

# Aliso: A Journal of Systematic and Evolutionary Botany

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Volume 23 | Issue 1

Article 28

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2007

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### Recommended Citation

Jacobs, Surrey; Bayer, Randall; Everett, Joy; Arriaga, Mirta; Barkworth, Mary; Sabin-Badereau, Alexandru; Torres, Amelia; Vázquez, Francisco; and Bagnall, Neil (2007) "Systematics of the Tribe Stipeae (Gramineae) Using Molecular Data," *Aliso: A Journal of Systematic and Evolutionary Botany*: Vol. 23: Iss. 1, Article 28.

Available at: <http://scholarship.claremont.edu/aliso/vol23/iss1/28>

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# Systematics of the Tribe Stipeae (Gramineae) Using Molecular Data

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## SYSTEMATICS OF THE TRIBE STIPEAE (GRAMINEAE) USING MOLECULAR DATA

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### ABSTRACT

Internal transcribed spacer (ITS) sequences have been determined for a wide range of stipoid grasses (Poaceae, Pooideae, Stipeae). *Nardus* was confirmed as the most appropriate outgroup. *Anisopogon* is consistently included among the stipoid genera. *Lithachne* and *Oryza* form a clade and are clearly not close to Stipeae, and there is no support for including *Brachyelytrum* within Stipeae. *Ampelodesmos* and *Diarrhena* do appear among the core taxa in some analyses, but their positions are unstable and the evidence for retaining them is limited. So far there is inadequate support for rejecting them from Stipeae, so they should be included in any comprehensive study of the tribe. The ITS phylogeny supports a narrow interpretation of *Jarava*, one that includes only species with clear adaptations to anemophilous diaspore dispersal. There is no support for *Achnatherum* s.l. being a monophyletic group, nor are there any clear and consistent groups within it. *Nassella*, *Hesperostipa*, and *Piptochaetium* remain well supported. The data support some internal groupings within *Nassella*, but the sample size is small. It may be worthwhile investigating subgeneric relationships within *Nassella*. *Anemanthele* always appears associated with, and sometimes within, *Austrostipa*, but its position is inconsistent. We recommend continuing to recognize it at the generic level because of its distinctive morphological characters. *Stipa* s.s. shows some cohesion, but the results also suggest that some species currently included in the genus do not belong in it, suggestions that are supported by other studies. There has been no advance in understanding *Piptatherum*. The data support some of the subgeneric groupings within *Austrostipa*, but suggest that others should be combined. *Austrostipa* subgen. *Falcatheae* is well supported, in part by a shared deletion. Additional species of *Stipa* s.s. and *Piptatherum* are being sequenced to broaden the sampling of these two genera.

Key words: *Achnatherum*, Gramineae, ITS, *Nassella*, Pooideae, *Stipa*, Stipeae, systematics.

### INTRODUCTION

The tribe Stipeae (Gramineae, Pooideae) has had a fluid circumscription, possibly due, at least partially, to the effect of reduced spikelet structure on the number of available distinctive morphological characters. A list of the generic names that have been used in Stipeae may be found at <http://herbarium.usu.edu/stipeae/genera.htm>. Barkworth (1990, 1993) reviewed the use of these names prior to 1990. Her account was updated in Jacobs et al. (2000).

Recently, however, the major area of uncertainty has been in the circumscription of *Stipa* s.l. itself. Generic segregates of *Stipa* are gradually being accepted with many treatments recognizing the new or revised genera (Torres 1997; Peñailillo 2002; Wheeler et al. 2002; Valdés-Reyna and Barkworth 2002; Barkworth et al. in press), but the disposition of many species is still under active investigation, for example, the recent reinstatement of *Macrochloa* Kunth and the description of *Celtica* F. M. Vázquez & Barkworth (Vázquez and Barkworth 2004). The situation posed by limited morphological characters has been helped by the availability of nuclear DNA sequence data, particularly internal transcribed

spacer (ITS) studies (Jacobs et al. 2000). This contribution updates the story for Stipeae following recent additions to the species sampled for ITS. Given the continuing controversies and changes in generic interpretation within the tribe, it seems appropriate to review the present situation before discussing the results of the recent ITS studies.

It should be noted that some taxonomists (e.g., Curto 1998; Renvoize 1998) do not accept the dismemberment of *Stipa*, preferring to retain the genus in a sense very similar to that used by Bentham (1882) and Hackel (1887).

Recognition and interpretation of *Achnatherum* remains the most difficult aspect of the taxonomy of Stipeae. Keng (cited in Tsvelev 1977) was the first to expand *Achnatherum* to include a substantial number of Asian species, an expansion that was endorsed by Tsvelev (1977). Barkworth (1993) later expanded it further to include many North American and Mexican taxa, plus a few South American taxa. Matthei et al. (1998) expanded it again to include several South American species that Barkworth (1993) had not considered, including some previously positioned in *Jarava* (including the type). They also pointed out that *Jarava* (Ruiz and Pavón

1794) has priority over *Achnatherum* (Palisot de Beauvois 1812). Acceptance of Matthei et al.'s (1998) circumscription would require transferring all species of *Achnatherum* to *Jarava*.

Jacobs and Everett (1997) also recommended recognition of *Jarava*, but in a narrower sense than that adopted by Matthei et al. (1998), restricting it to South American species distinguished by having nonplumose awns, lemmas that are usually thinner than those in other stipoid genera and have an apical pappus of long hairs, and paleas that are much shorter than the lemmas. More recently, Peñailillo (2002) concluded that *Achnatherum* and *Jarava*, at least as represented in South America, are closely related, but that they should be treated as separate genera, with *Jarava* being interpreted as including species with long hairs on their lemmas or awns or with other adaptations for wind dispersal of their diaspores such as cleistogamous axillary aerial panicles. In his synopsis of the genus, Peñailillo (2002) listed 53 species, all of which traditional treatments would place in *Stipa* subgen. *Jarava* (Ruiz & Pav.) Trin. & Rupr., *Pappostipa* Speg., or *Ptilostipa* Speg. All species of *Jarava* (sensu Peñailillo 2002) are confined to South America except one, *J. speciosa* (Trin. & Rupr.) Peñailillo, that also grows in northwestern Mexico and the southwestern United States.

*Oryzopsis* is another genus whose interpretation remains controversial. Many taxonomists (e.g., Bentham 1882; Hackel 1887; Hitchcock 1935, 1951; Johnson 1945; Sývulescu et al. 1972) have interpreted it as including both Eurasian and North American taxa, but several others (e.g., Tsvelev 1976; Tutin et al. 1980; Cope 1982) have restricted the genus to North American taxa.

Freitag (1975), after comparing the morphology of the North American *Oryzopsis asperifolia* (the type species of *Oryzopsis*) with Eurasian species of *Oryzopsis* s.l., concluded that the Eurasian species belonged in *Piptatherum*, proposing three sections (*Mileaceum* Roshev. ex Freitag, *Piptatherum*, and *Virescentia* Roshev. ex Freitag). He also drew attention to the isolation of *P. miliaceum* (L.) Coss. from other members of the genus. Several taxonomists have since followed Freitag in placing the Eurasian species of *Oryzopsis* s.l. in *Piptatherum* (e.g., Tsvelev 1976; Tutin et al. 1980; Cope 1982).

Freitag (1975) did not examine any North American species of *Oryzopsis* other than *O. asperifolia*. Developmental and morphological data support its treatment as a unispecific genus (Kam and Maze 1974; Barkworth 1983; Kuo et al. 1983; Barkworth and Everett 1987; Everett 1990). If such a treatment is accepted, the problem becomes one of determining where the excluded North American species belong. Barkworth (1993) placed some of them in *Piptatherum*, some in *Achnatherum*, and left the status of three species (*O. canadensis* (Poir.) Torr., *O. exigua* Thurb., and *O. pungens* (Torr.) Hitchc.) unresolved.

