Re-evaluation of the taxonomic status of *Hackelochloa* (Poaceae) based on anatomical and phenetic analyses

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Hackelochloa is a pantropical genus of plant in the Poaceae, in which only two species have been included, *H. granularis* and *H. porifera*. Despite several morphological differences, notably the more prominent sculpturing of the lower glume and the larger dimensions of several quantitative traits, *H. porifera* has been reduced to the synonymy of *H. granularis*. Moreover, the status of the genus itself has been questioned, with a regional revision of the genus proposing inclusion of its members in the genus *Mnesithea*. In the present study, we investigated a range of morphological and anatomical attributes to assess critically the generic delimitation between *Hackelochloa* and *Mnesithea*. In clustering analysis, *H. granularis*, *H. porifera* and *Mnesithea* species were clearly resolved as three distinct groups with an R-value of 0.98114. Likewise, three clusters representing these taxonomic units were revealed using principal component analysis (PCA), with the first two principal components highlighting key qualitative and quantitative characters. Scanning electron microscopy was used to reveal different patterns of sculpturing on the lower glumes in the two putative species and the ecological significance of these differences is inferred. The outline of leaf transverse sections, presence of parenchymatous tissue in the midrib region, number of adjacent bundles and number of chlorenchyma layers in the culm were also found to be diagnostic anatomical characters. This study supports the recognition of *H. porifera* as distinct from *H. granularis* and provides evidence that the genus *Hackelochloa* should be maintained. © 2016 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2016, **181**, 224–245.

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INTRODUCTION

Hackelochloa Kuntze is a pantropical genus in the Poaceae, tribe Andropogoneae, subtribe Rottboelliinae (Clayton & Renvoize, 1986; Watson & Dallwitz, 1992). Its distribution covers the Old World and New World tropics (Clayton & Renvoize, 1986). Only two species, *H. granularis* (L.) Kuntze and *H. porifera*

(Hack.) D.Rhind, have been recognised in the genus (Hooker, 1897; Rhind, 1945; Bor, 1960; Clayton & Renvoize, 1982, 1986; Sreekumar & Nair, 1991; Watson & Dallwitz, 1992; Shukla, 1996; Moulik, 1997; Noltie, 2000; Bixing & Phillips, 2006; Traiperm, 2007). Despite the wide geographical distribution of the genus, the two species co-occur in only a few countries in tropical Asia, including Myanmar, Thailand, Vietnam, parts of southwestern China and India (Hooker, 1897; Rhind, 1945; Bor, 1960; Clayton & Renvoize, 1982, 1986; Sreekumar & Nair, 1991;

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Watson & Dallwitz, 1992; Shukla, 1996; Moulik, 1997; Noltie, 2000; Bixing & Phillips, 2006; Traiperm, 2007).

In terms of its morphology, *Hackelochloa* exhibits several diagnostic features in the structure of its spikelet pair, including the fusion of the rachis internode and pedicel into a single unit, unique sculpturing on the surface of the lower glume of the sessile spikelet and the overall shape of the spikelet pair (Bor, 1960; Clayton & Renvoize, 1986; Clark & Pohl, 1996; Noltie, 2000; Traiperm, 2007). Although *Hackelochloa* can therefore be morphologically distinguished from other genera, its two species closely resemble each another, at least superficially. However, in nature, *H. porifera* significantly exceeds *H. granularis* in several quantitative traits, such as the dimensions of the leaf blade, leaf sheath and overall plant height. Furthermore, more elaborate sculpturing on the lower glume in *H. porifera* also supports discrimination between these two species (Hooker, 1897; Bor, 1960; Noltie, 2000; Bixing & Phillips, 2006; Traiperm, 2007).

Previous taxonomic studies have proposed different justifications for the separation or combination of the two species, causing problems for the recognition of the genus as a whole. Bor (1960) examined specimens of *H. porifera* from a restricted selection of provenances (India, Myanmar, Vietnam and China) and concluded that spikelet dimension and the prominence of lower glume sculpturing could be used to adequately differentiate *H. porifera* from *H. granularis*. In contrast, Veldkamp, de Koning & Sosef (1986) raised objections to the separation of the two species. Their comparative investigation of the holotype of *H. porifera* and two specimens from Sumatra that exhibit large (1.5–1.7 mm long) and well decorated glumes caused them to reject the notion that spikelet size and glume sculpturing are satisfactory diagnostic features and they concluded that the concepts are, at best, extreme manifestations of the same entity. In the absence of other supplementary distinguishing characters to divide the species, *H. porifera* has subsequently been treated as a synonym of *H. granularis* (Veldkamp *et al.*, 1986).

Further appraisal at the generic level led to *H. granularis* being merged under the related genus *Mnesithea* Kunth, with the new scientific name *M. granularis* (L.) de Koning & Sosef proposed by Veldkamp *et al.* (1986) to accommodate the single remaining species. Other authors have followed Veldkamp *et al.* (1986) in maintaining a broad circumscription of *Mnesithea* (Soreng *et al.*, 2015). Hitherto, *Hackelochloa* had been maintained as distinct from *Mnesithea* based on the spherical shape of the lower glume and the fusion of rachis internode and pedicel.

Subsequent transfer of the morphologically intermediate *Heteropholis annua* Lazarides, which has a subglobose lower glume (similar to that of *Hackelochloa*) that is free of the rachis internode (as in *Mnesithea*), to *Mnesithea* [as *M. annua* (Lazarides) de Koing & Sosef] appeared to provide yet weightier justification for the obsolescence of *Hackelochloa* (Veldkamp *et al.*, 1986).

Despite the few recent molecular studies of Andropogoneae and related tribes (Mathews *et al.*, 2002; Skendzic, Columbus & Cerros-Tlatilpa, 2007; Teerawatananon, Jacobs & Hodkinson, 2011; Morrone *et al.*, 2012), the phylogenetic placement of *Hackelochloa* and its relationships in Rottboelliinae have not been closely scrutinised. Skendzic *et al.* (2007) sampled *H. granularis* (which they treated as *Hackelochloa*) but not *Mnesithea* in their analysis of the Andropogoneae, with the former forming part of an unresolved polytomy with *Rottboellia aurita* Steud., *Coelorachis rugosa* (Nutt.) Nash and *Hemarthria uncinata* R.Br. (all also members of Rottboelliinae), with a number of representatives of other subtribes, in their individual ITS and *trnL-F* trees. Their combined analysis failed to find support for a monophyletic Rottboelliinae, with *Elionurus tripsacoides* Humb. & Bonpl. ex Willd. as sister to a large clade of members of assorted subtribes, including *H. granularis* (Skendzic *et al.*, 2007). In their study of Panicoideae, which made use of the same two markers plus *atpB-rbcL*, Teerawatananon *et al.* (2011) found *H. granularis* (again, treated as *Hackelochloa*) to form a well-supported clade with *Hemarthria pratensis* (Balansa) Clayton and *H. longiflora* (Hook.f.) A.Camus and this clade in turn to fall sister to *Mnesithea formosa* (R.Br.) de Koning & Sosef, the sole member of *Mnesithea* to be included. This relationship between *Hackelochloa* and *Hemarthria* R.Br. was unexpected and at odds with the findings of earlier morphological studies (Clayton & Renvoize, 1986), further confusing the picture. Moreover, *Elionurus* Humb. & Bonpl. ex Willd., *Eremochloa* Buse and *Phacelurus* Griseb., also members of Rottboelliinae, did not cluster together, providing a stronger indication that the subtribe is polyphyletic (Teerawatananon *et al.*, 2011). Corroboration for the inclusion of *Hackelochloa* in *Mnesithea* is therefore still lacking, with its placement in subtribe Rottboelliinae remaining as ambiguous as it is for several other currently included genera.

Clearly there is a need for fresh studies utilising alternative sources of information to re-evaluate the taxonomic status of *Hackelochloa*. We set out to investigate aspects of the micromorphology and anatomy of the two species of *Hackelochloa* in comparison with a broader range of *Mnesithea* species than has been included in any of the molecular

phylogenetic studies. Our aims were to examine the hypotheses that: (i) explicit, non-continuous characters exist that may be adequately used to resolve *H. granularis* as distinct from *H. porifera* and (ii) *Hackelochloa* and *Mnesithea* can be sufficiently well circumscribed in their own right to be regarded as independent members of Rottboelliinae. We performed external morphological and internal anatomical techniques to probe structures including the leaf epidermis, leaf and culm transverse section, followed by UPGMA analysis and principal component analysis (PCA) in an attempt to elucidate the status of *H. granularis* and *H. porifera* in relation to *Mnesithea*. Given the extensive geographical distribution of *Hackelochloa*, we included a wide range of specimens sourced from continental tropical Asia.

MATERIAL AND METHODS

PHENETIC ANALYSES

Plant materials

Thirty-three accessions of *H. granularis* and 11 accessions of *H. porifera* from India, China, Myanmar, Vietnam and Thailand deposited at BKF, K and QBG were used in this study (Table 1). Based on the generic delimitation presented by Veldkamp *et al.* (1986), 15 individuals representing eight *Mnesithea* species were also examined to assess putative differences between *Hackelochloa* and *Mnesithea*. Furthermore, two specimens of *Ophiuros*, also included in Rottboelliinae, were included as an outgroup to help clarify the degree of separation between these two related genera. Information on ecology and phenology was also gleaned from the specimens.

Character examination

Twenty-eight characters (G1–28) were used to examine relationships between *Mnesithea*, *Hackelochloa* and *Ophiuros*, and 17 characters (S1–17) were used to discriminate putative taxa in *Hackelochloa* (Tables 2 and 3). These characters focused on features of the leaf, inflorescence and spikelet; some were quantitative traits measured with a ruler and some were qualitative traits scored through observation. For the quantitative traits, a mean value was calculated from six to ten replicate observations of healthy organs. The shapes of some structures were described using standard ratios in order to reduce bias from observations (Dilcher, 1974; Simpson, 2006). Each morphological character state was then assigned a discrete number to construct a data matrix for further analysis. To score quantitative characters, scatter plots were constructed in Minitab version 16 (2010) to determine clear-cut ranges of each character.

Data analysis

Two cluster analyses were conducted: UPGMA and PCA. To construct the dendrogram, the data matrix derived from character scoring of 61 operational taxonomic units (OTUs) was analysed using SIMQUAL module to calculate a similarity matrix with SM (simple matching) coefficient. The SAHN module was used to construct a UPGMA tree, and the Graphics module was used to visualise the dendrogram in NTSYS-pc version 2.1 (Rohlf, 2000). To assess the distributions and extents of OTUs in the score plots, PCA was conducted in Minitab version 16 (2010). In addition, significant distinguishable characters for field identification were determined with support from the values of the eigenvectors derived from the first and second components of PCA.

MICROMORPHOLOGICAL ANALYSIS

Scanning electron microscopy (SEM) of lower glumes

Details of the lower glumes of the sessile spikelets were examined in specimens referable to either *H. granularis* or *H. porifera* (Table 1) under an Olympus SZ40 stereomicroscope. All fixations were performed by immersing samples in 70% (v/v) ethanol for 5 min. The samples were sonicated to remove debris and then critical-point dried. After that, the samples were coated in platinum–pallidium using a Hitachi E-102 Ion Sputter and then examined in a HITACHI S-2500 SEM.

