



Segregation of *Leptatherum* from *Microstegium* (Andropogoneae, Poaceae) confirmed by Internal Transcribed Spacer DNA sequences

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Key words

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Abstract Phylogenetic analyses of *Microstegium* (Andropogoneae, Poaceae) and some other Andropogoneae species were conducted inferred from the ITS/5.8S sequences. As a result of this study, *Microstegium* is polyphyletic. There are two rather distant monophyletic clades, one with *Microstegium nudum* (type of *Leptatherum*), *M. japonicum* and *M. somae*, the other one with *M. ciliatum*, *M. fauriei*, *M. geniculatum* and *M. vimineum* (type of *Microstegium*). Therefore we feel justified to reinstate the genus *Leptatherum* and three new combinations, *Leptatherum boreale*, *L. nudum* and *L. somae*, are proposed.

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INTRODUCTION

The genus *Microstegium* Nees belongs to the tribe *Andropogoneae* of the family *Poaceae* and contains about 25 species. They are widely distributed in the tropical and subtropical Old World (Clayton & Renvoize 1986, Koyama 1987, Watson & Dallwitz 1992, Hsu 2000). They are creeping or rambling annual or perennial plants with broadly linear to lanceolate leaf-blades, inflorescences of 1–many subdigitate racemes with a fragile rachis; internodes filiform to clavate or inflated; sessile spikelet: lower glume dorsally with a deep groove or a broadly concave median channel, the margins sharply inflexed and usually keeled; upper glume often shortly awned; lower floret well developed, reduced to a palea or absent; upper lemma linear to cordate and usually with long awns, usually accompanied by a small palea; stamens 3, rarely 2; pedicelled spikelet resembling the sessile one, occasionally slightly smaller and male (Clayton & Renvoize 1986).

The genus was first distinguished by Trinius (1832) as *Pollinia* Trin., a name previously used by Sprengel (1815) for what now is *Chrysopogon* Trin., so it is a later homonym and illegitimate. *Microstegium* was erected by Nees (1836) with as the only species and thus the type, *M. willdenovianum* Nees, now to be called *M. vimineum* (Trin.) A. Camus. The original specimen, Wallich 8838 in B is lost, but there are duplicates in BM, CAL, E, G, K (microfiche IDC 7394), L and P.

Nees (1841a, b) described another genus, *Leptatherum* Nees, with as type *L. royleanum* Nees, now *M. nudum* (Trin.) A. Camus. In most subsequent publications this has been regarded as a

synonym of *Microstegium*. However, Tzvelev (1966) considered it to be a distinct section of *Microstegium*, based on the slender and hairless racemes, abruptly acute glume apex and concave dorsal face of the lower glumes. He included *M. nudum* and *M. japonicum* (Miq.) Koidz. (as a subspecies) in it. In these species the stamens are 2 per floret, while they are usually 3 in the species of *Microstegium*, only *M. tenue* (Trin.) Hosok. has 1.

Hayata (1918) created the genus *Polliniopsis* Hayata for *P. somae* Hayata (usually and erroneously written as '*somai*'). He regarded it as distinct because of the binate spikelets which are both pedicelled, and in the 2-aristate lower glume, and an upper glume, lower lemma and upper lemma each with a long awn. So a single spikelet has 3 awns. There are 2 stamens per floret. Ohwi (1942) regarded it as belonging to *Microstegium*, and made the combination *M. somae* (Hayata) Ohwi for it. Koyama (1987) went even further and regarded it as a subspecies of *M. japonicum*, subsp. *somae* (Hayata) T. Koyama.