Curto (1998) recommended including *Oryzopsis canadensis* in *Stipa* sect. *Lasiagrostis* (Link) Hack. (= *Achnatherum*) and leaving *O. pungens* and *O. exigua* with *O. asperifolia* in *Oryzopsis*. He noted that all three species have fused styles and short glumes, a combination of characteristics not observed in other members of Stipeae. Dorn (2001), on the other hand, transferred *O. canadensis*, *O. exigua*, and *O. pungens* to *Piptatherum*. Morphological and anatomical ob-

servations suggest that the inclusion of *O. exigua* in *Piptatherum* is more problematic than inclusion of *O. canadensis* and *O. pungens* (M. E. Barkworth unpubl. data). The ITS data analyses by Jacobs et al. (2000) separated *O. asperifolia* from the other species of *Oryzopsis* s.l., placing it in a clade sister to a clade containing the two Eurasian species (as *Piptatherum*) examined, not in a clade with the other North American species.

No new genera have been published since Jacobs et al. (2000), but it has been drawn to our attention that the name *Nicoraella*, which Torres published in 1997, is a superfluous name for *Anatherostipa* Peñailillo, published in 1996. There will, however, probably be more changes in generic interpretation within the tribe in the near future.

Following on from Jacobs et al. (2000) the number of taxa sampled was increased in an attempt to answer some of the questions raised in that study. Of particular interest (for both evolutionary and nomenclatural reasons) were the relationships between (i) *Achnatherum* s.s. and *Jarava*, (ii) *Oryzopsis* and *Piptatherum*, and (iii) the remaining species in *Stipa* s.s.

#### MATERIALS AND METHODS

##### *Plant Material*

DNA was extracted from 123 samples representing 96 species and 11 genera of stipoid grasses (*Achnatherum*, *Ane-manthele*, *Anisopogon*, *Austrostipa*, *Hesperostipa*, *Jarava*, *Nassella*, *Oryzopsis*, *Piptatherum*, *Piptochaetium*, and *Stipa* s.s.), as well as one species each of *Ampelodesmos*, *Brachyelytrum*, *Diarrhena*, *Lithachne*, *Nardus*, and *Oryza*. Of this last group of six genera, *Nardus* and *Oryza* were included only as outgroups and the remaining four genera were included as they have been, at some stage or other, often only by word of mouth, suggested as being part of, or related to, *Stipa*. We thought it may be possible to shed light on such suggestions, or simply test these genera as potential outgroups. Some stipoid species were represented by several duplicate and a few triplicate samples. Vouchers are mostly lodged at NSW and UTC; details of voucher and source material are in Table 1.

##### *DNA Extraction, Amplification, and Sequencing*

Previous DNA extractions (those whose sequences have been previously reported, or for those prepared for those studies, but not used in the analyses) were as described in Jacobs et al. (2000). For the more recent samples, total DNA was isolated as outlined in Bayer et al. (1996). Recalcitrant DNA was purified with Qiaquick® PCR Purification Columns (QIAGEN Pty. Ltd., Clifton Hill, Victoria, Australia). The ITS region was amplified via the polymerase chain reaction (PCR) using *Taq* DNA polymerase. The PCR reaction mixture consisted of 5 µl of 10× reaction buffer, 3 µl of 25mM magnesium chloride solution, 4 µl of a 1.25 mM dNTP solution in equimolar ratio, 25 pmol of each primer, 10–50 ng of template DNA, and 1.0 unit of polymerase in a total volume of 50 µl. The PCR samples were heated to 94°C for 3 min prior to the addition of DNA polymerase to denature unwanted proteases and nucleases. The ITS double-stranded PCR products were produced via 30 cycles of de-

naturation (94°C for 1 min), primer annealing (48°C for 1 min), and extension (72°C for 2 min). A 7 min final extension cycle at 72°C followed the 30<sup>th</sup> cycle to ensure the completion of all novel strands. The ITS region was amplified using primers ITS1 (Hsiao et al. 1994) and ITS4 (White et al. 1990). Herbarium specimens were amplified in two fragments, using the internal primers ITS2 and ITS3 (White et al. 1990) in conjunction with ITS1 and ITS4, respectively.

The double-stranded PCR products were then used as templates in cycle-sequencing reactions. The sequencing primers were the same as the PCR amplification primers. The double-stranded PCR products were sequenced using the dideoxy chain termination method with the use of the Big Dye Terminator RR Kit<sup>®</sup> (Perkin-Elmer Applied Biosystems, Wellesley, Massachusetts, USA) and an ABI automated sequencer in the Division of Plant Industry, CSIRO. Sequencing reactions used 55°C annealing temperatures. The cycle-sequencing protocol followed manufacturer's instructions. Sequences were assembled using Sequencher<sup>™</sup> 3.0 (Gene Codes Corporation, Ann Arbor, Michigan, USA).

#### DNA Sequence Analysis

Sequences were first aligned using ClustalW (Australian National Genomic Information Service, University of Sydney, New South Wales). Multiple alignment parameters were set at the default values: gap-opening penalty of 10, gap-extension penalty of 5, and gap-separation penalty of 8. Sequences were secondarily manually aligned using MacClade vers. 4.03 (Maddison and Maddison 2001) and analyzed using PAUP\* vers. 4.0b10 (Swofford 2002). To detect and remove random pairing a stepwise alignment strategy was used (Hsiao et al. 1998, 1999). This strategy required aligning sequences of closely related groups first, then aligning the full sequence data set manually. Sequences of newly added taxa or taxa of questionable affinities were aligned variously with the sequences of the other taxa, each time followed by a parsimony analysis. If such taxa proved topologically unstable in these analyses, every attempt was made to find potentially pairing or closely related sequences to optimize the alignment and provide more robust results. The final alignment was further optimized manually. This process eliminated most of the random pairings of taxa and was critical for sequence alignment of the variable DNA region. Some sequences obtained from different sources proved consistently different. The differences in most cases could be reduced to a single base position and the effect removed by deleting that single position from the analysis. Gaps were treated as additional characters if the gaps occurred in more than one species and if there appeared to be any possibility of taxonomic information in the character. The gaps that were not scored were mostly in the outgroup genera. The sequence alignments were too large to be included here, but they are available upon request to Surrey Jacobs or Joy Everett.

We used several options in PAUP\* to analyze the data matrix. Heuristic searches were used initially but took several days and the results obtained rarely differed substantially from those obtained using distance methods. In most analyses the neighbor-joining option was used, and the data were resampled via jackknifing with 10,000 replicates and

37% deletion. As well, for *Austrostipa*, the most-parsimonious trees were determined using a heuristic search algorithm with random taxon entry, 5000 replicates, saving only 100 trees each replicate, tree-bisection-reconnection (TBR) branch swapping, and the random-stepwise-addition option generating a majority-rule tree.

Various genera were used as outgroups—*Brachyelytrum*, *Lithachne*, *Lygeum*, *Nardus*, and *Oryza*—both singly and in various combinations. Results from Hsiao et al. (1999) indicated all of these genera were potential outgroups. In practice, all produced similar results. *Nardus* was the genus we used most frequently as the outgroup because its sequence was closest to those of the stipoids and could be aligned with more confidence.