ANATOMICAL ANALYSES

Leaf epidermis

Representative materials of the two species of *Hackelochloa* were selected for anatomical investigation from the fresh samples collected in Thailand (Table 1). Three duplicates of leaf tissues removed at mid-laminar position were partitioned from intact leaves on abaxial and adaxial surfaces. All preparations were made by scraping leaves with a razor blade by hand and the samples were then stained with Safranin O. To make permanent slides, each section was placed on a slide and mounted with DePeX artificial mounting medium.

Leaf and culm anatomy

Leaf samples were obtained from mid-laminar and marginal positions of leaf blades. Culms were cut into 5–10-mm pieces. The samples were fixed in 70% (v/v) ethyl alcohol for at least 24 h. To remove air bubbles in the tissue, 2-min suctions alternating with 1-min pauses were performed five times or until the sample was fully submerged in solution. The samples were then dehydrated by transferring them to tertiary butyl alcohol (TBA) and ethyl

Table 1. List of specimens examined for the phenetic analyses, including both the cluster analysis and principal component analysis

Species	Location	Collector name	Collector number	Herbarium	SEM study	Anatomical study
H. granularis (L.) Kuntze	India, Andhra Pradesh	*J.L.G. van der Maesen*	4977	K	×	×
	India, Tamil Nadu, Tiruchi	*C. Manohoran*	19540	K	×	×
	China, Kwagsi, Kwei-Lin	*W.T. Tsang*	28030	K	×	×
	China, Hunan, Yizhang	*Xiao Bai-Zhong*	4593	K	×	×
	China, Guangdong Province, Ying-tak district	*Y.K. Wang*	2841	K	×	×
	Vietnam, Ha Giang	*N.T. Hiep, N.Q. Binh, I. Averyanov & P. Cribb*	NTH 3561	K	×	×
	Myanmar, Myitkyina	*R.O. Belcher & K. Bunchuai*	BC555	K	×	×
	Thailand, Chiang Mai Mae Rim	*BGO Staff*	6935	QBG	×	×
	Thailand, Chiang Mai	*BGO Staff*	7379	QBG	✓	×
	Thailand, Chiang Mai, Doi Inthanon	*H. Koyama*	T-61246	QBG	×	×
	Thailand, Loei, Phu Kradueng	*H. Koyama*	T-61379	BKF	×	×
	Thailand, Chiang Mai	*J.F. Maxwell*	87-1277	BKF	×	×
	Thailand, Chiang Mai, Doi Suthep	*J.F. Maxwell*	88-1066	BKF	×	×
	Thailand, Chiang Mai	*K. Iwatsuki & N. Fukuoka*	T-10392	BKF	×	×
	Thailand, Chiang Rai, Doi Tung	*K. Iwatsuki*	T-11135	BKF	×	×
	Thailand, Chiang Mai, Doi Suthep	*K. Iwatsuki*	T-9458	BKF	×	×
	Thailand, Chiang Mai	*L. Kethirun & P. Traiperm*	008	BKF	✓	✓
	Thailand, Chiang Mai, Doi Chiang Dao	*M. Norsaengsri*	894	QBG	×	×
	Thailand, Chiang Mai	*M. Norsaengsri*	1690	QBG	✓	×
	Thailand, Chiang Mai	*M. Norsaengsri*	4090	QBG	×	×
	Thailand, Chiang Mai	*M. Norsaengsri et al.*	2166	QBG	✓	×
	Thailand, Chiang Mai, Mae Rim	*P. Traiperm*	299	BKF	✓	✓
	Thailand, Chiang Mai	*P. Traiperm*	303	BKF	✓	✓
	Thailand, Chiang Mai	*P. Traiperm*	310	BKF	✓	✓
	Thailand, Chiang Mai	*Shunsuke Tsugaru*	T-61739	QBG	×	×
	Thailand, Chiang Mai, Mae Rim, Ban Mae Maa	*W. Nanakorn et al.*	4789.1	QBG	×	×
	Thailand, Loei, Phu Luang	*W. Nanakorn et al.*	2636	QBG	✓	×
	Thailand, Loei, Phu Kradueng	*S. Phusomsaeng & K. Bunchuai*	23	BKF	×	×
	Thailand, Chiang Mai, Doi Suthep	*Th. Sørensen, K. Larsen & B. Hansen*	4378	BKF	×	×
	Thailand, Lampang	*T. Shimizu*	T-10596	BKF	×	×
	Thailand, Loei, Phu Kradueng	*T. Smitinand*	1989	BKF	×	×
	Thailand, Nong Khai, Sri Chiang Mai	*T. Smitinand*	11645	BKF	×	×
	Thailand, Chiang Mai, Ang Kang	*Umpai*	517	BKF	×	×

Table 1. *Continued*

Species	Location	Collector name	Collector number	Herbarium	SEM study	Anatomical study
H. porifera (Hack.) D.Rhind	Vietnam, Tonkin	*B. Balansa*	4940	K	×	×
	Myanmar, Tenasserim Division, Tavoy District	*J. Keenan, U Tun Aung & R.H. Rule*	1824	K	×	×
	Myanmar, Myitkyina, Tang H'pre Area	*R.O. Belcher*	KC 819	K	×	×
	Thailand, Kanchanaburi	*L. Kethirun & P. Traiperm*	009	BKF	✓	✓
	Thailand, Chiang Mai	*M. Norsaengsri*	737	QBG	✓	×
	Thailand, Chiang Mai	*M. Norsaengsri*	1847	QBG	✓	×
	Thailand, Kanchanaburi, Thong Pha Phoom	*M. Norsaengsri*	2896	QBG	×	×
	Thailand, Chiang Mai	*P. Traiperm*	311	BKF	✓	✓
	Thailand, Tak, Tha Song Yang	*R. Pooma et al.*	7564	BKF	×	×
	Thailand, Tak, Tha Song Yang	*R. Pooma et al.*	7564	QBG	×	×
	Thailand, Chiang Mai	*S. Lægaard*	21684	BKF	✓	×
Mnesithea cancellata (Ridl.) Ridl.	Thailand, Chiang Mai, Doi Suthep	*J.F. Maxwell*	87-856	BKF	×	×
	Thailand, Loei, Phu Kradueng	*L. Kethirun & P. Traiperm*	031	BKF	×	×
M. formosa (R.Br.) de Koning & Sosef	Indonesia, Pulau Trangan	*M. van Balgooy & J. Mamesah*	6337	K	×	×
M. geminata (Hack.) Ridl.	Malaysia, Pahang, Nenasi	*E.J.H. Corner*	29882	K	×	×
M. glandulosa (Trin.) de Koning & Sosef	Thailand, Chiang Mai	*L. Kethirun & P. Traiperm*	014	BKF	×	×
M. laevis (Retz.) Kunth	Thailand, Songkhla	*Kai Larsen*	141546	BKF	×	×
	Thailand, Kamphaengphet	*T. Smitinand*	5960	BKF	×	×
	Thailand, Chiang Mai	*L. Kethirun & P. Traiperm*	015	BKF	×	×
M. mollicoma (Hance) A.Camus	Thailand, Chiang Mai, Doi Suthep	*J.F. Maxwell*	93-19	BKF	×	×
	Thailand, Chiang Mai, Doi Suthep	*J.F. Maxwell*	88-1106	BKF	×	×
	Thailand, Phitsanulok, Phuhinrongkla	*L. Kethirun & P. Traiperm*	006	BKF	×	×
M. rottboellioides (R.Br.) de Koning & Sosef	Malaysia, Sarawak, Rajang River	*J. & M.S. Clemens*	21884	K	×	×
M. striata (Nees ex Steud.) de Koning & Sosef	Thailand, Tak, Mae Sot	*L. Kethirun & P. Traiperm*	007	BKF	×	×
	Thailand, Chiang Mai, Thung Khao Phuang	*L. Kethirun & P. Traiperm*	020	BKF	×	×
	Thailand, Chiang Mai	*L. Kethirun & P. Traiperm*	021	BKF	×	×

Table 1. *Continued*

Species	Location	Collector name	Collector number	Herbarium	SEM study	Anatomical study
Ophiuros exaltatus (L.) Kuntze	Thailand, Petchabun, Nam Nao National Park	*P. Traiperm*	*176*	BKF	×	×
O. megaphyllus Stapf ex Haines	Thailand, Saraburi, Ban Nawng Bua	*Put*	*1150*	BK	×	×

alcohol in a concentration series of 50, 70, 80, 95 and 100% (v/v). Each sample was then embedded in a paraffin block. A Leica SM2000 R sliding microtome was used to section the paraffin block transversely. Xylene was applied to dissolve paraffin out of the sample ribbon. Before staining, the samples were rehydrated in ethyl alcohol series. The samples were stained with Safranin O and then counter-stained with Fast Green (Johansen, 1940). Anatomical terminology follows Ellis (1976, 1979) and Metcalfe (1960).

TAXONOMIC TREATMENT

Herbarium specimens of the two species of *Hackelochloa* were studied from the herbaria AAU, BK, BKF, BM, C, K, K-W, KKU, L and QBG. In addition, fresh materials of the two species were collected from several locations in Thailand during the course of this study. Type specimens were verified directly at the herbaria visited where possible or from digital images available online if necessary and relevant literature was consulted.

RESULTS

PHENETIC ANALYSES

Cluster analysis

The dendrogram was constructed from the similarity matrix for *Hackelochloa*, *Mnesithea* and *Ophiuros* species (Fig. 1). The cophenetic correlation of the similarity matrix or R-value was 0.98114, indicating strong support for the clustering method. The dendrogram clearly resolved three clusters: one containing all *H. granularis* specimens; one containing all *H. porifera* specimens; and one comprising the majority of the *Mnesithea* species, the two *Ophiuros* species plus *M. laevis* (Retz.) Kunth and *M. formosa* (R.Br.) de Koning & Sosef. *Mnesithea formosa* was excluded from the group containing the majority of *Mnesithea* species and was placed basal in the cluster containing the two *Ophiuros* species and *M. laevis*. The group containing *H. granularis* specimens had a high internal similarity coefficient of 0.86 and the group containing *H. porifera* specimens had a high internal similarity coefficient of 0.80; the two clusters were separated by a similarity coefficient of c. 0.53. The two clusters containing all *Mnesithea* and *Ophiuros* species were widely separated from the two *Hackelochloa* clusters by a low similarity coefficient of 0.22.