A generic distinction between *Microstegium* and *Leptatherum* was indicated by Spangler (Spangler et al. 1999, Spangler 2000) in a survey of the phylogeny of chloroplast gene *ndhF* sequences of *Andropogoneae*. Here *M. vimineum* and *M. nudum* were not in one monophyletic clade but were separated far apart in the cladogram. These two species are the types of *Microstegium* and *Leptatherum*, respectively. A weakness in their strict consensus tree of *ndhF* gene is that most internal nodes have only relatively low support measures (Spangler et al. 1999). However, in addition to *ndhF* gene, two other nuclear genes, *waxy* (Mason-Gamer et al. 1998) and phytochrome B (Mathews et al. 2002), show the same pattern of short internal branch lengths, suggesting a similar pattern of evolution. This repeated pattern across genes and genomes made Spangler et al. confident that their topology of *ndhF* gene tree for *Andropogoneae* is the correct one rather than one reflecting coincidental convergence (Spangler et al. 1999). In another word, the genus *Microstegium* is polyphyletic (Mathews et al. 2002). Though we knew the distinction between the *M. nudum* group and the other species of *Microstegium*, this result was quite astonishing. We even suspected that the author(s) might have misidentified their materials.

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In this study, we wanted to elucidate the phylogeny of the *M. nudum* group and some representative species of *Microstegium* and to see if we could repeat or refute Spangler et al.'s observation. Since the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron (18S-5.8S-26S) has been used for plant molecular systematic research at the species level (Alvarez & Wendel 2003) with a good effectiveness of species-level discrimination and technical ease, a large body of sequence data already exists in the GenBank for this region (Kress et al. 2005). In addition, it has been suggested as a potential plant barcode locus (Stoeckle 2003, Kress et al. 2005). Therefore we chose it as a marker to achieve the above objectives.

MATERIAL AND METHODS

Taxa sampling

A total of 23 accessions of ITS sequences of seven *Microstegium* species were processed. Seven were of *M. ciliatum* (Trin.) A. Camus, two of *M. vimineum*, two of *M. fauriei* (Hayata) Honda, one of *M. geniculatum* (Hayata) Honda, three of *M. nudum*, four of *M. somae* and four of *M. japonicum* (see Table 1 for details). All vouchers were deposited in the herbaria of the Endemic Species Research Institute (TAIE) and the National Museum of Natural Science (TNM), Taiwan.

Molecular methods

Leaves dried in silica gel or taken from herbarium specimens were frozen with liquid nitrogen and crushed using a mortar and pestle. The total DNA was extracted using a modified cetyltrimethyl-ammonium bromide (CTAB) extraction procedure (Murray & Thompson 1980).

The ITS region, which includes the ITS1, 5.8S rDNA and ITS2, was amplified by PCR with primers we designed based on the 26S rRNA gene and 18S rRNA gene of some other grasses. The sequence of forward primer, IT-11:5'-TCG TAA CAA GGT TTC CGT AGG T-3', is located at the 3' end of 18S rRNA gene, and the reverse one, IT-8:5'-GTAAGT TTC TTC TCC GCT-3', at the beginning sequence of 26S rRNA gene.

The protocols for the PCR were as follows: we used a 50 µl mixture containing 40 mM Tricine-KOH (pH 8.7), 15 mM

KOAc, 3.5 mM Mg(OAc)₂, 3.75 µg/ml BSA, 0.005 % Tween 20, 0.005 % Nonidet-P40, four dNTPs (0.2 mM each), primers (0.5 µM each), 2.5 units of Advantage 2 DNA polymerase (Clontech), 10 ng genomic DNA, and a 50 µl volume of mineral oil. The PCR mixture for amplifying the ITS region included 10 % dimethylsulfoxide (DMSO) to reduce problems related to the secondary structure and efficiency of PCR primer binding. Amplification reactions were completed in a dry-block with two-step thermal cycles (Biometra). In the first step, the mixture was incubated at 94 °C for 3 min, then it underwent 10 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min. The second step was carried out by the following process: 30 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min, with a final extension for 10 min at 72 °C. These PCR products were detected by agarose gel electrophoresis (1.0 %, w/v in TBE), stained with 0.5 µg/ml ethidiumbromide, and finally photographed under UV light exposure.

These DNAs were directly sequenced following the method of dideoxy chain-termination using an ABI377 automated sequencer with the Ready Reaction Kit (PE Biosystems, California) of the BigDye™ Terminator Cycle Sequencing. Sequencing primers were the same as those used for PCR. Each sample was sequenced two or three times to confirm the sequences. These reactions were performed as recommended by the manufacturers.