After the initial analyses more detailed analyses were conducted on some of the clades. Some sequences that appeared problematic were identified in the earlier analyses and omitted from the subsequent analyses in an attempt to obtain results for hypotheses generation. One of these sequences was for *Austrostipa stipoides* that did not appear stable in any analysis. Another was the second sample of *A. rudis*, which was a large partial sequence but clearly had to be deleted when results from the other two samples were included. For detailed analysis of the more derived clades, *Hesperostipa* was a logical genus to use as an outgroup (Jacobs et al. 2000).

#### RESULTS AND DISCUSSION

Aligning ITS proved fairly straightforward for all of the Stipeae core taxa, and for *Nardus*. The other genera caused a few more problems in alignment, with *Lithachne* and *Oryza* being the most difficult, but even here we were satisfied with the final alignment. There were 654 bases with 39 insertions/deletions (indels) scored, making a total of 693 characters. There were six incomplete sequences, for *Austrostipa geoffreyi*1, *A. rudis*3, *A. scabra*3, *Nassella argentinensis*, *N. hyalina*, and *Piptatherum racemosum*2.

*Nardus* was confirmed as the most appropriate outgroup based on the sampling used in this study. Hsiao et al. (1999), based on ITS, found that the core stipoid genera (*Achnatherum*, *Austrostipa*, *Nassella*, *Oryzopsis*, *Piptatherum*, *Piptochaetium*, and *Stipa*) were part of a monophyletic lineage that also included *Ampelodesmos*, *Anisopogon*, and *Diarrhena*. *Brachyelytrum*, *Lithachne*, *Nardus*, and *Oryza* diverged before the clade above diverged from other pooid genera. The larger data set in this study and the more rigorous scoring of indels support the inclusion of *Anisopogon* among the stipoid genera. *Lithachne* and *Oryza* form a clade and are clearly not close to Stipeae (Fig. 1). There is no support for including *Brachyelytrum* within Stipeae.

*Ampelodesmos* and *Diarrhena* do appear among the core taxa in some analyses (Fig. 1, 2), but their positions are unstable and the evidence for retaining them is weak using morphological data. Likewise, there is inadequate support for rejecting them from Stipeae, and they should be included in any comprehensive study of the tribe. These data thus only weakly support Decker's (1964) suggestion of retaining *Ampelodesmos* within Stipeae. The mobility of the two genera probably reflects our inability thus far to sequence species that are closely related to either, and their relationships

Table 1. Study taxa and source/voucher information. Some of the earlier studies (e.g., Hsiao et al. 1999) recorded minimal information for the vouchers; these data are presented as recorded. Some sequences were taken from the literature and some from GenBank, and the relative information is presented for these. For more recent vouchers, information is provided on collector and number (or date or locality if number not available) and the herbarium (in parentheses) where the main specimen is lodged.

Taxon	Source/Voucher
<i>Achnatherum</i> P. Beauv.	
<i>A. capense</i> P. Beauv.	California (K)
<i>A. contractum</i> (B. L. Johnson) Barkworth	<i>Jacobs &amp; Barkworth 4515</i> (UTC)
<i>A. coronatum</i> (Thurb.) Barkworth	<i>Thurber s. n.</i> (K)
<i>A. editorum</i> (Fourn.) Valdés-Reyna	Los Linos, Mexico (UTC)
<i>A. eminens</i> (Cav.) Barkworth	<i>Hsiao 151</i> (UTC)
<i>A. hendersonii</i> (Vasey) Barkworth	<i>Maze &amp; Maze 2</i> (UTC)
<i>A. hymenoides</i> (Roem. & Schult.) Barkworth	<i>Barkworth 05.125</i> (UTC)
<i>A. inebrians</i> (Hance) Keng	China (K)
<i>A. lemmonii</i> (Vasey) Barkworth	<i>Maze &amp; Maze 7</i> (UTC)
<i>A. lobatum</i> (Swallen) Barkworth	<i>Barkworth 4734</i> (UTC)
<i>A. nelsonii</i> (Scribn.) Barkworth	(1) <i>Garden 7-58</i> (UTC)
	(2) <i>Maze &amp; Maze 1</i> (UTC)
<i>A. perplexum</i> Hoge & Barkworth	<i>Barkworth 99.134</i> (UTC)
<i>A. pinetorum</i> (M. E. Jones) Barkworth	<i>Barkworth 99.109</i> (UTC)
<i>A. robustum</i> (Vasey) Barkworth	<i>Barkworth 99.122</i> (UTC)
<i>A. sibiricum</i> (L.) Keng ex Tzvelev	Mongolia (K)
<i>A. speciosum</i> (Trin. & Rupr.) Barkworth	<i>Barkworth 99.138</i> (UTC)
<i>A. splendens</i> (Trin.) Nevski	<i>Jiji s. n.</i> , China (K)
<i>Ampelodesmos</i> Link	
<i>A. mauritanica</i> (Poir.) T. Durand & Schinz	150-90.00982 K (K)
<i>Anemanthele</i> Veldkamp	
<i>A. lessoniana</i> Veldkamp	(1) CHR 499639 (CHR)
	(2) <i>Connor s. n.</i> , 12 Feb 2003 (NSW)
<i>Anisopogon</i> R. Br.	
<i>A. avenaceus</i> R. Br.	<i>Jacobs 8480</i> (NSW)
<i>Austrostipa</i> S. W. L. Jacobs & J. Everett	
Subgen. <i>Arbuscula</i> S. W. L. Jacobs & J. Everett	
<i>A. acrociliata</i> (Reader) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 7041</i> (NSW)
	(2) <i>Jacobs 8416</i> (NSW)
<i>A. breviglumis</i> (J. M. Black) S. W. L. Jacobs & J. Everett	<i>Jacobs 8469</i> (NSW)
<i>A. platychaeta</i> (Hughes) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 7039</i> (NSW)
	(2) <i>Jacobs 7054</i> (NSW)
Subgen. <i>Aulax</i> S. W. L. Jacobs & J. Everett	
<i>A. setacea</i> (R. Br.) S. W. L. Jacobs & J. Everett	<i>Jacobs 8449</i> (NSW)
Subgen. <i>Austrostipa</i>	
<i>A. densiflora</i> (Hughes) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 8461</i> (NSW)
	(2) <i>Jacobs 8648</i> (NSW)
	(3) <i>Jacobs 4646</i> (NSW)
<i>A. mollis</i> (R. Br.) S. W. L. Jacobs & J. Everett	<i>Jacobs 6919</i> (NSW)
<i>A. semibarbata</i> (R. Br.) S. W. L. Jacobs & J. Everett	<i>Jacobs 6951</i> (NSW)
Subgen. <i>Bambusina</i> S. W. L. Jacobs & J. Everett	
<i>A. ramosissima</i> (Trin.) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 8467</i> (NSW)
	(2) Living Collections Record 860980 (NSW)
	(3) <i>Everett 1800</i> (NSW)
<i>A. verticillata</i> (Nees ex Spreng.) S. W. L. Jacobs & J. Everett	<i>Jacobs 8475</i> (NSW)
Subgen. <i>Ceres</i> S. W. L. Jacobs & J. Everett	
<i>A. aristiglumis</i> (F. Muell.) S. W. L. Jacobs & J. Everett	<i>Jacobs 8468</i> (NSW)
<i>A. bigeniculata</i> (Hughes) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 7062</i> (NSW)
	(2) <i>Jacobs 8459</i> (NSW)
<i>A. blackii</i> (C. E. Hubb.) S. W. L. Jacobs & J. Everett	<i>Jacobs 8456</i> (NSW)
<i>A. gibbosa</i> (Vickery) S. W. L. Jacobs & J. Everett	<i>Jacobs 8455</i> (NSW)
Subgen. <i>Eremophilae</i> S. W. L. Jacobs & J. Everett	
<i>A. eremophila</i> (Reader) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 7057</i> (NSW)
	(2) <i>Jacobs 8415</i> (NSW)
<i>A. puberula</i> (Steud.) S. W. L. Jacobs & J. Everett	<i>Jacobs 8441</i> (NSW)

Table 1. Continued.