Principal component analysis

In the PCA of 28 morphological characters assessed in 61 specimens belonging to *Hackelochloa*,

Table 2. Morphological characters investigated in cluster and principal component analyses and weightings of each character in the first two principal components in the analysis of 61 OTUs of *Hackelochloa*, *Mnesithea* and *Ophiuros* (Appendix 1)

Morphological characters		Components 1	Components 2
G1	Length of leaf sheath	0.244	0.200
G2	Length of leaf blade	0.212	0.140
G3	Width of leaf blade	0.064	0.528
G4	Ratio of length of leaf blade to width of leaf blade	0.136	−0.050
G5	Length of raceme	0.367	−0.060
G6	Length of spatheole	0.264	0.042
G7	Shape of rachis internode	0.123	−0.038
G8	Length of rachis internode	0.308	−0.018
G9	Length of spikelet	0.178	0.239
G10	Shape of lower glume of sessile spikelet	0.242	0.033
G11	Presence of winged apex on lower glume	0.096	0.045
G12	Presence of appendages on lower glume	0.009	0.018
G13	Surface of lower glume of sessile spikelet	0.047	−0.003
G14	Surface of the base of lower glume	0.047	−0.003
G15	Shape of the base of lower glume	0.192	0.202
G16	Length of lower glume of sessile spikelet	−0.116	0.040
G17	Presence of sculpture on sessile spikelet	−0.113	0.162
G18	Types of sculpturing on lower glume of sessile spikelet	−0.095	0.171
G19	Shape of upper glume of sessile spikelet	0.242	0.033
G20	Length of upper glume of sessile spikelet	0.298	0.064
G21	Presence of pedicelled spikelet	−0.035	0.109
G22	Length of lower glume of pedicelled spikelet	−0.183	0.437
G23	Length of pedicelled spikelet	0.117	0.363
G24	Length of pedicel	−0.087	−0.073
G25	Number of glumes on pedicelled spikelet	−0.079	0.201
G26	Presence of fused pedicel and rachis internode	−0.087	−0.073
G27	Ratio of length of lower glume of pedicelled spikelet to length of pedicelled spikelet	−0.385	0.319
G28	Ratio of length of pedicel to length of pedicelled spikelet	0.129	0.014
Eigenvalue		12.205	3.077
Cumulative % of eigenvalue		61.5	77.0

Mnesithea and *Ophiuros*, the first two principal components accounted for 61.5 and 15.5% of total variance, respectively (Fig. 2, Table 2). Seven characters received high positive loadings in the first component: length of leaf sheath (G1); length of raceme (G5); length of spatheole (G6); length of rachis internode (G8); shape of lower glume of sessile spikelet (G10); shape of upper glume of sessile spikelet (G19); and length of upper glume of sessile spikelet (G20). A high negative loading was exhibited for the ratio of length of lower glume of pedicelled spikelet to length of pedicelled spikelet (G27). High positive loadings of the second component representing four characters were associated with separation of three distinct clusters: width of leaf blade (G3); length of lower glume of pedicelled spikelet (G22); length of pedicelled spikelet (G23);

and ratio of length of lower glume of pedicelled spikelet to length of pedicelled spikelet (G27).

In the PCA focusing only on specimens referable to *Hackelochloa*, the first two principal components accounted for 72.1 and 12.9% of total variance, respectively (Fig. 3 and Table 3). In the first component, the following 12 characters received high positive loadings in separating two distinct clusters that reflected the two species: length of leaf blade (S2); width of leaf blade (S3); ratio of length of leaf blade to width of leaf blade (S4); length of raceme (S5); number of spikelets per raceme (S6); length of spatheole (S7); shape of lower glume of sessile spikelet (S10); length of lower glume of sessile spikelet (S11); shape of base of lower glume (S12); types of sculpturing on lower glume of sessile spikelet (S13); length of upper glume of sessile spikelet (S14); and

Table 3. Morphological characters investigated in cluster and principal component analysis and weightings of each character in the first two components in the analysis of 44 OTUs of *Hackelochloa* (Appendix 1)

Morphological characters		Components 1	2
S1	Length of leaf sheath	0.144	−0.101
S2	Length of leaf blade	0.282	0.294
S3	Width of leaf blade	0.427	0.729
S4	Ratio of length of leaf blade to width of leaf blade	0.207	−0.070
S5	Length of raceme	0.207	−0.070
S6	Number of spikelets per raceme	0.207	−0.070
S7	Length of spatheole	0.270	−0.191
S8	Length of rachis internode	0.191	−0.101
S9	Length of spikelet	0.185	−0.142
S10	Shape of lower glume of sessile spikelet	0.207	−0.070
S11	Length of lower glume of sessile spikelet	0.364	0.017
S12	Shape of base of lower glume	0.207	−0.070
S13	Types of sculpturing on lower glume of sessile spikelet	0.207	−0.070
S14	Length of upper glume of sessile spikelet	0.308	−0.331
S15	Shape of upper glume of sessile spikelet	0.207	−0.070
S16	Length of lower glume of pedicelled spikelet	0.156	−0.327
S17	Length of pedicelled spikelet	0.146	−0.234
Eigenvalue		4.257	0.7635
Cumulative % of eigenvalue		72.1	85.0
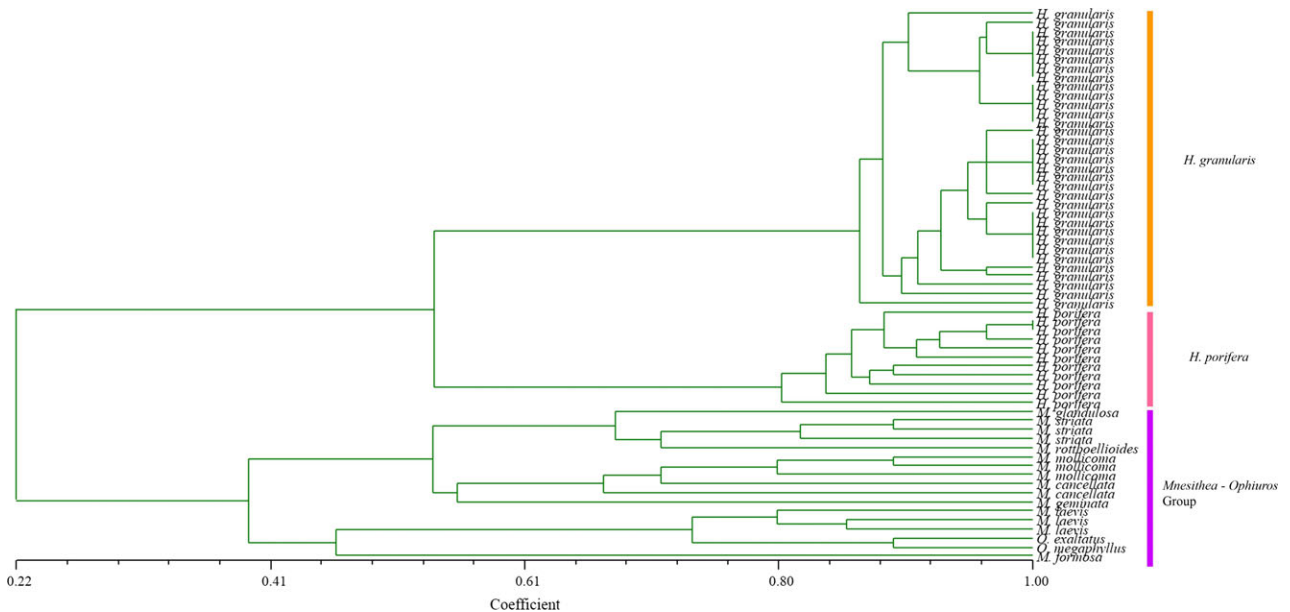
Figure 1. Dendrogram from the cluster analysis for *Hackelochoa*, *Mnesithea* and *Ophiuros* using UPGMA algorithm.

shape of upper glume of sessile spikelet (S15). In the second principal component, high positive loadings were given to characters S2 and S3. The following three characters received high negative loadings: length of upper glume of sessile spikelet

(S14); length of lower glume of pedicelled spikelet (S16); and length of pedicelled spikelet (S17).

The scatter plot of the first two principal components resolved three clusters, corresponding to *H. granularis*, *H. porifera* and a group comprising
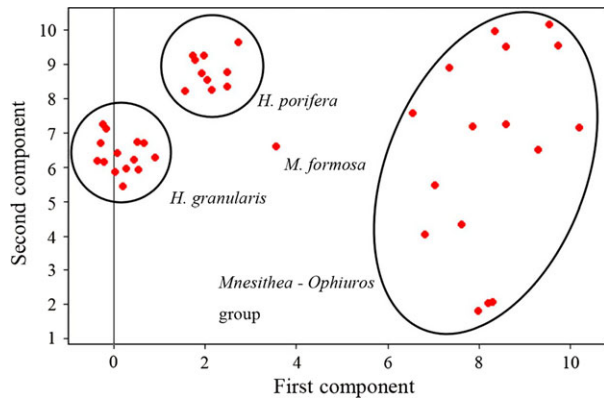
Figure 2. Principal component analysis score plot of first and second components of *H. granularis*, *H. porifera*, *Mnesithea* and *Ophiuros* based on 28 morphological characters.
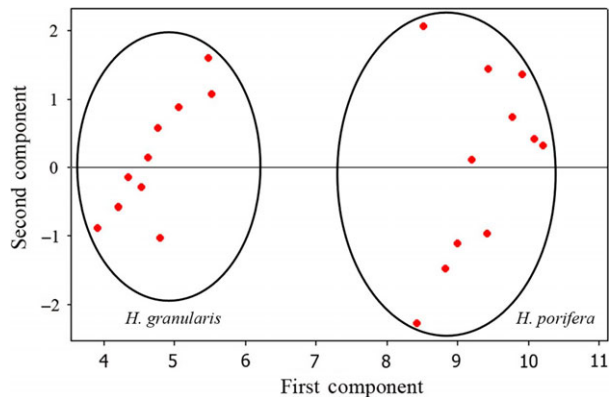

Figure 3. Principal component analysis score plot of first and second components of *H. granularis* and *H. porifera* based on 17 morphological characters.

Mnesithea and *Ophiuros* (Fig. 2). OTUs in the *Mnesithea–Ophiuros* cluster were more loosely grouped than those in the two *Hackelochloa* clusters and *M. formosa* fell outside its main range. Furthermore, *M. laevis* and *Ophiuros* species were also grouped away from the majority of the cluster. The clusters representing *H. granularis* and *H. porifera* were both clearly isolated from the *Mnesithea–Ophiuros* cluster and each was differentiated from the other. A second plot of the first two components applied to 17 characters scored only in specimens referred to as *Hackelochloa* (Fig. 3) further confirmed the separation of the two species. In this plot, two clusters representing *H. granularis* and *H. porifera* were widely separated by the first component. Scattering of OTUs along the second component was suggestive of variation within either species.

MICROMORPHOLOGICAL ANALYSIS

SEM of lower glumes

Distinct sculpturing of the lower glumes in both *Hackelochloa* species was observed through SEM. In *H. granularis*, the lower glume of the sessile spikelet has a hemispherical shape (Fig. 4A) and the surface of the glume is covered with numerous minute tubercles (Fig. 4B). In contrast, *H. porifera* possessed an oblong lower glume (Fig. 4C) and, instead of tubercles, the surface had a series of ridges that protrude from the glume in a reticulate pattern (Fig. 4D). In general, the lower glume in *H. porifera* was larger than that in *H. granularis*. These differences in lower glume morphology could be used to consistently differentiate *H. porifera* from *H. granularis*; some of these characters were utilised in the subsequent phenetic analyses.

