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Barcode of nuclear ribosomal internal transcribed spacer regions (ITS) as a useful tool to recognize a newly naturalized and potentially invasive weed, *Chloris pilosa* Schumach. (Poaceae), in Taiwan

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ABSTRACT: An unknown grass was found naturalized in central Taiwan recently. We tried to identify it based on its morphological characters and could recognize it as a taxon of grass genus *Chloris.* Nevertheless it is obviously different from the other species of the genus which have been reported to occur in Taiwan. Based on its nuclear ribosomal internal transcribed spacer (ITS) sequence, through the approaches of BLAST, phylogeny reconstruction and statistic tests for alternative topologies of phylogeny, we could make sure of its identity further and recognize it as the species *Chloris pilosa* Schumach. precisely. The species is native to equatorial Africa and this is the first report about its occurrence in Asia. A key to the species of genus *Chloris* in Taiwan, morphological description and illustrations of *C. pilosa* are also provided in this article.

KEY WORDS: barcode, Chlorioideae, *Chloris pilosa*, ITS, naturalized weed, Poaceae, Taiwan.

INTRODUCTION

The grass genus *Chloris* Swartz (Poaceae: Chloridoideae: Cynodonteae) comprises about 60 species throughout tropical and warm-temperate regions of the world (Sun and Phillips, 2006; Kellogg, 2015; Peterson *et al.*, 2015; Soreng *et al.*, 2017). We can recognize this genus by the terminal inflorescences with several spike-like racemes borne digitately or in whorls, and also by laterally compressed spikelet which consists of one fertile floret and 1–2 sterile florets (Sun and Phillips, 2006; Chen *et al.*, 2011a).

In Taiwan, there are three native and three additional exotic naturalized species of the genus (Hsu, 2000; Jung *et al.*, 2009; Chen *et al.*, 2011a). Recently, we found a few populations of an unusual taxon of *Chloris* in central Taiwan. It was obviously different from the other known species of the genus in Taiwan by two minute awns on spikelet, and strong gibbous keel on fertile floret.

In this study, we would like to figure out the identity of the uncertain taxon. Besides the identification based on morphological characters, we also conducted molecular analyses in order to confirm our conclusion. The internal transcribed spacer (ITS) region of nuclear ribosomal cistron (18S-5.8S-26S) has been commonly used in molecular systematic research of Poaceae because of good effectiveness of species-level discrimination and technical ease (Hodkinson *et al.*, 2002; Álvarez and Wendel, 2003; Blattner, 2004; Catalán *et al.*, 2004; Essi *et al.*, 2008; Hand *et al.*, 2010; Peterson *et al.*, 2010, 2014; Syme *et al.*, 2013; Wang *et al.*, 2017). We also have elucidated the relationship between grass genera *Leptatherum* and *Microstegium* based on the phylogeny of ITS sequences successfully (Chen *et al.*, 2009). Furthermore, the ITS phylogeny was also helpful to identify a cryptic species of the genus *Microstegium* in Taiwan (Chen *et al.*, 2011b). It has also been recommended as a core DNA barcode of seed plants (Stoeckle, 2003; Kress *et al.*, 2005; Li *et al.*, 2011; Hollingsworth, 2011). Meanwhile, a large dataset of ITS sequences has been uploaded into GenBank (Kress *et al.*, 2005; Feliner and Rosselló, 2007). Therefore we chose it as an ideal barcode to figure out the identity of the uncertain taxon in this study.

After confirming the identity of the uncertain taxon, more information about its taxonomy would be provided for identification, including a key to species of genus *Chloris* in Taiwan, morphological description, illustration and photos, etc.

MATERIALS AND METHODS

Morphological examination

Besides the uncertain taxon which we would like to figure out, we also examined quite a few specimens which were identified as *Chloris* in the herbaria of National Taiwan University (TAI), Endemic Species Research Institute (TAIE), and Forestry Research Institute (TAIF) for the purposes of identification and comparison. Specimens examined for this study were shown in Table S1 as supplementary.

Taxa sampling for ITS phylogeny

Total of 14 accessions from Taiwan were sampled in this study, including four of uncertain taxon, two *Chloris virgata*, two *Chloris barbata*, two *Chloris gayana*, one

Table 1. Samples collected in Taiwan and the other Genbank accessions used in the study.