Taxon	Source/Voucher
Subgen. <i>Falcateae</i> S. W. L. Jacobs & J. Everett	
<i>A. drummondii</i> (Steud.) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 7060</i> ("normal") (NSW) (2) <i>Jacobs 8420</i> (velutinous) (NSW)
<i>A. nitida</i> (Summerh. & C. E. Hubb.) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 7037</i> (NSW) (2) <i>Jacobs 8413</i> (NSW)
<i>A. nodosa</i> (S. T. Blake) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 7058</i> (NSW) (2) <i>Jacobs 8444</i> (NSW)
<i>A. scabra</i> (Lindl.) S. W. L. Jacobs & J. Everett subsp. <i>falcate</i> (Hughes) Vickery, S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 8457</i> (NSW) (2) <i>Everett 1804</i> (NSW) (3) <i>Jacobs 8462</i> (NSW)
<i>A. trichophylla</i> (Benth.) S. W. L. Jacobs & J. Everett	<i>Jacobs 8442</i> (NSW)
Subgen. <i>Lancea</i> S. W. L. Jacobs & J. Everett	
<i>A. echinata</i> (Vickery, S. W. L. Jacobs & J. Everett) S. W. L. Jacobs & J. Everett	<i>Jacobs 8446</i> (NSW)
<i>A. flavescens</i> (Labill.) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 6927</i> (NSW) (2) <i>Kharis 17</i> (NSW)
<i>A. velutina</i> (Vickery, S. W. L. Jacobs & J. Everett) S. W. L. Jacobs & J. Everett	<i>Jacobs 8421</i> (NSW)
Subgen. <i>Lanterna</i> S. W. L. Jacobs & J. Everett	
<i>A. nullanulla</i> (J. Everett & S. W. L. Jacobs) S. W. L. Jacobs & J. Everett	<i>Jacobs 8436</i> (NSW)
<i>A. vickeryana</i> (J. Everett & S. W. L. Jacobs) S. W. L. Jacobs & J. Everett	<i>Jacobs 8424</i> (NSW)
Subgen. <i>Lobatae</i> S. W. L. Jacobs & J. Everett	
<i>A. geoffreyi</i> S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 8465</i> (NSW) (2) <i>Jacobs 7030</i> (NSW)
<i>A. juncifolia</i> (Hughes) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 8466</i> (NSW) (2) <i>Jacobs 7017</i> (NSW)
<i>A. stipoides</i> (Hook. f.) S. W. L. Jacobs & J. Everett	<i>Jacobs 8448</i> (NSW)
Subgen. <i>Longiaristatae</i> S. W. L. Jacobs & J. Everett	
<i>A. compressa</i> (R. Br.) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 6921</i> (NSW) (2) <i>Jacobs 6933</i> (NSW)
Subgen. <i>Petaurista</i> S. W. L. Jacobs & J. Everett	
<i>A. elegantissima</i> (Labill.) S. W. L. Jacobs & J. Everett	(1) <i>Jacobs 6978</i> (NSW) (2) <i>Jacobs 7059</i> (NSW) (3) <i>Martyn 5</i> (NSW)
<i>A. tuckeri</i> (F. Muell.) S. W. L. Jacobs & J. Everett	<i>Everett 1622</i> (NSW)
Subgen. <i>Tuberculatae</i> S. W. L. Jacobs & J. Everett	
<i>A. muelleri</i> (Tate) S. W. L. Jacobs & J. Everett	<i>Jacobs 8453</i> (NSW)
<i>A. pubescens</i> (R. Br.) S. W. L. Jacobs & J. Everett	<i>Jacobs 8481</i> (NSW)
<i>A. rudis</i> (Spreng.) S. W. L. Jacobs & J. Everett subsp. <i>rudis</i>	(1) <i>Jacobs 8460</i> (NSW) (2) <i>Jacobs 4677</i> (NSW) (3) <i>Jacobs 8649</i> (NSW)
<i>Brachyelytrum</i> P. Beauv.	
<i>B. aristosum</i> (Michx.) P. Beauv. ex Trel.	<i>Muenschler &amp; Maguire 1909</i> (UTC)
<i>Diarrhena</i> P. Beauv.	
<i>D. americana</i> P. Beauv.	<i>Hsiao s. n.</i> , 4 Nov 1995 (UTC)
<i>Hesperostipa</i> (M. K. Elias) Barkworth	
<i>H. comata</i> (Trin. & Rupr.) Barkworth subsp. <i>comata</i>	<i>Barkworth 4554</i> (UTC)
<i>H. neomexicana</i> (Thurb. ex Coult.) Barkworth	<i>Barkworth 5118</i> (UTC)
<i>Jarava</i> Ruiz & Pav.	
<i>J. ichu</i> Ruiz & Pav.	<i>Renvoize &amp; Flores 5301</i> (K)
<i>J. plumose</i> (Trin.) S. W. L. Jacobs & J. Everett	<i>Arriaga 596</i> (BA)
<i>J. pseudoichu</i> (Caro) F. Rojas	<i>Anton &amp; Connor 240</i> (K)
<i>J. pungens</i> (Nees & Meyen) Matthei	BA 79989 (BA)
<i>Lithachne</i> P. Beauv.	
<i>L. humilis</i> Soderstr.	<i>USU s. n.</i> , GenBank AF019787 (US)

Table 1. Continued.