ANATOMICAL ANALYSES

Leaf epidermis

In general, *H. granularis* (Fig. 5A, C) and *H. porifera* (Fig. 5B, D) resembled one another in the anatomy of their leaf epidermis. The species possess two types of epidermal cells: long cells and short cells. Long cells are rectangular with an undulating anticlinal wall and a smooth periclinal wall. In both adaxial and abaxial surfaces, long cells were found to be wider in the intercostal zone than those in the costal zone or near the margins. Short cells are compressed with silica bodies on either side and are solitary between long cells. In the costal zone, silica bodies are commonly dumbbell-shaped, whereas in the intercostal zone cross-shaped silica bodies are mainly paired with short cells. In stomatal complexes, stomata are surrounded by triangular subsidiary cells and prominent dumbbell-shaped guard cells were found. Stomatal complexes are present on both abaxial and adaxial surfaces, but they are more abundant on the abaxial surface. Microhairs are present on both surfaces comprising distal cells tapering towards the apex and parallel-sided base cells. Unicellular trichomes with lignified walls composed of several basal cells. Quantitative variation between species and within individuals was surveyed. In *H. granularis*, three or four rows of stomata were found on the adaxial surface, and nine to 11 rows of stomata were found in intercostal zone on the abaxial surface. In contrast, *H. porifera* has four or five rows of stomata on the adaxial surface and eight to 12 rows of stomata on the abaxial surface. The length of microhairs in *H. granularis* ranged from 26 to 46 μm on the adaxial surface and 27 to 39 μm on the abaxial surface and in *H. porifera* they ranged from 23 to 51 μm on the adaxial surface and 21 to 41 μm on the abaxial surface.
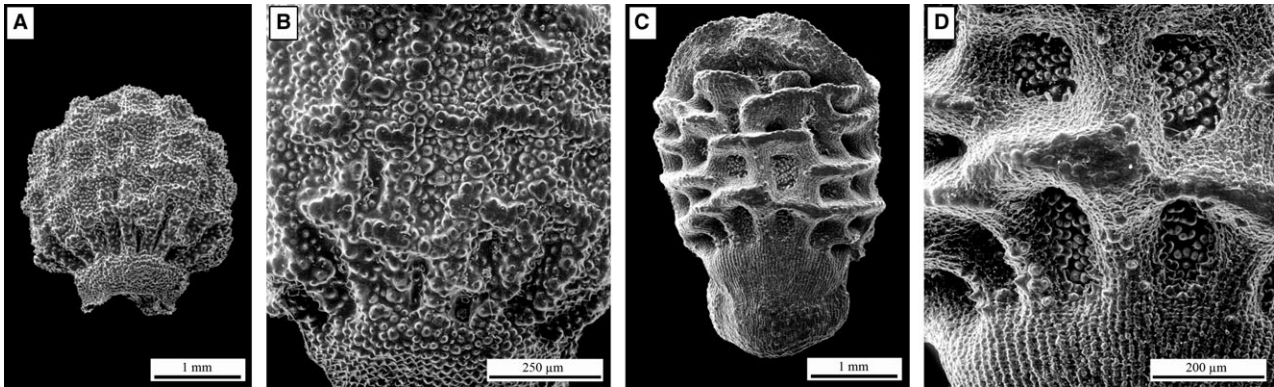
Figure 4. Micromorphology of lower glume of sessile spikelet in *H. granularis* and *H. porifera*. (A) Hemispherical glume with tubercle-covered surface in *H. granularis*. (B) Close-up of (A). (C) Oblong glume with reticulate ornamentation in *H. porifera*. (D) Close-up of (C).

Leaf transverse section

In *H. granularis*, the outline of the lamina is an open V-shape, with two straight arms set at an angle of 90° to one another. The keel region is flat in outline. There are four to six-second-order vascular bundles arranged along the two sides of the median bundle (Fig. 5E). The transverse section of the leaf blade in *H. porifera* is similar to that of *H. granularis*, although the former has a more emphasised keel structure, with a lamina thickness of *c.* 200 μm. The keel in *H. porifera* is V-shaped in outline and is complemented by an aggregation of colourless parenchyma cells superior to the median bundle and with prominent projections on the abaxial side. As a result of these projections, one median bundle and seven or eight small bundles are incorporated in the keel region (Fig. 5F).

In general, other structures observed in transverse sections were found to be similar to one another in the two species. No ribs or furrows were present on both the adaxial or abaxial surfaces, but a slightly undulating surface was observed on the abaxial side. The keel was not well developed to developed, with the midrib projecting abaxially, and it had a group of a few parenchyma cells situated above the median bundle. In addition, one median bundle and several smaller bundles were incorporated in the keel region. A girdle of triangular sclerenchyma tissue connects the median bundle to the abaxial surface. However, the other first-order bundles in the lamina were joined to abaxial and adaxial surfaces by sclerenchyma girdles. No sclerenchyma cells are present in other parts of the blade. All bundles were positioned in the central part of the blade. Vascular bundle sheaths form a single layer in first-order and third-order bundles, but they are interrupted by a sclerenchyma girdle in the median bundle. On the epidermis, bulliform cells are the main cell type.

Spherical bulliform cells mostly cover abaxial and adaxial surfaces. However, inflated bulliform cells were also found as an isolated group projecting above the level of the epidermis in some regions of the blade. Prickles are infrequent on both surfaces, but large ones were observed at the margins of the blade. Stomata are located on both adaxial and abaxial surfaces.

Culm transverse section

Most features of the culm were found to be similar in the two species. For instance, transverse sections of the culm in both species were elliptical in shape, with a diameter of *c.* 2 mm (Fig. 6A, C). Indentation was apparent as a result of the groove running along the length of the culm. Typical anatomy of a monocot stem is present in both species. The epidermis is simple with only one epidermal layer. Underneath the epidermis, a ring of sclerenchyma surrounds the internal culm layer. The cortex is mostly covered with parenchyma cells, with closed collateral vascular bundles scattered in parenchymatous tissue. There are no trichomes or crystals present in the culm of either species. However, in between the epidermal layer and sclerenchyma ring, there is a discontinuous column of chlorenchyma cells that is interrupted by sclerenchyma girdles projecting from the sclerenchyma ring. In *H. granularis*, this consists of three or four layers (Fig. 6B) but in *H. porifera* it consists of one or two layers (Fig. 6D).

DISCUSSION

Hackelochloa granularis vs. Hackelochloa porifera

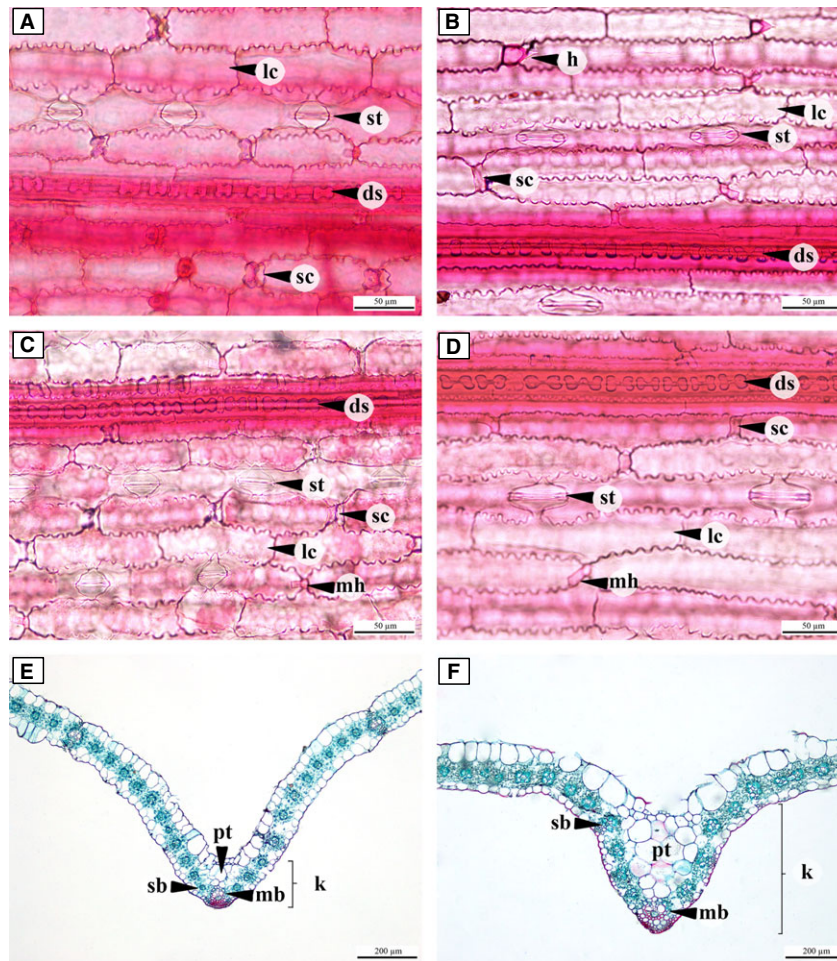The length of most structures (especially the spikelet and raceme) and the sculpturing of the lower glume

Figure 5. Leaf anatomy in *H. granularis* and *H. porifera*. (A, C) Organisation of adaxial and abaxial leaf epidermis in *H. granularis*. (B, D) Organisation of adaxial and abaxial leaf epidermis in *H. porifera*. (E) Transverse leaf section of *H. granularis*. (F) Transverse leaf section of *H. porifera*; note the pronounced keel region. ds, dumbbell silica bodies; h, hook; k, keel; lc, long cell; mb, median bundle; mh, micro-hair; pt, parenchymatous tissue; sb, second-order bundles; sc, short cell; st, stomata.

are two major criteria for discriminating *H. granularis* and *H. porifera* (Hooker, 1897; Bor, 1960; Noltie, 2000; Bixing & Phillips, 2006; Traiperm, 2007). However, the use of only these two criteria is somewhat weak evidence on which to establish two species of *Hackelochloa*. Until now, there has been no further supplementary evidence beyond these gross morphological aspects. In the present study, anatomical and phenetic analyses have provided further support for the recognition of *H. granularis* and *H. porifera* as separate species.

Morphological observation is the first step in the process of determining the taxonomic status of a particular genus and in Poaceae the morphology of the lower glume is an important character (Clayton & Renvoize, 1986; Clark & Pohl, 1996). In Andropogoneae, the lower glume is incorporated in the dispersal unit, which is composed of the rachis internode and a sessile or fertile spikelet (Clayton & Renvoize, 1986; Shouliang *et al.*, 2006; Skendzic *et al.*, 2007).