Taxa	GenBank accession number	Voucher or reference	Locality
Accessions of the unknown taxon	MK246190	*Chen Chih-Hui 7473*	Taiwan, Nantou
(=*Chloris pilosa*)	MK246191	*Chen Chih-Hui 7476*	Taiwan, Nantou
	MK246192	*Chen Chih-Hui 7477*	Taiwan, Nantou
	MK246193	*Chen Chih-Hui 7478*	Taiwan, Zhongliao
Chloris pilosa	KP873266	Peterson *et al.*, 2015	unknown
	KP873267	Peterson *et al.*, 2015	Nigeria
	KP873268	Peterson *et al.*, 2015	Tanzania
Chloris gayana	MK246196	*Chen Chih-Hui 5015*	Taiwan, Fuxing
	MK246197	*Chen Chih-Hui 6554*	Taiwan, Fuli
	KP873252	Peterson *et al.*, 2015	-
	KP873253	Peterson *et al.*, 2015	-
	KP873254	Peterson *et al.*, 2015	-
Chloris virgata	MN240431	*Chen Chih-Hui 7572*	Taiwan, Pingtung
	MN240432	*Chen Chih-Hui 7573*	Taiwan, Pingtung
	KP873295	Peterson *et al.*, 2015	-
	KP873297	Peterson *et al.*, 2015	-
	KP873294	Peterson *et al.*, 2015	-
	KP873299	Peterson *et al.*, 2015	-
	GU359323	Peterson *et al.*, 2010	-
Chloris divaricata	KP873242	Peterson *et al.*, 2015	-
Chloris divaricata var. *cynodontoides*	KP873241	Peterson *et al.*, 2015	-
Chloris pycnothrix	KP873273	Peterson *et al.*, 2015	-
	KP873275	Peterson *et al.*, 2015	-
Chloris barbata	MK246194	*Chen Chih-Hui 6294*	Taiwan, Lanyu
	MK246195	*Chen Chih-Hui 6802*	Taiwan, Liuqiu
	KP873226	Peterson *et al.*, 2015	-
Cynodon dactylon	MK246198	*Chen Chih-Hui 6773*	Taiwan, Zhuangwei
	MK246199	*Chih-Hui Chen 6823*	Taiwan, Liuqiu
	GU359243	Peterson *et al.*, 2010	-
Cynodon dactylon var. *pilosus*	KP873313	Peterson *et al.*, 2015	-
Cynodon nlemfuensis	KP873325	Peterson *et al.*, 2015	-
	KP873322	Peterson *et al.*, 2015	-
	KJ768881	Peterson *et al.*, 2015	-
Enteropogon dolichostachyus	MK246200	*Chen Chih-Hui 5786*	Taiwan, Chunri
	MK246201	*Chen Chih-Hui 6131*	Taiwan, Qishan
	KP873371	Peterson *et al.*, 2015	-
	KP873372	Peterson *et al.*, 2015	-
Eleusine indica	MK246202	*Chen Chih-Hui 6819*	Taiwan, Liuqiu
	KP873354	Peterson *et al.*, 2015	-
	KP873355	Peterson *et al.*, 2015	-
	KP873356	Peterson *et al.*, 2015	-

Cynodon dactylon, two *Enteropogon dolichostachyus*, and one *Eleusine indica*. The leaf materials were preserved in silica-gel. All vouchers were deposited in the herbarium of Endemic Species Research Institute, Taiwan (TAIE). Not only the above samples which were collected by ourselves in the field of Taiwan, but we also included some accessions of ITS sequences of the above species from GenBank. Detailed information of vouchers and accession numbers of the materials from GenBank were listed in Table 1.

DNA extraction, PCR amplification and DNA sequencing

Total genomic DNAs were isolated using taco total DNA extraction kit-320 (GeneReach Biotechnology Crop., Taichung, Taiwan). Dried leaf materials were taken in about 1 cm square and ground using a mortar and pestle with liquid nitrogen. The powder was mixed with 600 μL lysis buffer and shaken for one hour. The mixture was then transferred to 96-well extraction plate. The plate loaded with reagents provided in the kit and samples were installed in the taco nucleic acid automatic extraction system. The system then extracted total genomic DNAs automatically. The ITS/5.8S region, including ITS1, 5.8S, and ITS2, was amplified by PCR using the forward primer, IT-11: 5'-TCG TAA CAA GGT TTC CGT AGGT-3', and the reverse one, IT-8: 5'-GTA AGT TTC TTC TCC GCT-3' (Chen *et al.*, 2009; Chen *et al.*, 2011b). The PCR amplifications were performed in total 20 μL reaction volumes containing 5 μL genome DNAs extraction (20X diluted), 2 μL 10X PCR buffer, 3.2 μL 2.5 mM dNTP, 1.2 μL 25 mM $MgCl_2$, 1 μL DMSO, 0.2 μL Supertherm Taq, 1 μL each of 2 μM primers and 5.4 μL ddH_2O. The thermal cycles were performed with 95°C for 5 min, followed with 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, and final extension in 72°C for 7 min. The PCR products were cleaned up with spin column of the kit. The Sanger Sequence reaction were done using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Sequencing primers

were the same as those used for PCR. The sequencing products then were analyzed by DNA analyzer 3730xl (Applied Biosystems).