Taxon	Source/Voucher
<i>Nardus</i> L.	
<i>N. stricta</i> L.	Pavek & Survey Crew, 25 Oct 1988 (UTC)
<i>Nassella</i> E. Desv.	
<i>N. argentinensis</i> (Speg.) Peñailillo	Arriaga 602 (BA)
<i>N. cernua</i> (Stebbins & A. Löve) Barkworth	Tilden 79, 104 (UTC)
<i>N. charruana</i> (Arechav.) Barkworth	Sánchez s. n. (UTC)
<i>N. curviseta</i> (Hitc.) Barkworth	Renvoize & Flores 5212 (K)
<i>N. hyaline</i> (Nees) Barkworth	Arriaga 601 (BA)
<i>N. lepida</i> (Hitc.) Barkworth	Barkworth 3147 (UTC)
<i>N. leucotricha</i> (Trin. & Rupr.) Pohl	Houck s. n., GenBank L36520
<i>N. nardoides</i> (Phil.) Barkworth	Renvoize & Flores 5278 (K)
<i>N. neesiana</i> (Trin. & Rupr.) Barkworth var. <i>neesiana</i>	(1) Jacobs 8464 (NSW) (2) Arriaga 595 (BA)
<i>N. pulchra</i> (Hitc.) Barkworth	Barkworth 2444 (UTC)
<i>N. tenuissima</i> (Trin.) Barkworth	Mueller 783s (UTC)
<i>N. trichotoma</i> Hackel ex Arechav.	118-78, 21 Sep 1994 (K)
<i>N. viridula</i> (Trin.) Barkworth	Barkworth 99.137 (UTC)
<i>Oryza</i> L.	
<i>O. sativa</i> L.	Takaiwa et al. 1985
<i>Oryzopsis</i> Michx.	
<i>O. asperifolia</i> Michx.	Barkworth 89.002 (UTC)
“Pappostipa” (= <i>Stipa</i> subgen. <i>Pappostipa</i> Speg.)	
<i>S. humilis</i> Cav. var. <i>humilis</i>	Arriaga 598 (BA)
<i>S. vaginata</i> Phil. fo. <i>contracta</i> Roig	Arriaga 599 (BA)
<i>S. vaginata</i> fo. <i>immersa</i> Roig	Arriaga 597 (BA)
<i>Piptatherum</i> P. Beauv	
<i>P. canadensis</i> (Poir.) Dorn	Hsiao s. n. (UTC)
<i>P. exiguum</i> (Thurb.) Dorn	UTC 187825 (UTC)
<i>P. laterale</i> (Regel) Roshev.	Garden 4-73 (UTC)
<i>P. micranthum</i> (Trin. & Rupr.) Barkworth	Jacobs 4513 (NSW)
<i>P. miliaceum</i> Coss.	Barkworth & Vázquez, 15 Mar 2000 (UTC)
<i>P. paradoxum</i> (L.) P. Beauv.	000-69.1902, 21 Sep 1994 (K)
<i>P. pungens</i> (Torr. ex Spreng.) Dorn	Sprenyi s. n. (UTC)
<i>P. racemosum</i> (Sm.) Barkworth	(1) Aiken s. n. (K) (2) Clark 1646 (ISC), Civille 276 (UTC)
<i>P. shoshoneanum</i> (Curto & Douglass M. Hend.) P. M. Peterson & Soreng	
<i>P. songaricum</i> (Trin.) Roshev.	Hsiao 199 (UTC)
<i>Piptochaetium</i> J. Presl & C. Presl	
<i>P. setosum</i> (Trin.) Arechav.	Barkworth 5172 (UTC)
<i>P. stipoides</i> (Trin. & Rupr.) Hack. ex Arechav. var. <i>stipoides</i>	Arriaga 594 (BA)
<i>Stipa</i> L.	
<i>S. baicalensis</i> Roshev.	Mongolia (K)
<i>S. barbata</i> Desf.	SE Spain, 24 Sep 1994 (K)
<i>S. capillata</i> L.	Badarau & Pendea, May 2001 (UTC)
<i>S. glareosa</i> P. Smirnov	(1, 2) Mongolia (K)
<i>S. juncea</i> Lam.	151-87.01267, W Mediterranean, 21 Sep 1994 (K)
<i>S. lessingiana</i> Trin. & Rupr.	Badarau & Pendea, May 2001 (UTC)
<i>S. parviflora</i> Nees	Spain (K)
<i>S. tirsia</i> Stev.	(K)

still remain unresolved. It is possible relevant species are now extinct.

The differences between sequences from replicate samples of the same species were surprisingly large in some cases. This was most obvious in *Austrostipa* where there was the most multiple sampling. The greatest infraspecific variation

was 12 base pairs (bp) for the three samples of *A. densiflora*, followed by eight for the three samples of *A. rudis* and six for both sets of three samples each of *A. elegantissima* and *A. ramosissima*. Nine species with two samples each differed by 1–11 bp. The differences between samples of the same species were not much less than those between species.



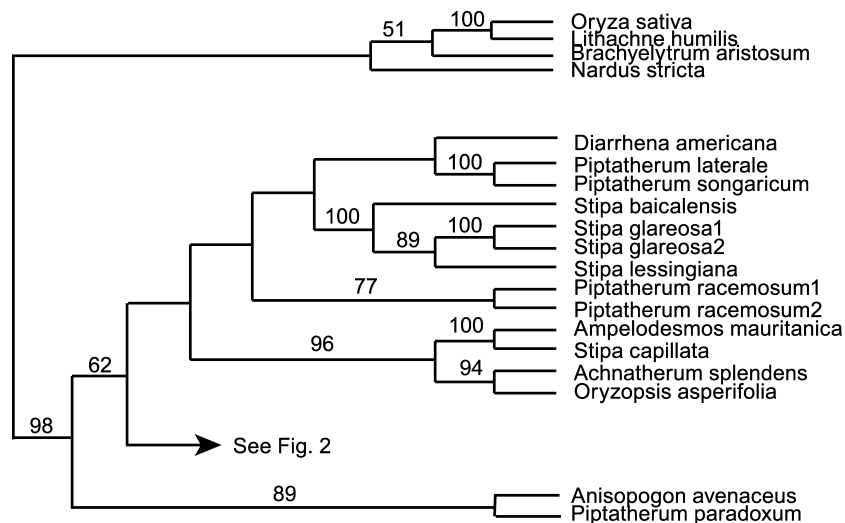


Fig. 1. Neighbor-joining tree showing the basal portions of the tree in Fig. 2, including the positions of the contentious genera *Diarrhena*, *Ampelodesmos*, and *Anisopogon*. Jackknife values >50% are included.

### Achnatherum

In Jacobs et al. (2000) the results supported the concept of *Achnatherum* being distinct from *Stipa* s.s., while suggesting that *Achnatherum*, whether interpreted in its traditional sense or in the expanded sense, is not monophyletic. While there is limited support for the clade as a whole, there is virtually no support for any internal structure. The more recent analyses demonstrate that the North American *Achnatherum* species sort into two weakly supported clades (clade 1: *A. contractum*, *A. coronatum*, *A. hymenoides*, *A. lobatum*, and *A. speciosum*; clade 2: *A. hendersonii*, *A. lemmonii*, *A. nelsonii*, and *A. robustum*) and are not monophyletic in (Fig. 2, 3). There are three other more or less consistent clades (Fig. 2, 3):

- (i) One of *A. editorum* and *A. eminens*, both of which grow in Mexico with *A. eminens* extending into the southwestern United States. This clade also includes *Austrostipa stipoides*, which has a very different sequence that does not appear to readily align with anything. It is quite possible that *A. stipoides* is either a hybrid or polyploid and more sequences are required to determine what the situation is here.
- (ii) A Eurasian clade represented here by *A. inebrians* and *A. sibiricum*, both of which are Asian species.
- (iii) A third clade comprises *Jarava* s.s. (*J. ichu*, *J. plumosa*, *J. pseudoichu*, and *J. pungens*).

*Achnatherum perplexum*, *A. pinetorum*, *A. splendens* (with *Oryzopsis asperifolia*), *Stipa capillata*, and *S. parviflora* are separately unstable with regard to position in different analyses (Fig. 2, 3).

It is not immediately obvious from these analyses what the best taxonomic groupings are. The jackknife values are low, but there is more support for recognition of *Jarava* s.s. than for the alternative broader circumscription (Peñailillo 2002), some suggestion of a core group of North American species, the possibility of a Eurasian group, but there are too many single outliers to place any confidence in any extrap-

olation yet. Further sampling is required to sort out the optimum treatment.

There is no support for the suggestion that *Achnatherum contractum* is a hybrid between *Achnatherum* and *Piptatherum* (Schechter and Johnson 1968; Schechter 1969) though, of course, a single ITS sequence will not necessarily detect that event.

### Anemanthele

The monotypic *Anemanthele* is distinguished by its single stamen and characteristic hilum, though there is some doubt about the hilum character being as distinct as originally stated (Veldkamp 1985; Everett 1990). Barkworth and Everett (1987) included *Anemanthele* in their concept of *Achnatherum*; Jacobs and Everett (1997) retained it as distinct. Various ITS analyses show it included in *Austrostipa* (Fig. 2–4), but never in a consistent position. Because of the instability and the morphological differences, we suggest retaining *Anemanthele*, at least for now.