According to our SEM results, the lower glumes of *H. granularis* and *H. porifera* have tubercled and reticulate architectures, respectively. Similar distinction of lower glume patterning has been reported by (Hooker, 1897; Bor, 1960; Noltie, 2000; Bixing & Phillips, 2006; Traiperm, 2007). Bixing & Phillips (2006) observed shallow pits and a fine granular surface on the lower glumes in *H. granularis*, vs. a ridged or honeycombed surface on that of *H. porifera*. In addition to surface sculpturing, the shape of the lower glume has been used in grass classification, e.g. in *Avena* L. (Ladizinsky, 2012), *Schizachyrium* Nees (Peichoto, 2006) and *Tridens* Roem. & Schult.
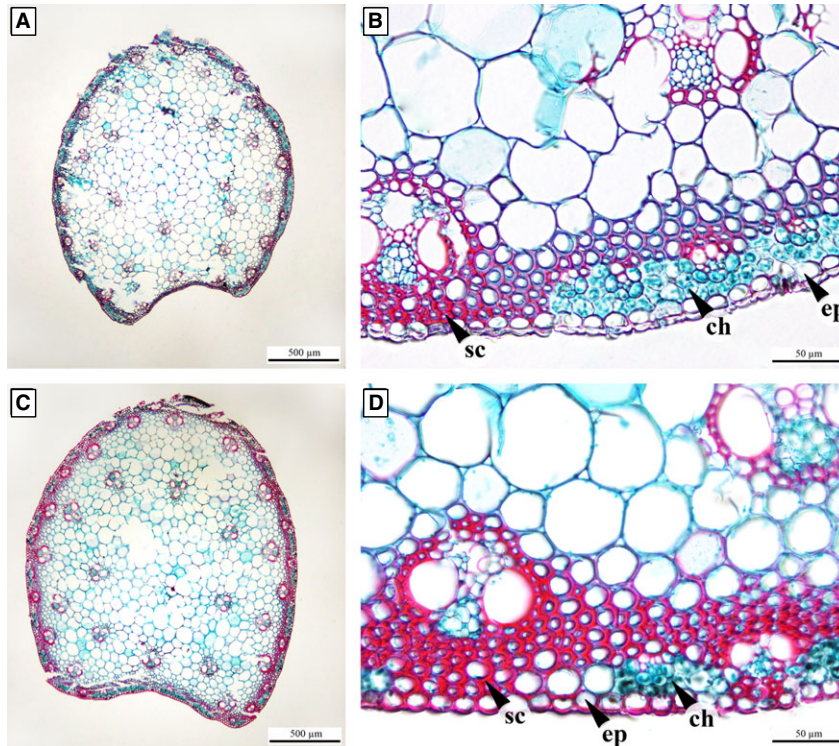
Figure 6. Culm anatomy in *H. granularis* and *H. porifera*. (A) Transverse culm section of *H. granularis* showing outer red sclerenchyma ring with scattered vascular bundles. (B) Detail of anatomy beneath outer sclerenchyma ring (sc) in *H. granularis*, showing chlorenchymatous tissue (ch) made up of three or four layers, and epidermal layer (ep). (C, D) Same for *H. porifera*, with one or two layers of chlorenchymatous tissue.

(Acedo & Llamas, 2003). In *H. granularis*, it has a hemispherical shape, whereas in *H. porifera* it is oblong. The distinction between these two outlines was based on the length:width ratio, which Simpson (2006) found to be an adequate means of overcoming descriptive ambiguities. Our results confirm that the lower glume of *H. granularis* has a ratio of 1:1 (spherical), whereas that of *H. porifera* is 3:2 (oblong). These ratios were consistent among the samples examined. As such, there is little doubt that glume sculpturing as observed in SEM and lower glume shape offer sound candidate characters for species identification. Moreover, the sculpturing itself can be inferred from field observations. SEM examination elucidated that the reticulate ribs in *H. porifera* are an extension of the nerves originating from the base of the lower glume. In *H. granularis*, these nerves are slightly swollen but never extend to form distinct ornamentation as in *H. porifera*. This higher degree of protrusion in *H. porifera* gives rise to the complex honeycombed pattern referred to by Bixing & Phillips (2006). Details of the nerves on the lower glume are commonly utilised in separating genera and species of Poaceae. For example, extensions or appendages to these nerves have been uti-

lised in distinguishing the genera *Cottea* Kunth, *Pappophorum* Schreb. and *Tridens* Roem. & Schult. (Clark & Pohl, 1996) and the number of nerves is used as a key taxonomic criterion in distinguishing members of *Andropogon* L. (Zanin & Longhi-Wagner, 2011) and *Schizachyrium* (Peichoto, 2010). As a modification of the nerves at the base of the lower glume, the observed differences in lower glume sculpturing in *Hackelochloa* are hypothesised here to have an evolutionary significance related to anthesis and dispersal and are therefore likely to be taxonomically valuable.

The upper glume is less modified than the lower glume, but is nevertheless informative in a taxonomic context for classification of *Hackelochloa*. From our observations, the shape of the upper glume can be readily divided into two forms: orbiculate in *H. granularis* and elliptic in *H. porifera*, as defined by length:width ratios of 1:1 and 3:2, respectively (Dilcher, 1974; Simpson, 2006). Previous publications have overlooked the utility of this characteristic in the identification of *Hackelochloa* (Bixing & Phillips, 2006).

Leaf and culm anatomy also represents a significant tool for grass classification (Ellis, 1976, 1979;

Ma, Peng & Li, 2005; Siqueiros-Delgado, 2007; Traiperm, 2007; Namaganda, Krekling & Lye, 2009; Stuessy, 2009; Traiperm *et al.*, 2011). This study is the first to establish the internal structure of selected organs in *Hackelochloa*. Utmost among these is the leaf keel. Ellis (1976) defined the term 'keel' as the region in which the median bundle is associated with parenchyma; it can be composed of more than one bundle. Keel characters have been used to distinguish four species of *Chloris* Sw. from each other (Fisher, 1939). Our study revealed that the arrangement of parenchyma cells in the keel region was completely different between the two species of *Hackelochloa*. In *H. porifera*, the keel is prominent, protruding on the abaxial surface and forming a V-shaped structure. In *H. granularis*, it is flattened, being composed of less parenchyma situated above the median bundles. In addition, the number of second-order bundles in the keel of *H. porifera* is greater than in that of *H. granularis*.

The number of chlorenchyma layers in the culm (three or four in *H. granularis* and one or two in *H. porifera*) underscored these distinctions. Similar differences in chlorenchymatous tissue have been identified in other grass taxa, such as *Arundinella* Raddi, which differ in terms of the continuity of tissue and the number of layers (Sanchez, Arriaga & Ellis, 1990). The number of chlorenchyma layers was even employed as one of the three characters to support the monophyly of two genera of Restionaceae, *Hopkinsia* W.Fitzg. and *Lyginia* R.Br. (Linder, Briggs & Johnson, 2000). The disparity in culm chlorenchyma layers observed in the present study might therefore be interpreted as a significant attribute in separating *H. granularis* and *H. porifera*. A sclerenchymatous ring surrounding the internal part of the culm was common to both species and probably related to their xerophytic habit, since the suberin that coats the sclerenchyma prevents water evaporation (Metcalfe, 1960; Fahn, 1990).

The leaf epidermis of both species was similar in structure and arrangement. Dumbbell-shaped guard cells, dumbbell-shaped silica bodies, macro hairs, long cells and short cells were frequently encountered in both species. Dumbbell-shaped guard cells regulate water loss and solute concentration in xerophytic plants (Franks & Farquhar, 2007) and are thought to be a more advanced character state derived from kidney-shaped guard cells (Hetherington & Woodward, 2003).

In terms of quantitative traits, the maximum measurements of stomata length and macro hairs length were greater in *H. porifera* than in *H. granularis*. Significant differences (*t*-test, $P < 0.00$) were found in the length of leaf sheath, the length of the raceme, the length of the rachis internode, the length of the spatheole, the length of the spikelet and the number of spikelets per raceme as visualised in box plots (Fig. 7) and were supported by PCA (Figs 2 and 3). These differences were not influenced by the origin of the plants. It is plausible that *H. porifera* is a polyploid form of *H. granularis*, but at present there is no cytogenetic or molecular confirmation of genomic relationships between the two.

Veldkamp *et al.* (1986) combined *H. porifera* with *H. granularis* based on an examination of two specimens from Sumatra at the Rijksherbarium (*Rahmat si Boeea 6284* and *de Voogd 1517*), without consulting specimens from India, China, Vietnam, Myanmar or Thailand. Our results indicate that the same two Sumatran specimens should be referred to *H. porifera*, given the ridged sculpturing of the lower glumes and the larger overall stature of the plants. Therefore, we infer that *H. porifera* occurs only in India, China, Vietnam, Myanmar, Thailand (Hooker, 1897; Rhind, 1945; Bor, 1960; Sreekumar & Nair, 1991; Shukla, 1996; Moulik, 1997; Noltie, 2000; Bixing & Phillips, 2006; Traiperm, 2007) and Indonesia (based on the two Sumatra specimens), whereas *H. granularis* is, in contrast, widely distributed throughout tropical and warm temperate regions of the World (Hooker, 1897; Achariyar, 1921; Camus & Camus, 1922; Ridley, 1925; Bor, 1938, 1960; Rhind, 1945; Gardner, 1952; Senaratna, 1956; Rotar, 1968; Clayton & Renvoize, 1982, 1986; Sreekumar & Nair, 1991; Watson & Dallwitz, 1992; Shukla, 1996; Moulik, 1997; Noltie, 2000; Duistermaat, 2005; Bixing & Phillips, 2006; Traiperm, 2007).

Status of Hackelochloa

Veldkamp *et al.* (1986) identified *Heteropholis annua* Lazarides as taxonomically intermediate between *Hackelochloa* and *Mnesithea* and concluded that *Hackelochloa* should be considered congeneric with the latter. Several other authors have continued to recognise *Hackelochloa* as a distinct genus (Gilliland, 1971; Bixing & Phillips, 2006; Traiperm, 2007; Teerawatananon *et al.*, 2011; Kellogg, 2015), but its status remains ambiguous. The molecular evidence to hand is equivocal, with the phylogenetic analyses presented by Teerawatananon *et al.* (2011) suggesting that *Hackelochloa* (represented by *H. granularis*) and *Mnesithea* (represented by *M. formosa*) are not monophyletic. Those authors also stated that *Hackelochloa* is the only genus in subtribe Rottboelliinae with globose-shaped, wingless lower glumes. In a cladistic analysis based on morphological and anatomical characters, Kellogg & Watson (1993) revealed *Hackelochloa* to be divergent from *Mnesithea* in both their most-parsimonious and consensus trees. Evidence from cytogenetic studies also
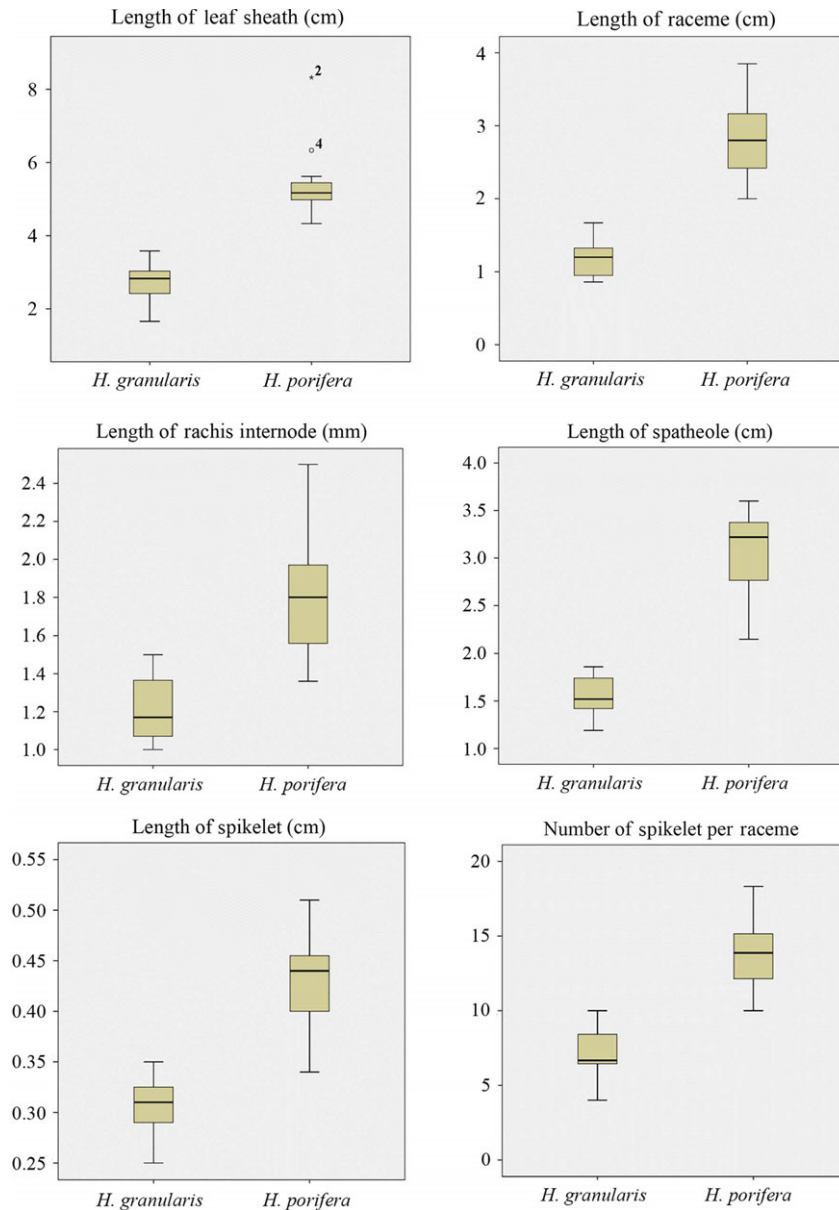
Figure 7. Box plots of significant quantitative characters in *H. granularis* and *H. porifera*.