Sequence alignment and phylogenetic analysis

In addition to the 23 accessions of *Microstegium*, the ITS sequences of 31 accessions of *Andropogoneae* and fifteen accessions of *Panicaceae* were obtained from GenBank.

Alignment of obtained sequences was first aided by using the program Clustal W multiple alignment in BioEdit (Hall 1999), and adjusted manually. The aligned data matrix and tree files are available from the first author.

Phylogenetic trees were constructed using two methods, neighbour-joining (NJ) (Saitou & Nei 1987) and maximum parsimony (MP) (Swofford et al. 1996). Both NJ and MP analyses were conducted using MEGA v4 (Tamura et al. 2007), and rooted by the *Panicaceae* accessions. For the NJ analysis, a rooted tree was generated from the distance matrix calculated by the two-parameter method of Kimura (1980). The MP tree was obtained using the Close-Neighbour-Interchange algorithm (Nei & Kumar 2000) with search level 3 (Felsenstein 1985, Nei & Kumar 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). The strict consensus parsimonious tree was then constructed. Supporting levels for nodes in both analyses were assessed with a bootstrap analysis (Felsenstein 1985) using 1 000 replicates.

RESULTS

The 23 new sequences produced in this study have been submitted to GenBank (see Table 1 for their accession numbers). The dataset had a total of 618 bp of aligned ITS sequence for each taxon with 326 variable characters and 249 of those were parsimony informative (40.3 % of the total ITS sequence length). The MP analysis resulted in 33 most parsimonious trees (length = 761), with consistency index (CI) of 0.442336, and retention index (RI) of 0.768485, for parsimony informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches.

The topologies of NJ tree and MP bootstrap consensus tree are as Fig. 1 and Fig. 2, respectively. In both trees, the species name and accession number of all the OTUs were given.

Table 1 Information of the 23 *Microstegium* samples used in this study.

Taxa	Sample number	Voucher information	Deposit herbarium	GENBANK accession number
<i>Microstegium vimineum</i> group				
<i>M. ciliatum</i>	S22	Wu 17-033	TNM	EU489066
	S28	S90933	TNM	EU489068
	S42	Huang 62	TNM	EU489069
	S46	S90921	TNM	EU489070
	W39	Chen 5639	TAIE	EU489077
	W53	Chen 5737	TAIE	EU489086
	W56	Chen 5741	TAIE	EU489087
<i>M. fauriei</i>	W42	Chen 5642	TAIE	EU489079
	W60	Chen 5799	TAIE	EU489088
<i>M. geniculatum</i>	W61	Chen 5802	TAIE	EU489071
<i>M. vimineum</i>	W44	Chen 5644	TAIE	EU489080
	W50	Chen 5628	TAIE	EU489083
<i>Microstegium nudum</i> group				
<i>M. japonicum</i>	W45	Chen 5623	TAIE	EU489081
	W46	Chen 5624	TAIE	EU489082
	W52	Chen 5630	TAIE	EU489085
	W65	Chen 5690	TAIE	EU489076
<i>M. nudum</i>	S24	Liu 890367	TNM	EU489067
	W34	Chen 5634	TAIE	EU489073
	W40	Chen 5640	TAIE	EU489078
<i>M. somae</i>	W32	Chen 5631	TAIE	EU489072
	W35	Chen 5635	TAIE	EU489074
	W36	Chen 5636	TAIE	EU489075
	W51	Chen 5629	TAIE	EU489084

