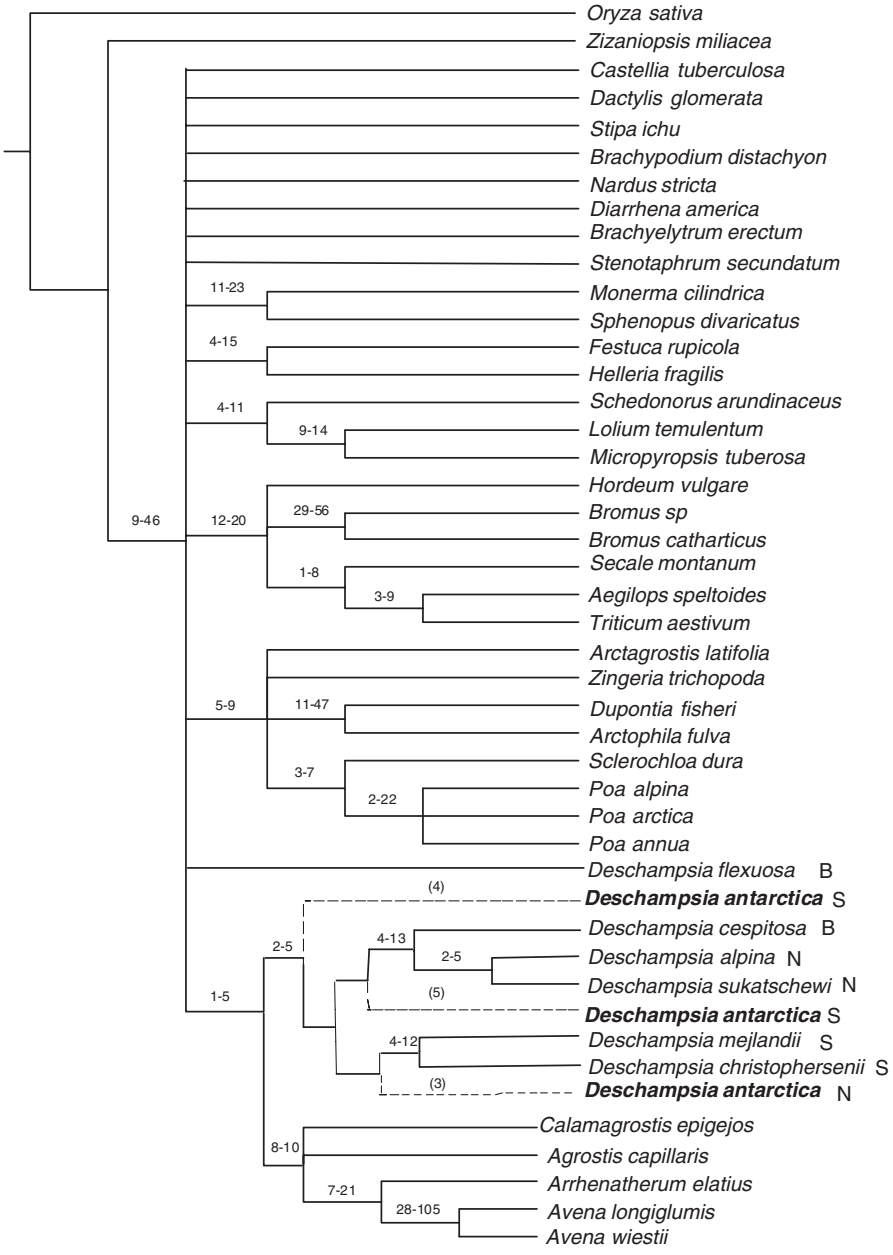

Fig. 1. Summary tree of all parameter sets. In dashed lines is shown the alternative positions of *D. antarctica* (see text). In brackets: number of conditions on which this position is present. *S* Southern hemisphere; *N* Northern hemisphere; *B* both hemispheres