### Austrostipa

About half the species of *Austrostipa* have now been sampled (37), and 15 of these have multiple samples, including five with three samples. As mentioned above, the infraspecific differences between sequences from replicate samples are surprisingly large in some cases, but replicates of a species mostly still grouped together or, at worst, separated only slightly. These species mostly have very large geographical ranges across the southern portion of Australia. The differences between sequences from the same species are often not much less than those between species. Consequently, it was not possible to obtain a strict consensus tree that was sufficiently resolved to be interpreted. This variation may lend support to our suggestion (Jacobs et al. 2000) that species of *Austrostipa* are comparatively recently evolved—because it may indicate that sequence homogenization is not yet complete within a species. Or, it may simply be related to the more intensive sampling. There is little support for

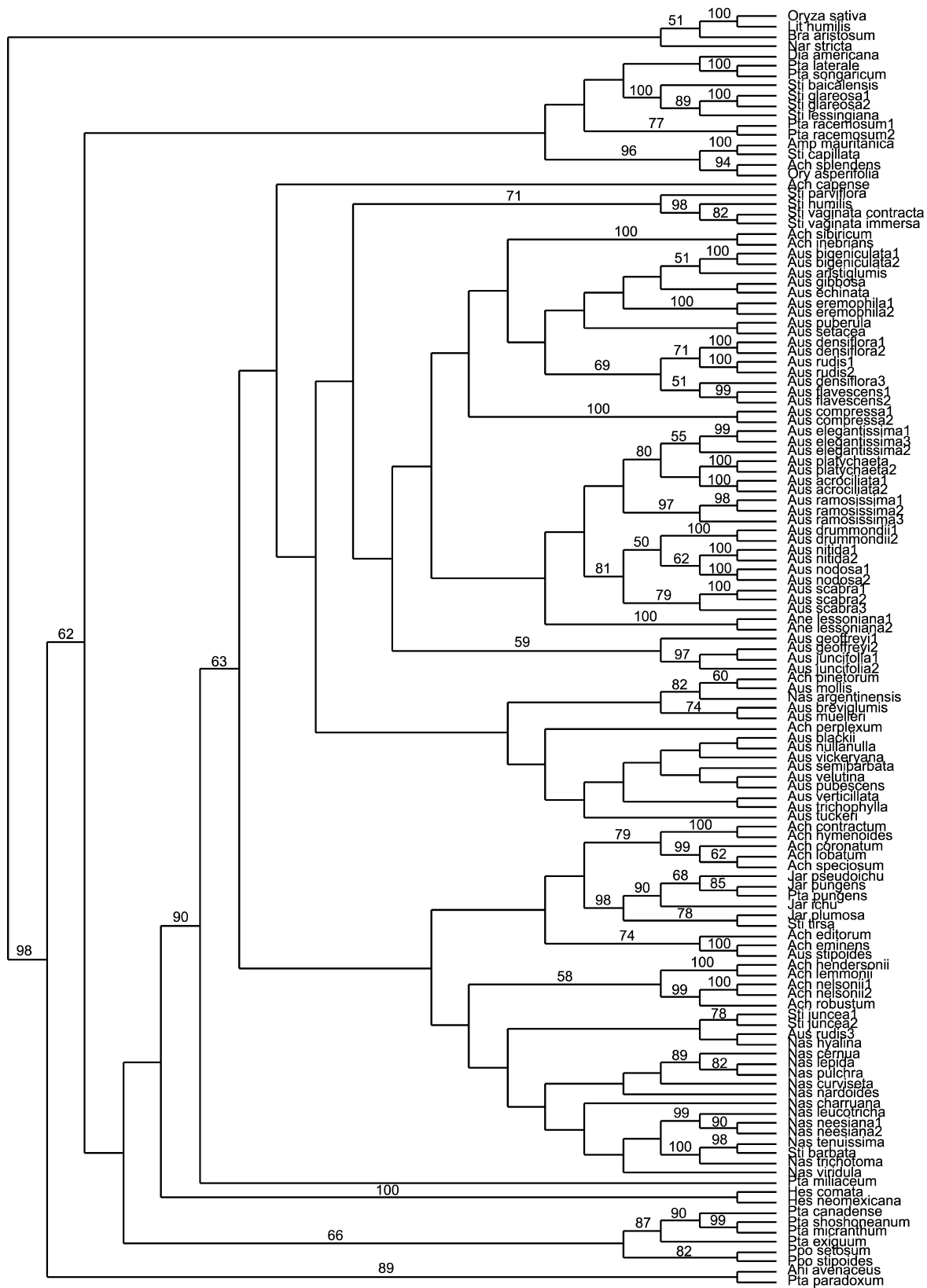


Fig. 2. Neighbor-joining tree derived from analysis of most of the samples. Jackknife values  $\geq 50\%$  are included. Ach = *Achnatherum*; Amp = *Ampelodesmos*; Ane = *Anemanthele*; Ani = *Anisopogon*; Aus = *Austrostipa*; Bra = *Brachyelytrum*; Dia = *Diarrhena*; Hes = *Hesperostipa*; Jar = *Jarava* s.s.; Lit = *Lithachne*; Nar = *Nardus*; Nas = *Nassella*; Ory = *Oryzopsis*; Pta = *Piptatherum*; Ppo = *Piptochaetium*; Sti = *Stipa* s.s.