suggest disparities, with *H. granularis* having a chromosome number of $2n = 14$ indicating a basic number of $x = 7$ for the genus (Celarier, 1957; Davidse & Pohl, 1974), vs. a basic chromosome number of $x = 9$ for *Mnesithea* (Davidse & Pohl, 1974). *Mnesithea laevis*, for instance, has a chromosome number of $2n = 18$ (Gould & Soderstrom, 1974) and *Coelorachis glandulosa* (Trin.) Stapf ex Ridl., as *M. glandulosa* (Trin.) de Koning & Sosef, was found to be hexaploid with $2n = 54$ (Celarier, 1957). It is likely that such differences in basic chromosome numbers will have taxonomic ramifications (Hilu, 2004).

In our PCA plot, *H. granularis* and *H. porifera* fell into two, non-overlapping groups (Figs 2 and 3). Some scattering was detected, representing intraspecific variation in quantitative traits such as the length of the spikelet, the length of the spatheole and the length of leaf sheath. In the *Mnesithea* cluster, most OTUs were widely dispersed within the group because of interference from multiple species included in the analysis. The presence of gaps again implies variation among species in the group. However, the *Hackelochloa* cluster was completely disconnected from the *Mnesithea* group. This also indicates that some characters chosen in this study

are meaningful for generic delimitation between *Hackelochloa* and *Mnesithea*, although phylogenetic studies are required to determine the evolutionary polarity of these characters.

The position of *M. formosa* in the PCA deviated from the rest of the *Mnesithea* group and the species was basal within a cluster comprising *M. laevis*, *Ophiuros exaltatus* (L.) Kuntze and *O. megaphyllus* Stapf ex Haines which fell basal to all other *Mnesithea* species included in our phenetic analysis. Its hairy lower glume and pedicel that is united with the rachis internode were found to be key characters in placing *M. formosa* outside the *Mnesithea* group towards *Hackelochloa*. Veldkamp *et al.* (1986) stated that the annual habit is shared by *M. formosa* and *H. granularis* and this was considered a significant factor linking *Hackelochloa* and *Mnesithea*, even though other species of *Mnesithea* are perennial.

Under the generic delimitation proposed by Veldkamp *et al.* (1986), several species of the genus *Rottboellia* L.f. were transferred to *Mnesithea*. For instance, *M. formosa* was derived from *R. formosa* R.Br. In addition, *Heteropholis annua* was assumed to be intermediate between *Hackelochloa* and *Mnesithea* because of its ambiguous characters, such as globose lower glume and straight plane of articulation, and it too was subsumed into *Mnesithea*. In terms of its morphology, *M. annua* has globose, sculpture-free lower glumes, whereas *M. formosa* has a hairy, ornamented lower glumes. Both species are also outstanding in the fusion of their rachis internode with the pedicel, a character that is uncommon in *Mnesithea* (Veldkamp *et al.*, 1986). The unconventional morphology of these two species suggests the possibility that they are not typical of *Mnesithea*, a perspective consistent with our finding that *M. formosa* was distinct from the other *Mnesithea* species included in our study.

Furthermore, *M. laevis* was included in the subgroup containing *Ophiuros* species rather than being placed with other *Mnesithea* species (Fig. 1). Two characters may be highlighted to explain this placement: only remnants of the pedicelled spikelets are present and the pedicel is assimilated into the rachis internode in both *M. laevis* and *Ophiuros* species These characters are at odds with other *Mnesithea* species which all otherwise have a pedicelled spikelet with a free pedicel. Mapping these and other characters assessed in this study onto a well sampled and well resolved phylogenetic tree will help determine which are of greatest phylogenetic utility.

The loadings of the first two components revealed the main diagnostic morphological characters separating *Mnesithea* and *Hackelochloa*, namely the shape of lower and upper glumes, the shape of rachis internode, the reduction of the pedicelled spikelet represented by the ratio of length of lower glume of pedicelled spikelet to length of pedicelled spikelet and the ratio of length of pedicel to length of pedicelled spikelet. In the dendrogram, the two *Hackelochloa* clusters were resolved as one discrete group with relatively high internal similarity, whereas the cluster containing the *Mnesithea* taxa were positioned farther apart with low similarity to the *Hackelochloa* cluster.

Hackelochloa shares two derived characters in the structure of the sessile spikelet: a hardened glume and an invaginated rachis internode. These two characters are thought to have evolved from the ancestral states in the tribe, namely membranous glumes and flat internodes (Preston *et al.*, 2012). From an evolutionary standpoint, *Hackelochloa* may have benefited from the hardened glume that is not digested by seed predators, thereby increasing its chances of seed dispersal (Wilkes, 1967). In Rottboelliinae, sister genera that display advanced states as in *Hackelochloa*, such as *Rottboellia*, have acquired novel characters from different transition stages, as indicated by the maximum likelihood tree inferred from combined analysis with *TGA1* sequences (Preston *et al.*, 2012). In *Zea mays* L., a mutation in *tga1* is involved in a regression to membranous glumes and flat rachis internodes. However, at present there is no evidence of how this gene affects glume and rachis internode development in other species. Nevertheless, the potential influence of this gene deserves more attention in its control of rachis internode invagination, especially because it could provide crucial insights into the evolution of *Hackelochloa* and therefore its taxonomic distinction from *Mnesithea*. In most species of *Mnesithea* (excluding *M. laevis*), the rachis internode is not hollowed and is held erect besides the sessile spikelet. In contrast, both species of *Hackelochloa* develop a cavity within the rachis internode, with the upper glume embedded within it (Traiperm, 2007).

Hackelochloa and *Mnesithea* exhibit bidirectional trends in the shape of the lower and upper glumes (Shouliang *et al.*, 2006). The lanceolate shape is defined by a ratio of 3:1 in *Mnesithea*, whereas the hemispherical and oblong shapes are defined by a ratio of 1:1 and 3:2 in *H. granularis* and *H. porifera*, respectively (Dilcher, 1974; Simpson, 2006). Furthermore, the sessile spikelet is shorter than the rachis internode in *Hackelochloa*, whereas the length of sessile spikelet exceeds the length of the rachis internode in *Mnesithea*.

As mentioned above, seed dispersal is a crucial process for the survival of grasses. This process requires the disarticulation of one dispersal unit from another along the raceme. As a consequence, the fragility of the raceme directly correlates with

the degree of disarticulation and the plane of the rachis internode plays an important role in disarticulation (Veldkamp *et al.*, 1986). A variety of rachis internode shapes may result in distinct planes of disarticulation, which affect the degree of raceme fragility. Therefore, the straight rachis internode in *Hackelochloa* vs. the clavate rachis internode in *Mnesithea* could underpin significant differences in the efficiency of seed dispersal in either genus.

Beyond contributing to the disarticulation of the dispersal unit, the fusion of rachis internode to the pedicel of the pedicelled spikelet provides another taxonomic distinction between *Hackelochloa* and *Mnesithea*. A completely fused pedicel can be observed in both *H. granularis* and *H. porifera*, whereas the pedicel in most species of *Mnesithea* is free of the rachis internode (Kellogg & Watson, 1993; Bixing & Phillips, 2006; Traiperm, 2007), except for *M. formosa* in which the pedicel is adnate to the rachis internode.

Either male or sterile florets are borne in the pedicelled spikelet. In some species, the pedicelled spikelet is well developed with male florets producing pollen. *Hackelochloa* has large pedicelled spikelets relative to the size of the sessile spikelet and pollen-producing male florets. In contrast, most species of *Mnesithea* possess highly reduced pedicelled spikelets. In all *Mnesithea* species except *M. rottboellioides* (R.Br.) de Koning & Sosef, which has prominent lower glumes on the pedicelled spikelet containing male or bisexual florets, the sterile lower glumes are greatly reduced or absence (Veldkamp *et al.*, 2013). As evidenced by the pedicelled spikelet, *Hackelochloa* shows a more plesiomorphic state than *Mnesithea* in bearing fertile and less-reduced pedicelled spikelets (Shouliang *et al.*, 2006). However, the shape of the lower glume, the free pedicel and the shape of the rachis internode all point towards the inclusion of *M. rottboellioides* within *Mnesithea*. In this study, the reduction of pedicelled spikelets was found to be neatly expressed in the ratio of the length of the lower glume of the pedicelled spikelet to the length of the pedicelled spikelet. Lower ratios are found in *Mnesithea*, except for *M. rottboellioides*. This indicates a high degree of reduction of the pedicelled spikelet in most species of *Mnesithea*. In contrast, *Hackelochloa* species have a higher ratio, implying more well developed pedicelled spikelets.

CONCLUSIONS

The results presented here support the view that *H. porifera* should be recognised at specific rank and so should lose its status as a synonym of *H. granularis*. Anatomical investigation revealed that keel structure is effective in resolving the taxonomic status of the two taxa. The outline of the keel, the presence of distinctive parenchyma cells, the number of second-order bundles and the number of chlorenchymatous layers in the culm are diagnostic characters. Furthermore, SEM and PCA showed that the sculpturing of the lower glume and the shape of the lower and upper glume of the sessile spikelet have characteristic states in the two species; both of these characters are suitable for field identification. In addition, quantitative traits, such as the length of the leaf sheath, raceme, lower glume, spatheole, rachis internode and spikelet, further support their discrimination. Moreover, the shape of the lower and upper glume, the shape of the rachis internode, reduction of the pedicelled spikelet and the nature of the fusion between rachis internode and pedicel are key characters that distinguish *Hackelochloa* from *Mnesithea* at the generic level. Detailed phylogenetic analyses are needed to provide clarity regarding relationships of *Hackelochloa* with other members of subtribe Rottboelliinae.