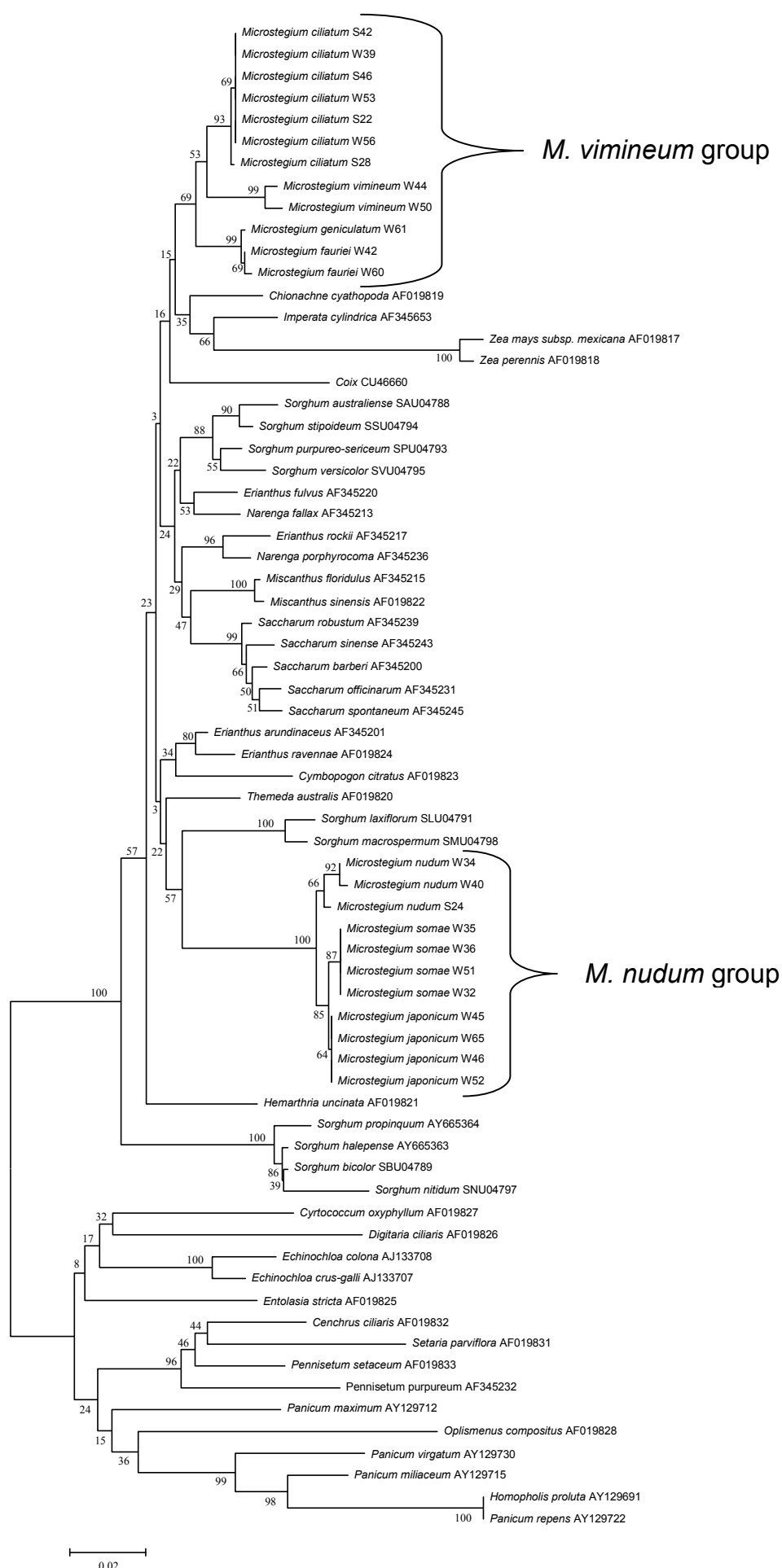


Fig. 1 Phylogenetic tree resulted from NJ analysis inferred from the ITS/5.8S sequences of *Microstegium* and some other *Andropogoneae* species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches.