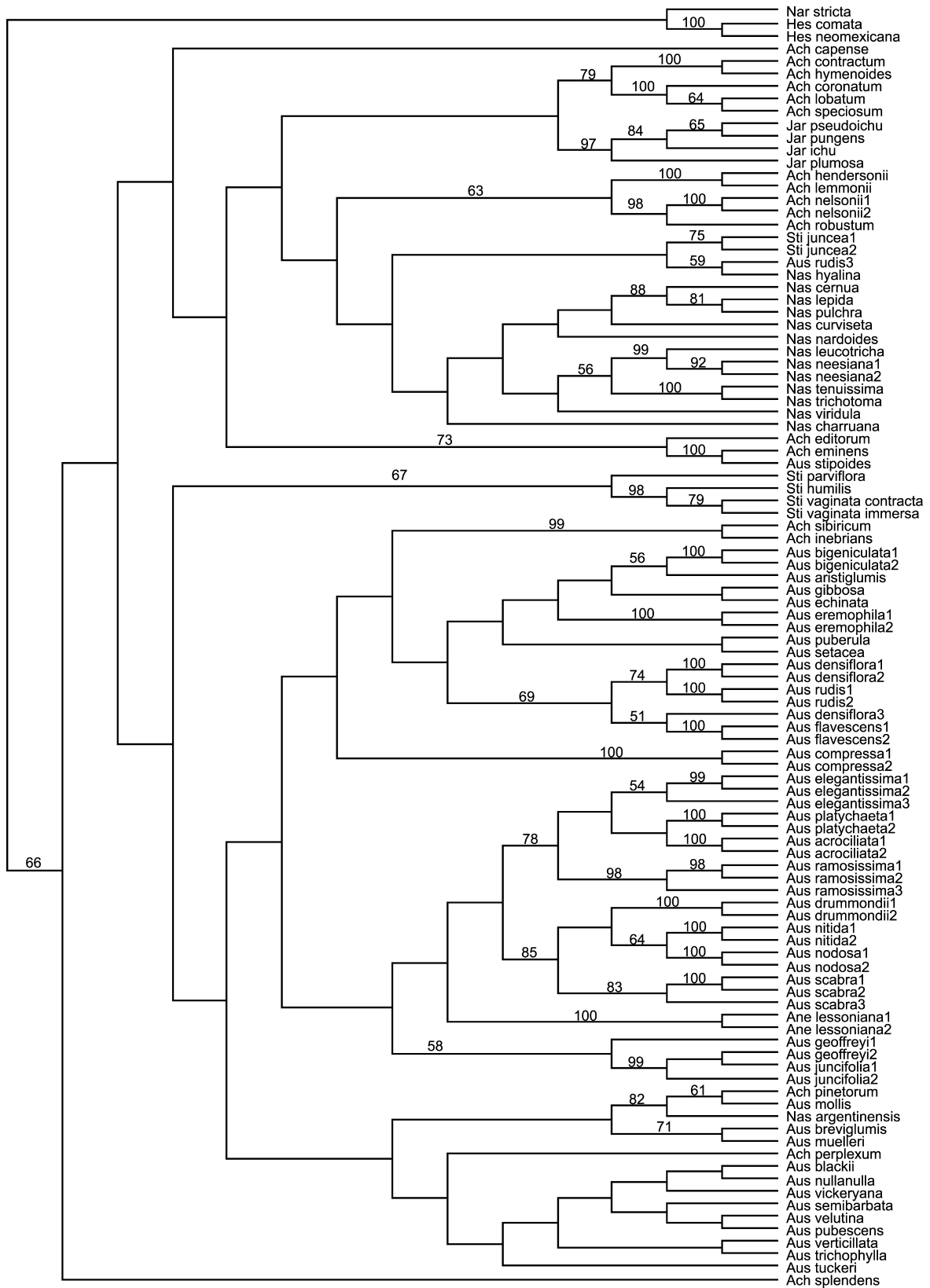


Fig. 3. Neighbor-joining tree derived from analysis of those species included in the contentious *Achnatherum* s.l. clade. Jackknife values >50% included. While there is limited support for the clade as a whole, there is virtually no support for any internal structure. Ach = *Achnatherum*; Ane = *Anemanthele*; Aus = *Austrostipa*; Hes = *Hesperostipa*; Jar = *Jarava* s.s.; Nar = *Nardus*; Nas = *Nassella*; Sti = *Stipa* s.s.

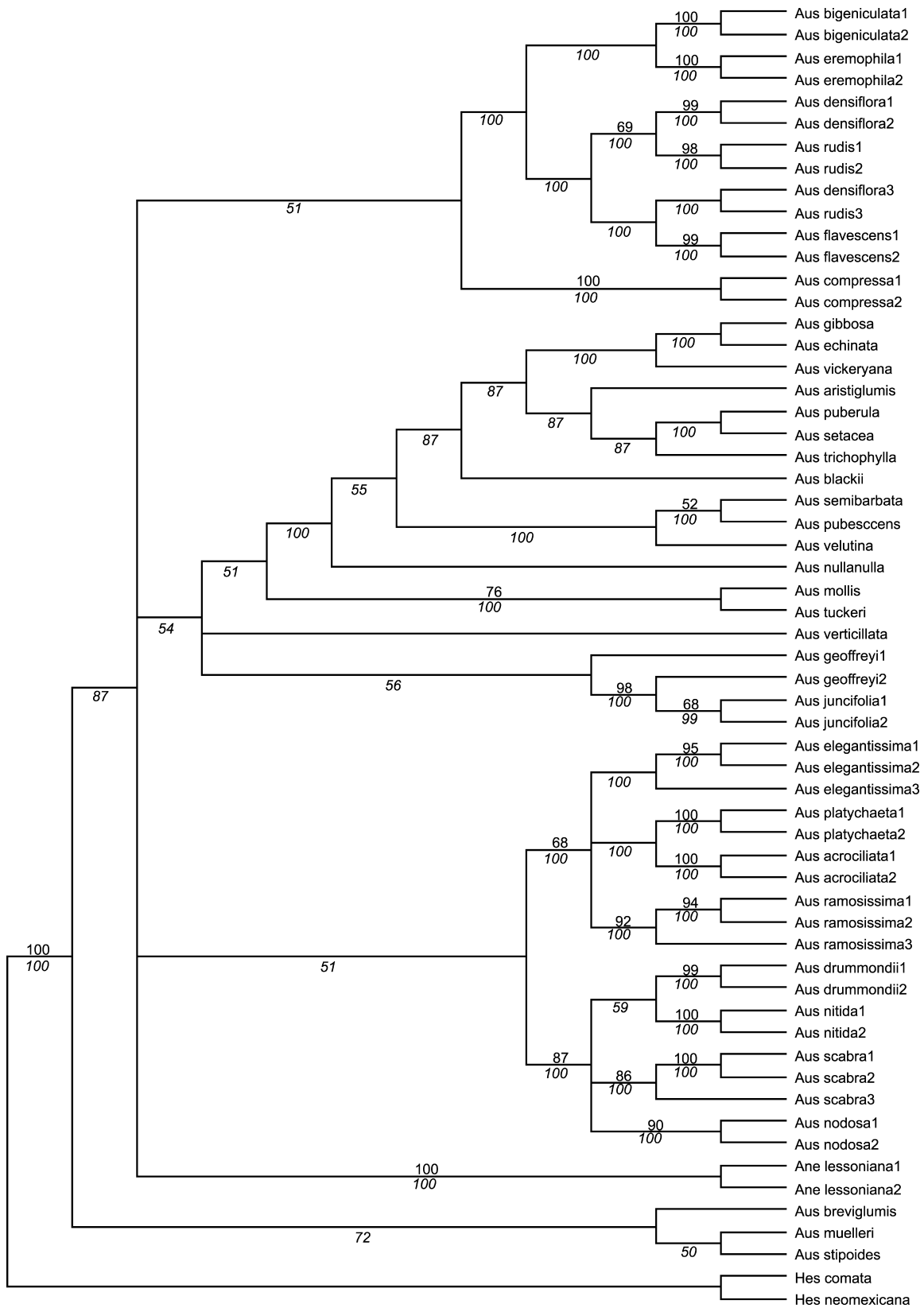


Fig. 4. Majority-rule tree derived from parsimony analysis of *Austrostipa* (9302 trees, 502 steps, CI = 0.568, RI = 0.756, RC = 0.487). Jackknife values >50% are shown above branches. Shown below branches is the percentage of all most-parsimonious trees exhibiting the clade. There is little support for any structure though the analysis has resolved some significant terminal groups. Ane = *Anemanthele*; Aus = *Austrostipa*; Hes = *Hesperostipa*.

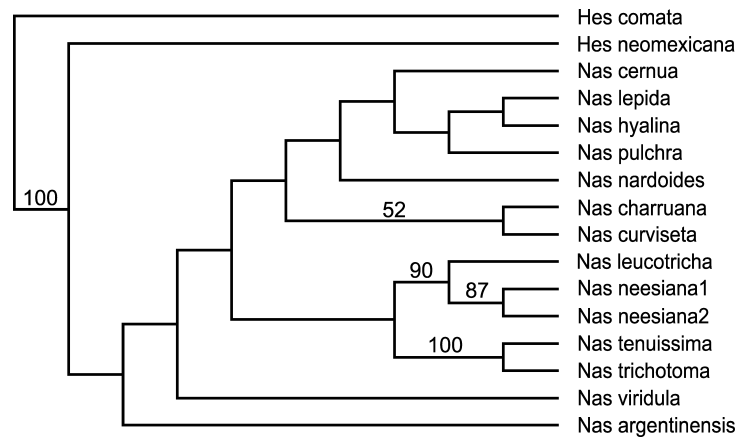


Fig. 5. Neighbor-joining tree derived from analysis of *Nassella*. While there is some jackknife support (values >50% shown) for some of the branches, such support is meaningless considering the low level of sampling. It does suggest there may be benefit in more intensive sampling. Hes = *Hesperostipa*; Nas = *Nassella*.

any structure; however, by using multiple replicates (5000) of 100 trees, it was possible to identify some consistent groupings (Fig. 4).