TAXONOMIC TREATMENT

HACKELOCHLOA KUNTZE, Revis. Gen. Pl. 2: 776 (1891). = *Rytilix* Raf. ex Hitchc., in U.S.D.A. Bull. (1915–23) 772: 278 (1920), nom. illeg. superfl.

Type species: H. granularis (L.) Kuntze
Annual, tufted or solitary. *Culms* erect. *Leaf sheath* loose; *ligules* membranous, ciliate; *leaf blade* linear or broadly linear. *Inflorescence* composed of several racemes, terminal or axillary, each raceme subtended by spatheole. *Spikelet* in pairs, sessile and the other one pedicelled. *Sessile spikelets* united with rachis; dorsally compressed. *Glumes*; lower glume swollen and hemispherical or broadly oblong, pitted or reticulate on the back; *upper glume* adhering to the cavity. *Lower floret*; lemma, hyaline; *palea* absent. *Upper floret*; fertile. *Pedicelled spikelet* neuter. *Pedicels* fused to rachis internode.

Distribution: A genus of two species distributed throughout the tropics. Growing in open, disturbed environments.

KEY TO THE SPECIES

1 Sessile spikelets subglobose; lower glume of sessile spikelet 0.8–1.3 mm long, pitted and tubercled on the back . . . .
   1. *H. granularis*

1 Sessile spikelets broadly oblong; lower glume of sessile spikelet 1.5–2.5 mm long, ridged and reticulate on the back . . . .
   2. *H. porifera*

HACKELOCHLOA GRANULARIS (L.) KUNTZE
(FIGS 8, 10A, B)

Revis. Gen. Pl. 2: 776 (1891). ≡ *Cenchrus granularis* L., Mant. Pl. 2: 575 (1771). ≡ *Manisuris granularis* (L.) L. f., *Nov. Gram. Gen.* 37, 40, pl. 1, f. 4–7 (1779). ≡ *Tripsacum granulare* (L.) Raspail, Ann. Sci. Nat., Bot. 5: 306 (1825). ≡ *Rytilix granularis* (L.) Skeels, U.S.D.A. Bur. Pl. Industr. Bull. 282: 20 (1913). ≡ *Rottboellia granularis* (L.) Roberty. Boissier 9: 79 (1960). ≡ *Mnesithea granularis* (L.) de Koning & Sosef, Blumea 31(2): 295 (1986). *Type*: India Orientalis, *Herb. Linn. 1217.12* (lectotype LINN!, designated by Clayton & Renvoize, Fl. Trop. E. Africa, Gramineae 3: 849 (1982).

Culms 15–80 cm tall, erect, subterete on the lower part, grooved on the upper part, glabrous. *Leaf sheath* 1.5–3.6 cm long, loose, flat, pilose with tubercle-based hairs; *ligules c.* 1 mm long, membranous with cilia; *leaf blade* 2.0–18.0 × 0.5–1.5 cm, linear,
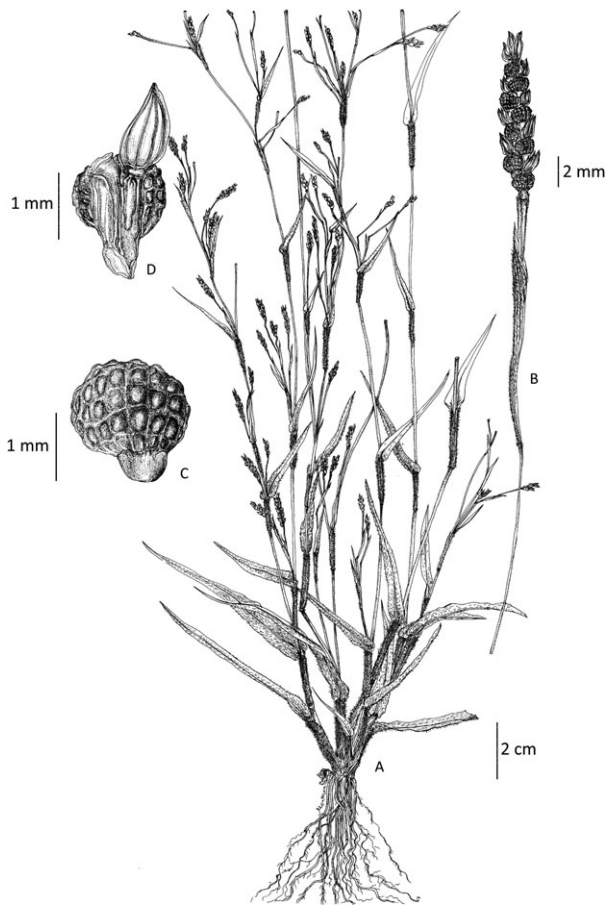

Figure 8. *Hackelochloa granularis*: A, plant; B, raceme; C, lower glume of the sessile spikelet; D, rachis with pedicelled spikelet. All line drawings were drawn by M. Norsaengsri.

pilose with tubercle-based hairs on both surfaces especially on the margins, base round or subcordate, apex acute. *Inflorescence* composed of several racemes, terminal and axillary, with one to five racemes, each raceme subtended by spatheole, raceme 5–17 mm long; *peduncle* 10–30 mm long; rachis 9–15 mm long, oblong, flattened, fragile at the nodes; adherent to upper glume of sessile spikelets, rachis internode tip transverse, cup-shaped; spikelets dorsally compressed. *Sessile spikelets* subglobose, united with rachis. *Glumes*; lower glume 0.8–1.3 mm long, turgidly swollen and hemispherical, coriaceous, pitted and tubercled on the back; upper glume 0.9–1.2 mm long, orbiculate, hyaline, enfolded, adhering to the cavity. *Lower floret*; lemma ovate, *c.* 0.8 mm long, hyaline; palea absent. *Upper floret*; lemma *c.* 0.8 mm long, ovate-obtuse, hyaline; palea *c.* 0.6 mm long, ovate, hyaline. *Anthers* three, *c.* 0.2 mm. *Pedicelled spikelet* neuter. *Pedicels* completely fused with rachis; united wholly; oblong. *Glumes*; lower glume *c.* 10 mm long, elliptic, four- or five-nerved, keeled, scabrous on keel; upper glume 6–8 mm long, elliptic, five- or six-nerved, enfolded, keeled and scabrous on back. *Caryopsis* orbicular, dorsally compressed.

Representative specimens examined: YEMEN. *P. Hein, H. Kürschner & M. Reisch YP751a* (C). JAPAN. *S. Hatasima 19154* (L); *S. Hatusima 20054* (L). CHINA. *Xiao Bai-Zhong 4593* (K); *K. Wang 2841* (K); *W.T. Tsang 28030* (K). INDIA. *J.L.G. van der Maesen 4977* (K); *G. Panigrahi 4270* (L); *A.P. Young s.n.* (L); *Walter N. Koelz 19068* (L); *K.M. Matthew & N. Venugopal 16813* (L), *C. Manoharan 19540* (K); *B.K. Mina 826* (L); *G. Panigrahi 20617* (L). MYANMAR. *R.O. Belcher BC 555* (K), PAKISTAN. *R.R. Stewart 23326* (L). BHUTAN. *R. Pradhan & T. Wangdi EG55, EG139* (E); *Wangdi & Kinzang 2002.S10* (E). SRI LANKA. *D. Clayton 5284* (L). THAILAND. *A.F.G. Kerr 2213* (E, K); *BGO. Staff 7, 4306, 6935, 7113, 7379* (QBG); *G. Murata et al. T-15609* (AAU, BKF, C, K, L, P); *H. Koyama T-61037* (BKF, L), *T.-61246* (BKF, QBG), *T-61379* (AAU, BKF, L); *J.F. Maxwell 00-316* (L), *75-983* (AAU, BK, L), *87-1277* (BKF, CMU, L), *88-1066* (AAU, BKF, CMU, L), *90-1032* (AAU, CMU, L), *92-432* (L), *96-1056* (BKF, L), *97-904* (BKF, L), *97-952* (BKF, L), *96-1478* (L); *K. Iwatsuki & N. Fukuoka T-10392* (BKF), *K. Iwatsuki et al. T-9458* (AAU, BKF, C, E, K, L), *T-11135* (AAU, BKF, C, E, L); *K. Larsen et al. 46828* (AAU, L); *L. Kethirun & P. Traiperm 008* (BKF), *M. Norsangsri 894, s.n.* (QBG), *1083* (QBG), *1690* (QBG), *4090* (QBG); *M. Norsangsri et al. 2166* (QBG), *M.N. Tamura T-60223* (BKF); *NH 5-46* (QBG); *P. Chantaranothai & J. Parnall 90/779* (AAU, K); *P. Palee 237* (BKF, L), *400* (L); *P. Traiperm 299, 303, 310* (BKF); *Put 3989* (BK, BM, K); *R. Wehner 41* (L); *S. Laegaard 21684* (AAU, L), *21690*

(AAU); *S. Phusomsaeng & K. Bunchai 23* (BKF, K, L); *S. Tsugaru T-61246* (BKF), *T-61739* (BKF, L); *T. Shimizu T-10596* (BKF, L); *T. Shimizu et al. T-10464* (BKF); *T. Smitinand 1989* (BKF, K), *3056* (BKF), *11645* (BKF, L); *Th. Sørensen et al. 4711* (C); *Th. Sørensen et al. 4378* (BKF, C, K), *4988* (C), *5038* (C, E, K); *W. Nanakorn et al. 2636* (QBG), *4451* (QBG), *4789.1* (QBG); *Umpai 517* (BKF); *Y. Paisooksantivathana y 2569-89* (BK). VIETNAM. *N.T. Hiep, N.Q. Binh, L. Averyanov & P. Cribb NTH 3561* (K), *N.M. Cuong 490* (L). INDONESIA. *W. Meuer 10201* (L); *Beguin 29* (L); *P. Buwalda 7410* (L); *F.W. Rappara 242* (L); *C.A. Backer 36023* (L); *C.A. Backer s.n.* (L); *Bakh Brink 6493* (L); *F.C. van Loenen 12* (L); *C.G.G.J. van Steenis 7741* (L); *S. Bloembergen 3164* (L); *Monod de Froideville 1941* (L); *Dr. Rant 767* (L); *P. Buwalda 4014* (L); *Kyma 1220* (L); *M. Jacobs 4623* (L); *W.J.J.O. de Wilde & B.E.E. de Wilde-Duyfjes 18949* (L); *J.A. Lörzing 13587* (L). MALAYSIA. *J. Sinclair, Kadim B. Tassim & Kapis B. Sisiron 9239* (L). PHILIPPINES. *A.D.E. Elmer 18237* (C); *Jose Vera Santos 7557* (L); *M. Ramos 2-107* (C);