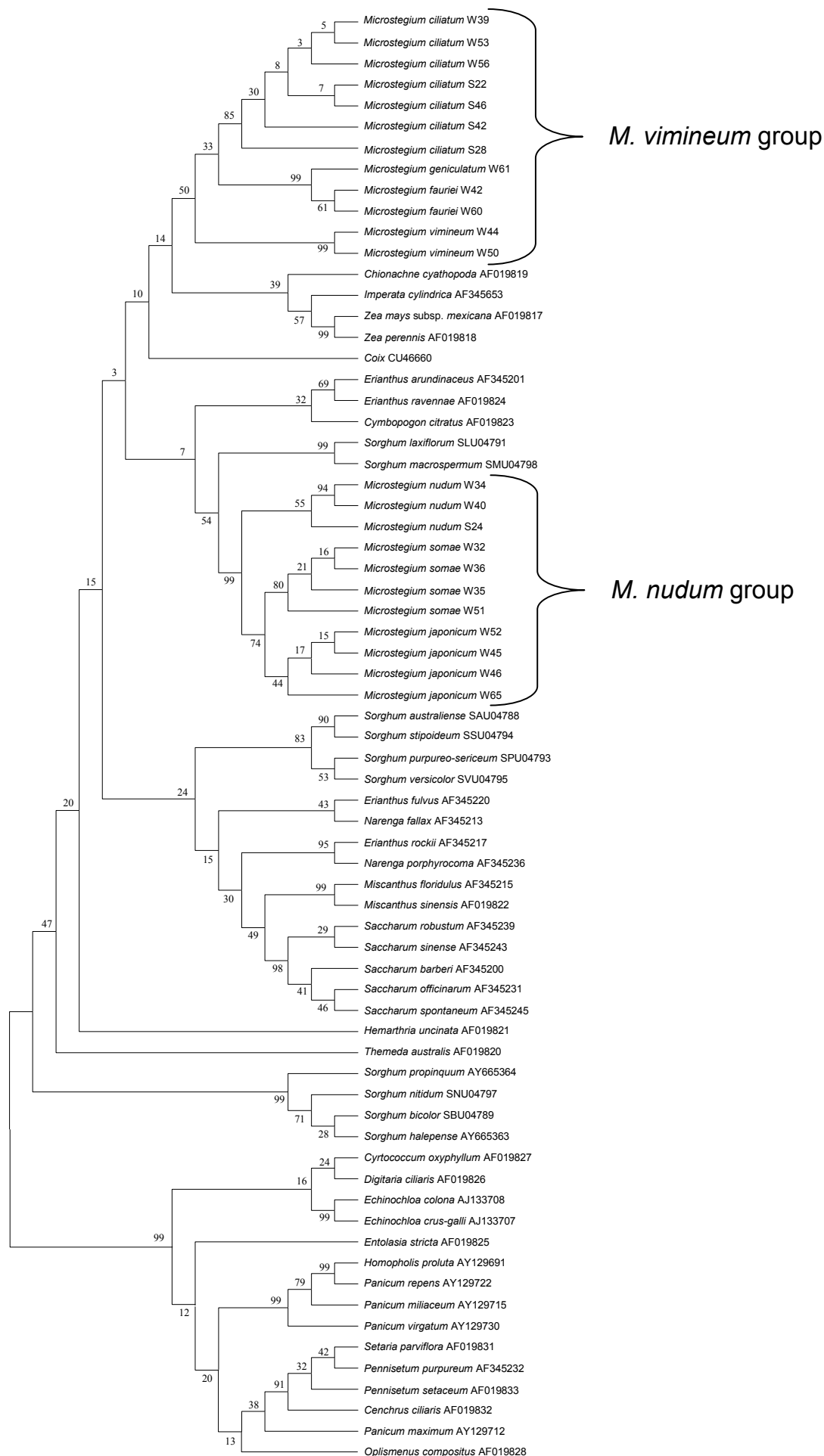


Fig. 2 Consensus parsimonious tree resulted from NJ analysis inferred from the ITS/5.8S sequences of *Microstegium* and some other *Andropogoneae* species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches.