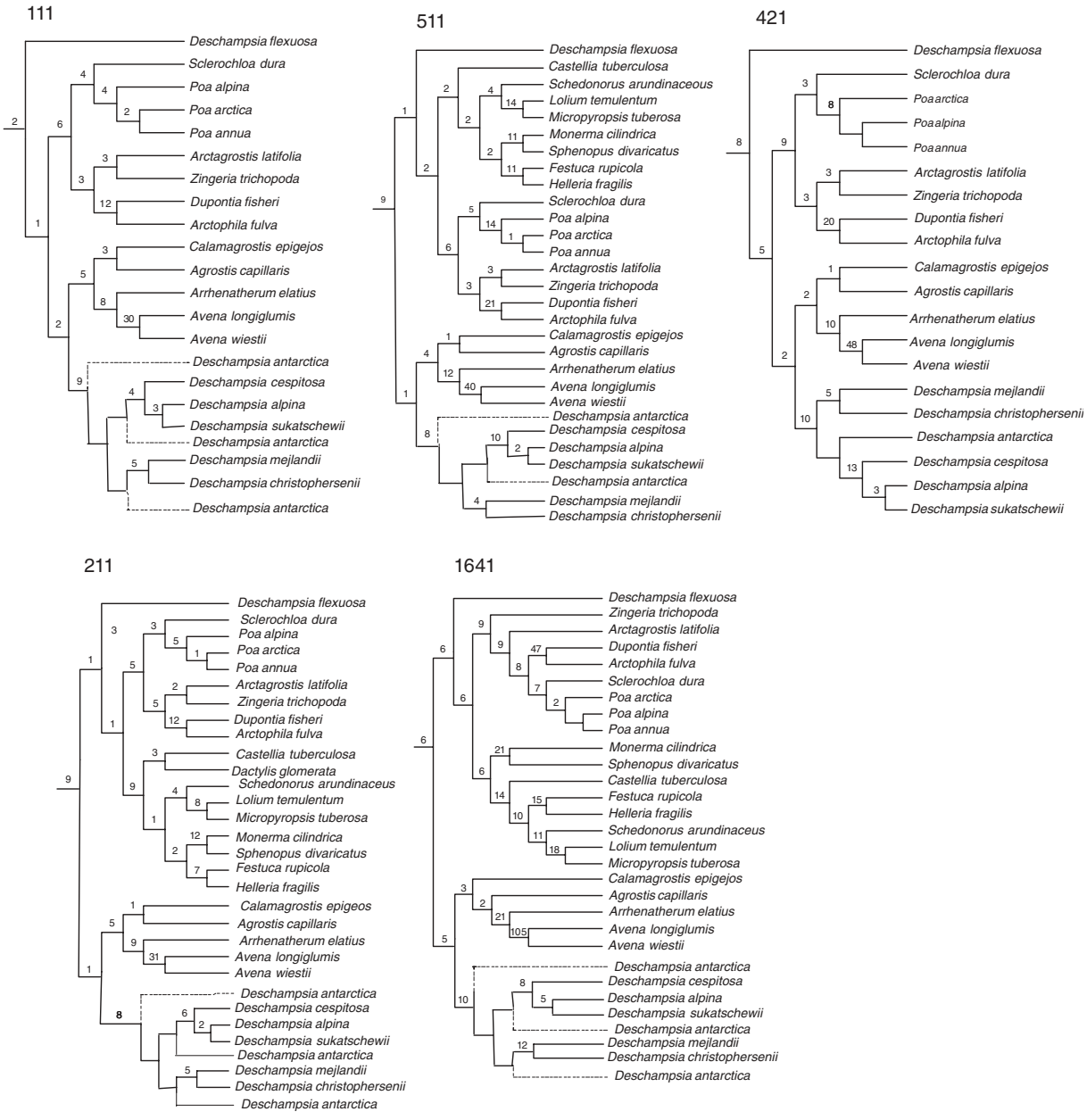

Fig. 2. Relationship between the *Deschampsia* species and Avenae, Poeae species under each parameter set. In dashed lines is shown the alternative positions of *D. antarctica* in the different optimal trees (see text). Numbers above branches represent Bremer support values

tives, more molecular markers, and combination of molecular data with the morphological data is needed to clarify their phylogenetic relationships.

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