F. Seidenschwarz L39 (L); *A.D.E. Elmer 10409* (L); *Eimer. D. Merrill 3094* (L); *Jose Vera Santos 7695* (L); *Dr. H. Conklin & del Rosario 42666* (L); *A.D.E. Elmer 11039* (L); *Jose Vera Santos 7594* (L); *Jose Vera Santos 4791* (L); *Jose Vera Santos 8131* (L). INDONESIA. *C. Kalkman BW 6397* (L); *J. Raynal 16,645* (L). PAPUA NEW GUINEA. *J.M. Simaga 37* (L); *D. Fryar 3615* (L); *E.E. Henty 14325* (L); *P.F. Stevens 50201* (L); *A. Clive Jermy 4412* (L); *H. Streimann & A. Kairo 45423* (L); *L.J. Brass 3701* (L); *A.N. Gillison NGF 22183* (L), *P.J. Darbyshire 697* (L); *E.E. Henty & P. Katik NGF 42914* (L); *M. Galore NGF 17566* (L). AUSTRALIA. *Einer Nielsen 559* (C); *S.T. Blake 13337* (L). IVORY COAST. *Adam Jacques-Georges 27157* (C). MALI. *Adam Jacques-Georges 28208* (C). SENEGAL. *Simon Lægaard, Jens E. Madsen & Sobéré A. Traoré 16839* (C); *Simon Lægaard, Jens E. Madsen & So`béré A. Traoré 16904* (C); *C. Vanden Berghen 4641* (C). TOGO. *F.J. Breteler 7166* (C). CAMEROON. *M.G. Latilo & B.O. Daramola 28798* (C). CONGO. *L. Liben 2406* (C); *A.B. Stam 66* (L). ETHIOPIA. *G.J.H. Amshoff 7486* (C); I. *Friis, Sally Bidgood, Fantahun Semon, Michael Jen-*
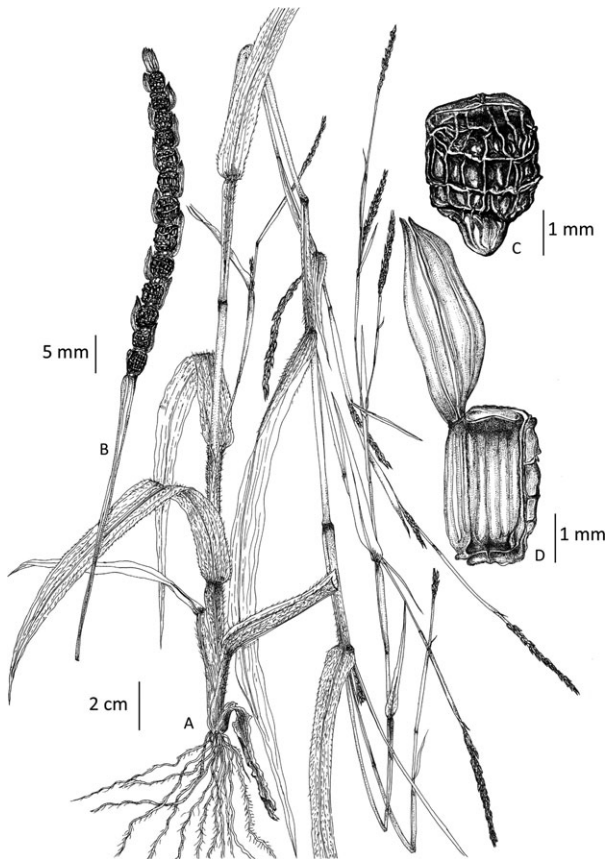

Figure 9. *Hackelochola porifera*: A, plant; B, raceme; C, lower glume of the sessile spikelet; D, rachis with pedicelled spikelet. All line drawings were drawn by M. Norsaengsri.

Figure 10. A, B. *Hackelochloa granularis*: A. habit (photograph by L. Kethirun); B. inflorescence (photograph by W. Tanming). C–E. *H. porifera*: C. habit; D and E. inflorescence (photographs by P. Traiperm).

sen & Menassie Gashaw 7706 (C); *M.G. Gilbert & Getachew A. 3075* (C). TANZANIA. *M. Batty 295* (C); *P.J. Greenway & Kanuri 15134* (C); *K. Vollesen 3627* (C); *A. Stolz 1240* (C). ZIMBABWE. *R.N. Davies 3027* (L). MADAGASCAR. *1906* (L). SOUTH AFRICA. *H. Faulkner 38* (L). DOMINICAN. *E.L. Ekman 13401* (C). HONDURAS. *C.L. Lundell 6693* (C). MEXICO. *A.S. Hitchcock 240* (C); *C.G. Pringle 11228* (L); *Fournier 6145* (C). PANAMA. *M. Nee 6933* (L); *M. Nee 7381* (L); *H. Pittier 3965* (C). CUBA. *A.H. Curtiss 493* (L). JAMAICA. *Wm. Harris 11334* (C); *Wm. Harris 12147* (C). BRAZIL. *H.S. Irwin, H. Maxwell & D.C. Wasshausen 21122* (C). PERU. *E. Ule. 6640* (L).

Distribution: Tropical, sub-tropical and warm temperate parts of the world.

Ecology: Scattered on grassy slopes in forest gaps and in disturbed areas. Flowering in May to March.

Used: *Hackelochloa granularis* is prescribed internally with a little sweet oil, in cases of enlarged spleen and liver (Bor, 1960).

HACKELOCHLOA PORIFERA (HACK.) D.RHIND (FIGS 9, 10C-E)

Grass. Burma 2: 77 (1945). ≡ *Manisuris porifera* Hack., Oesterr. Bot. Z. 41(2): 48 (1891). *Type*: Sikkim, Darjeeling, *C.B. Clarke 9752 A* (holotype W!, isotype K!).

Culms 50–200 cm tall, erect, subterete, glabrous. *Leaf sheath* 2.0–8.5 cm long, loose, flat, hirsute with tubercle-based hairs; *ligules* 2.5–3.5 mm long, membranous, ciliate; *leaf blade* 5.0–30.0 × 0.7–2.5 cm, broadly linear, hirsute with tubercle-based hairs on both surfaces, margins scabrous, base round, apex acute. *Inflorescence* composed of several racemes, terminal and axillary, with two to four racemes, each raceme 2.0–4.5 cm long; subtended by spatheole raceme; *peduncle* 3–8 cm long; rachis 13–25 mm long, oblong, flattened, fragile at the nodes; adherent to upper glume of sessile spikelets, rachis internode tip transverse, cup-shaped; spikelets dorsally compressed. *Sessile spikelets* broadly oblong, united with rachis. *Glumes*; lower glume 1.5–2.5 mm long, turgidly swollen, broadly oblong, coriaceous and robust, ridged and reticulate on the back; upper glume 0.9–1.8 mm long, elliptic, boat-shaped, membranous, adhering to the cavity. *Lower floret* barren; lemma *c.* 1 mm long, broadly ovate-obtuse, hyaline; palea absent. *Upper florets* fertile; lemma *c.* 1 mm long, broadly ovate-obtuse, hyaline; palea *c.* 0.8 mm long, ovate, hyaline. *Pedicelled spikelet* neuter. *Pedicels* completely fused with rachis; rachis 18–20 mm long, united wholly, oblong. *Glumes*; lower glume 3.0–3.5 mm long, narrowly ovate, five- or six-nerved, enfolded, scabrous on the margins; upper glume 2.8–3.0 mm long, elliptic, five-nerved enfolded, keeled and scabrous on the back. *Caryopsis* 1.0–1.2 mm in diam., orbicular, dorsally compressed,

Representative specimens examined: INDIA. *C.B. Clarke 9752 A* (K). VIETNAM. *B. Balansa 4940* (K). MYANMAR. *J. Keenan, U Tun Aung & R.H. Rule 1824* (K); *R.O. Belcher KC 819* (K). THAILAND. *L. Kethirun & P. Traiperm 009* (BKF); *M. Norsaengsri 737, 742, 1847, 2896* (QBG); *P. Traiperm 311* (BKF); *R. Pooma et al. 7564* (BKF), *S. Laegaard 21684, 21689* (AAU); *W. Nanakorn et al. 4453* (QBG). INDONESIA. *de Voogd 1517* (L); *Rahmat si Boeea 6284* (L).

Distribution: Tropical and sub-tropical parts of Asia.

Ecology: In bamboo forests and on open ground, from sea level to 1500 m altitude. Flowering in August to January.

ACKNOWLEDGEMENTS

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Appendix 1 Measurement ranges of quantitative traits examined in phenetic analysis

Quantitative traits	*H. granularis*	*H. porifera*	*Mnesithea*	*Ophiuros*
Length of leaf sheath	1.48–3.58 cm	4.33–8.32 cm	3.28–12.74 cm	5.93–7.04 cm
Length of leaf blade	4.05–17.65 cm	10.23–23.66 cm	13.87–79.57 cm	26.61–31.58 cm
Width of leaf blade	0.45–1.06 cm	0.73–1.40 cm	0.25–1.88 cm	0.55–1.02 cm
Ratio of length of leaf blade to width of leaf blade	7.08–19.61	11.34–26.96	22.55–75.58	14.64–30.86
Length of raceme	0.86–1.67 cm	2.07–3.85 cm	3.30–18.02 cm	7.70–9.61 cm
Length of spatheole	1.16–1.83 cm	2.15–3.60 cm	5.3–13.37 cm	4.50–5.03 cm
Length of rachis internode	0.96–1.50 mm	1.36–2.5 mm	2.08–5.10 mm	2.88–4.00 mm
Length of spikelet	2.50–3.50 mm	3.40–5.10 mm	3.70–8.80 mm	2.90–4.00 mm
Length of lower glume of sessile spikelet	0.88–1.04 mm	1.27–1.66 mm	1.93–5.20 mm	2.38–3.20 mm
Length of upper glume of sessile spikelet	0.96–1.20 mm	0.99–1.50 mm	1.72–4.70 mm	2.50–3.14 mm
Length of lower glume of pedicelled spikelet	1.31–3.50 mm	1.98–3.33 mm	Reduced–3.43 mm	Reduced
Length of pedicelled spikelet	2.60–4.67 mm	3.34–5.83 mm	3.80–8.76 mm	Reduced
Length of pedicel	0.94–2.00 mm	1.36–2.50 mm	2.12–4.73 mm	Reduced
Ratio of length of lower glume of pedicelled spikelet to length of pedicelled spikelet	0.46–0.75	0.55–0.60	0.00–0.56	No ratio*

Table . *Continued*

Quantitative traits	*H. granularis*	*H. porifera*	*Mnesithea*	*Ophiuros*
Ratio of length of pedicel to length of pedicelled spikelet	0.25–0.54	0.34–0.45	0.61–1.00	No ratio*

For the ratio between the length of the lower glume of the pedicelled spikelet and the length of the pedicelled spikelet, values for *Mnesithea* species are generally < 0.40, except *M. formosa* which has a ratio of 0.56. Furthermore, the length of the upper glume of the sessile spikelet and the rachis internode are > 2.0 mm and 2.5 mm, respectively, in most species of *Mnesithea*, but are lower than these values in *M. formosa*. Although the overall length of the pedicelled spikelet overlaps across genera, the ratio of the length of the lower glume of the pedicelled spikelet to the length of the pedicelled spikelet and the ratio of the length of the pedicel to the length of the pedicelled spikelet both indicate a less development in the pedicelled spikelet in *Mnesithea*, as compared with *Hackelochloa*.

* No ratio available for *Ophiuros* due to reduced pedicelled spikelets.