Though the topologies of two trees are not identical, they both demonstrate that the *Microstegium* are not in one monophyletic clade but are separated far apart in the cladogram. This result is in accordance with that of Spangler which was obtained from *ndhF* sequences (Spangler et al. 1999, Spangler 2000). In both NJ and MP trees, the *M. nudum* group formed a monophyletic clade with very strong bootstrap support (100 % in NJ tree and 99 % in MP trees). The other species, including *M. ciliatum*, *M. fauriei* and *M. geniculatum* and *M. vimineum* (hereafter *M. vimineum* group), formed another monophyletic clade but with low bootstrap support (only 69 % in NJ tree and 50 % in MP trees). Besides, the phenomenon of relatively low bootstrap supports for most internal nodes in both our NJ and MP trees is similar to Spangler et al.'s result, too.

DISCUSSION

In our results, the NJ and MP analyses both support the results of Spangler et al. (1999) that *M. vimineum* is widely separated from *M. nudum*, although we also were unable to recover a reasonable level of support for the intervening nodes. Similar to Spangler et al.'s result of *ndhF* gene (1999), our NJ and MP tree of ITS sequences have relatively low supports for most internal nodes. It is a problem to determine how the nodes are related to each other. Spangler et al.'s result and ours both indicate the level of molecular divergence within the tribe is low. However, at least, the clade of the *M. nudum* group are very well supported. This can correspond with Tzvelev's (1966) opinion that *Leptatherum* (incl. *M. somae*) can be distinguished from *Microstegium* at the infrageneric level.

All results obtained so far therefore indicate that *Microstegium* is non-monophyletic and consists of two separate clades. One of them is composed of the *M. nudum* group, the other of *M. vimineum* and allies. Although, in view of the low bootstrap supports, further confirmation is necessary, we think that our results in combination with those of Spangler (2000) and Mathews et al.'s (2002) opinion justify the reinstatement of *Leptatherum* as a distinct genus, necessitating three new combinations, *Leptatherum boreale* (Ohwi) C.-H. Chen, C.-S. Kuoh, Veldk., *L. nudum* (Trin.) C.-H. Chen, C.-S. Kuoh, Veldk. and *L. somae* (Hayata) C.-H. Chen, C.-S. Kuoh, Veldk.

KEY TO THE SPECIES

1. Ligule absent. Blades firm, base broadly rounded. Racemes drooping, rachis tenaceous. Sessile spikelets with a short pedicel, deciduous without the adjacent joint or pedicel of the pedicelled spikelet. — China, Taiwan, Japan, S. Korea 2
1. Ligule a glabrous membrane. Blades flaccid, base attenuate to slightly rounded. Racemes patent, rachis fragile. Sessile spikelets without a short pedicel, deciduous with the adjacent joint and pedicel of the pedicelled spikelet. — S Africa to Japan and New Guinea 2. *L. nudum*
2. Upper glume muticous. Lower lemma oblong, muticous. — China (Anhui, Hubei, Hunan, Jiangsu, Jiangxi, Zhejiang), Japan, S. Korea 1. *L. boreale*
2. Upper glume mucronate. Lower lemma lanceolate, awned. — China (Anhui, Fujian), Taiwan, Ryukyu Isl. . 3. *L. somae*

1. *Leptatherum boreale* (Ohwi) C.-H. Chen, C.-S. Kuoh, Veldk., *comb. nov.*

Microstegium boreale Ohwi, J. Jap. Bot. 12 (1936) 653. — *Microstegium nudum* (Trin.) A. Camus var. *boreale* (Ohwi) Ohwi (1937) 151. — *Microstegium japonicum* (Miq.) Koidz. var. *boreale* (Ohwi) Ohwi (1942) 156. — Type: Ohwi & Tagawa 727 (holo KYO).

Pollinia japonica Miq. (1866a) 290; (1866b) 178. — [*Pollinia japonica* Miq. var. *polystachya* Franch. & Sav. (1876) 190, nom. inval.]. — *Microstegium japonicum* (Miq.) Koidz. (1929) 394. — [*Microstegium vimineum* (Trin.) A. Camus var. *polystachyum* (Franch. & Sav.) Ohwi (1942) 156, nom. inval.]. — [*Pogonatherum glabratum* (Brongn.) Roberty subvar. *japonicum* (Miq.) Roberty (1960) 388, nom. inval.]. — [*Microstegium vimineum* (Trin.) A. Camus forma *polystachyum* (Franch. & Sav.) T.K. Koyama (1971) 65, nom. inval.]. — *Microstegium nudum* (Trin.) A. Camus subsp. *japonicum* (Miq.) Tzvelev (1976) 695. — Type: Keiske s.n. (holo L).