When Jacobs and Everett (1996) established *Austrostipa*, they also suggested a subgeneric classification with 13 subgenera. Testing subgeneric classifications is not easy with sequence data as the differences are often too few, and the potential problems caused by hybrid origins (multiple ITS copies) and multiple genomes (polyploidy) are most obvious although we have no direct evidence of this. This may be why the ITS data failed to recover the subgeneric classification suggested by Jacobs and Everett (1996) (Table 1, Fig. 2–4). Subgenus *Falcateae* (here represented by *A. drummondii*, *A. nitida*, *A. nodosa*, and *A. scabra*) is well supported as monophyletic, characterized in part by a four base-pair deletion. Subgenus *Lobateae* (*A. geoffreyi*, *A. juncifolia*) is supported, although *A. geoffreyi* has an incomplete sequence and one of the other species in the subgenus, *A. stipoides*, has a problematic sequence and will need to be resampled. The position of the sequence suggests that *A. stipoides* may have a hybrid origin. Subgenus *Longiaristatae* (*A. compressa*) is also distinct, but is only represented by one species here.

The ITS phylogeny also suggests that some subgenera could be reassessed and possibly some combined, for example subgen. *Austrostipa* (*A. densiflora*, *A. mollis*, *A. semi-barbata*) combined with subgen. *Tuberculatae* (*A. pubescens*, *A. rudis*), and subgen. *Bambusina* (*A. ramosissima*, *A. verticillata*) combined with subgen. *Arbuscula* (*A. acrociliata*, *A. breviglumis*, *A. platychaeta*). Species of subgen. *Austrostipa* and *Tuberculatae* have similar growth forms, similar-sized florets, and more or less similar lemma surfaces, and grow on sandy or sandstone-derived soils; the main differences between the two are the long-hairy awns and more densely pubescent lemmas of subgen. *Tuberculatae*. Species of both subgen. *Bambusina* and subgen. *Arbuscula* have a characteristic shrubby growth form often with multiple branching at the nodes, but there are differences in floret size and awn length between the two subgenera.

#### Hesperostipa

As in Jacobs et al. (2000) there is strong support for *Hesperostipa* (Fig. 2).

#### Jarava

*Jarava* was first recognized by Ruiz and Pavón (1794), but, until recently (Jacobs and Everett 1997), was treated as a section of the genus *Stipa* (Caro and Sánchez 1973) along with many genera now recognized as distinct. The diaspores are wind dispersed and have a range of morphological characters that distinguish them from the rest of *Achnatherum* (Jacobs and Everett 1997). The results to date do not support Peñailillo (2002) in his expansion of the circumscription of *Jarava* to include many South American stipoid species; the species in Fig. 3 he has transferred to *Jarava* are *A. speciosum*, *S. humilis*, and *S. vaginata*.

#### Nassella

*Nassella* remains monophyletic except for a single outlier (Fig. 2, 3), and that outlier (*N. argentinensis*) is an incomplete sequence. There is some evidence of subgeneric groupings, sufficient to suggest that further sampling may be informative (Fig. 5). While there is some jackknife support for some of the branches such support is meaningless considering the low level of sampling. Some species of *Stipa* s.s. are included in the clade (Fig. 2, 3) and this further supports the suggestion below that much work is yet to be done.

#### Oryzopsis and Piptatherum

*Oryzopsis* and *Piptatherum* have had a variety of treatments. Freitag (1975) in his treatment of *Piptatherum* included several species formerly placed in *Oryzopsis* and referred to a table comparing *Piptatherum* with the type species of *Oryzopsis*, *O. asperifolia*, and to another group of North American species of *Oryzopsis*. Kuo et al. (1983), Barkworth and Everett (1987) and Everett (1990) have all suggested that *Oryzopsis* should be monotypic and restricted to *O. asperifolia*. Our results here again support that suggestion (Fig. 2) although it does form a small well-supported clade that includes *Achnatherum splendens*, *Ampelodesmos mauritanica*, and *Stipa capillata*. These seem to be strange bedfellows and we can offer no explanation. Barkworth (1993) moved several *Oryzopsis* species to *Achnatherum* and others to *Piptatherum*. Our results support the separating of

*Oryzopsis* into more than one genus, but the polyphyly of *Achnatherum* indicates that more work is required to fully resolve the situation.

Jacobs et al. (2000) suggested that there was some support for the subgeneric groupings in *Piptatherum*. That support appears to have evaporated with the slightly larger data set (Fig. 1, 2). Although there is still no clear solution, species of *Oryzopsis* and *Piptatherum* are now tending to group together with some species of *Stipa* s.s. that diverge early from the rest of the species. There is little information on the grouping of *Piptatherum* species at present. Some of the groups have high jackknife values, but their relationships to each other are not clear.

#### “*Pappostipa*” (*Stipa* subgen. *Pappostipa*)

There is no support for including *Stipa* subgen. *Pappostipa* (*Stipa humilis* and *S. vaginata* in this analysis) with *Jarava* s.s. (Peñailillo 2002) (Fig. 2, 3). Indeed, there is no support for the monophyly of *Pappostipa* with the inclusion of *S. parviflora* in the clade. As many of the *Stipa* species appear alone in apparently unrelated clades this may be a reflection of insufficient sampling of *Stipa* s.s. rather than reflecting actual relationship.

#### Piptochaetium

As in Jacobs et al. (2000) there is strong support for *Piptochaetium* (Fig. 2), but so far there have only been sequences obtained for species with long-cylindrical florets. Sampling of some with almost spherical florets is required to confirm the monophyly of the genus.

#### *Stipa* *Sensu Stricto*

*Stipa* s.s. is represented by a small clade of three species in the analysis (*S. baicalensis*, *S. glareosa*, and *S. lessingiana*) while four other species (*S. barbata*, *S. capillata*, *S. juncea*, and *S. tirsia*) are scattered (Fig. 2), and their relative positions remain reasonably stable, altering only slightly with different analyses using different combinations of taxa in the data sets. The lack of monophyly is a surprising result as it has always been assumed that *Stipa* s.s. is a homogeneous group and the number of species sampled was based on that assumption. There is some reflection on geography; the first three species have a mainly Asian distribution, while *S. barbata*, *S. juncea*, and *S. tirsia* have a more European distribution, with *S. tirsia* connecting the two. *Stipa tirsia* belongs to sect. *Stipa*, whereas, all of the others belong to sect. *Leiostipa* Dumort. Vázquez and Barkworth (2004) recently reinstated *Macrochloa* and described *Celtica*, defined on morphological data, and studies are continuing on other species in this group. The results from the ITS analyses are not adequate to suggest anything more than the need to include more species from each of the sections of *Stipa* s.s. in any further analyses. There is obviously a need to examine even this new narrowly defined genus in more detail. The narrower interpretation of *Stipa* is defined by comparatively large one-flowered spikelets (usually >1 cm long), long, (>5 cm) long-hairy (pennate) awns, and lemmas not particularly indurate.

#### Overall Phylogeny

There is little support for the suggestion of two distinct lineages of American Stipeae (Barkworth and Everett 1987). All analyses suggest that, of the core genera, members of the *Piptatherum/Oryzopsis* complex are among the earliest-diverging lineages, along with some species of *Stipa* s.s. from the Americas and Eurasia distributed through the trees. *Hesperostipa* is next to diverge, and a logical genus to use as an outgroup when analyzing subsequent groups. All analyses indicate that *Austrostipa* is probably the most recently derived genus or group. The data clearly suggest a Eurasian origin for the tribe as the early branches are all essentially European and most of the next-diverging branches (with the exception of *Hesperostipa*) contain both European and New World species.

#### ACKNOWLEDGMENTS

We would like to thank Chris Quinn for his generous help in analyzing the data. This is a product of the Stipoid Working Group, a collaboration of people interested in the phylogeny and biology of the stipoid grasses. Details can be obtained from the author listed for correspondence.

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