Microstegium nudum (Trin.) A. Camus var. *shimidzui* Honda (1930) 407. — *Microstegium japonicum* (Miq.) Koidz. forma *shimidzui* (Honda) Ohwi (1942) 156. — Type: Shimizu s.n. 1929 (holo TI).

Note — The combination *Leptatherum japonicum* (Miq.) cannot be made, as there already is the heterotypic *L. japonicum* Franch. & Sav., a synonym of *L. nudum*, q.v. A line drawing plate of this species has been made by Chen & Kuoh (2007).

2. *Leptatherum nudum* (Trin.) C.-H. Chen, C.-S. Kuoh, Veldk., *comb. nov.*

Pollinia nuda Trin., Mém. Acad. Imp. Sci. St. Pétersbourg VI, Sci. Math. 2 (1832) 307. — [*Pollinia nuda* Trin. var. *genuina* Hack. (1889) 179, nom. inval.]. — *Eulalia nuda* (Trin.) Kuntze (1891) 775. — *Microstegium nudum* (Trin.) A. Camus (1921) 201. — [*Pogonatherum glabratum* (Brongn.) Roberty subvar. *nudum* (Trin.) Roberty (1960) 389, nom. inval.]. — Type: Wallich ex Hornemann in Herb. Trinius 56.1 (holo LE, drawing & fragm., IDC microfiche BT-16/1; this probably is Wallich Cat. 8831, microfiche IDC 7394, photo in BRI, as this is the only collection of this species in the Wallich herbarium; iso C, CAL, E, K, L, P).

Leptatherum royleanum Nees (1841a) 93; (1841b) 220; cited by Steud. (1854) 409] as "Jard. Annal. vii: 220". — Type: Herb. Royle 219 (holo B, lost?; iso CGE?, LIV?, W).

Psilopogon capensis Hochst. (1846) 117. — *Eulalia capensis* (Hochst.) Hochst. ex Steud. (1854) 412. — *Pollinia nuda* Trin. var. *capensis* (Hochst.) Hack. (1889) 179. — *Microstegium capense* (Hochst.) A. Camus (1921) 201. — Type: C. Krauss 92 (holo HBG).

Leptatherum japonicum Franch. & Sav. (1876) 190; (1878) 609. — Type: Savatier 1507, 2557 (syn P).

Pollinia arisanensis Hayata (1918) 74, t. 43. — *Microstegium arisanense* (Hayata) A. Camus (1921) 201. — Type: Faurie Dec. 1914 (holo TI).

Microstegium mayebarum Honda (1930) 405. — Type: Mayebara 334, 335, 345, 346, Miyagi 394, Sakaguchi 10 (syn TI).

Note — A line drawing plate of this species has been made by Hsu (1975).

3. *Leptatherum somae* (Hayata) C.-H. Chen, C.-S. Kuoh, Veldk., *comb. nov.*

Microstegium somae (Hayata) Ohwi, Acta Phytotax. Geobot. 11 (1942) 155. — *Polliniopsis somae* Hayata (1918) 76. — [*Ischaemum petiolare* (Trin.) Hack. var. *somae* (Hayata) Roberty (1960) 346, nom. inval.]. — *Microstegium japonicum* (Miq.) Koidz. var. *somae* (Hayata) T. Koyama (1962) 238. — *Microstegium japonicum* (Miq.) Koidz. subsp. *somae* (Hayata) T. Koyama (1987) 427, 516. — Type: Soma s.n. 1914 (holo TI).

Note — A line drawing plate of this species has been made by Hsu (1975). The name is usually misspelled as '*soma*'.

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