Sequence alignment and phylogenetic analysis

In order to figure out the identity of the unknown taxon, both BLAST and tree-base methods were used for molecular identification. In BLAST method, sequences of the unknown taxon were compared with GenBank database using NCBI MegaBLAST 2.8.0+ (Zhang *et al.*, 2000; Morgulis *et al.*, 2008). In tree-base methods, further 26 sequences belong to eleven taxa from GenBank were used for alignment and further phylogeny analysis (Table1). Hence, total 40 accessions belongs to four genera were involved to analysis with the species of *Chloris* as ingroup (26 accessions) and the other genera as outgroup (14 accessions). Sequences were aligned using MUSCLE function of MEGA7 (Edgar, 2004; Kumar *et al*., 2016) and edited manually using BioEdit version 7.2.5 (Hall, 1999) for correction and trimming.

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) phylogenies were reconstructed using PAUP* 4.0a161 (Swofford, 2002), PhyML v3.1 (Guindon and Gascuel, 2003) and MrBayes v3.2 (Ronquist *et al.*, 2012), respectively. Heuristic search of MP trees was conducted with 10000 random addition replicates under the setting of tree bisection-reconnection (TBR) branch swapping, MULPARS on, and saving no more than 3 trees per replicate with length greater than or equal to 10. Internal support was accessed by 1000 bootstrap replicates (Felsenstein, 1985) with 100 random addition replicates and same setting as for heuristic search in each bootstrap replicate.

The alignment matrix was applied to jModelTest 2.1.10 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012) to determine the substitution model used for reconstructing ML and BI phylogenies. The optimal model identified was TIM3ef+Γ (Posada, 2003) with the best Akaike Information Criteria (AIC) (Akaike, 1974; Posada and Buckley, 2004). While the TIM3 model cannot be implemented in either PhyML or MrBayes, the GTR model (Tavaré, 1986) and GTR+Γ model (Yang, 1994) were chosen for ML and BI analyses, respectively. ML phylogeny was reconstructed under the setting of fixed proportion of invariable sites and four substitution rate categories. Internal support was accessed by 1000 bootstrap replicates. BI phylogeny was analyzed with four Markov chain Monte Carlo (MCMC) simulation for 2,000,000 generations and sampled one per 1000 generations. The first 800 trees of sample trees were discarded as burn-in before the probability was calculated. Phylogenetic trees were visualized with TreeView (Page, 1996).

Tests of alternative tree topologies

In case of the low resolution of phylogenetic relationship, we used the program *baseml* of PAML v4.9i (Yang, 2007) and the programs *makermt*, *consel* and *catpv* of CONSEL v0.1j (Shimodaira and Hasegawa, 2001) to test alternative phylogenetic tree topologies with approximately unbiased test (AU), Kishino-Hasegawa test (KH), and Shimodaira-Hasegawa test (SH) (Shimodaira, 2002; Kishino and Hasegawa, 1989; Shimodaira and Hasegawa, 1999) in order to find out the most likely relationship among our uncertain samples and the other species of *Chloris* in Taiwan. The greater the p-value of the tests, the greater the probability that the tree is the true tree (Shimodaira, 2002).

RESULTS

Identification in herbaria

After checking some references (Anderson, 1974; Clayton *et al.*, 1974; Barkworth, 2003; Nightingale *et al.*, 2005.) and examining morphological characters of specimens, we thought the specimens fits the description of *Chloris pilosa* by Anderson (1974) and therefore *C. pilosa* could be the identity of the unknown taxon. Among the three herbaria mentioned above, specimens of *C. pilosa* were found only in TAIE. Its individuals from Taiwan have spreading long hair on pedicle, three florets and two awns on spikelet, and strong gibbous keel on fertile lowest floret. In addition, plant height and, especially, awn length on spikelet varied widely. We could divide the individuals of the *C. pilosa* from Taiwan into two types based on the variation. One type has prominent awns up to 6 mm long (vouchers: *Chen 7477* and *7478*, Figs. 1D and 4B) and the other one has awns shorter than 1 mm and becoming short mucros (vouchers: *Chen 7473* and *7476*, Figs. 1E and 4C).

Sequences' information and BLAST alignment

ITS sequences of all the samples from Taiwan were successfully amplified and generated from one-direction reads. They showed length variation from 637 to 651 bps and contributed to an alignment length of 659 characters. Within the 659 characters, 212 of them were variable and 198 of them were parsimony-informative. Polymorphic sites of uncertain samples, *C. pilosa* and *C. virgata* were shown in Table 2. Our uncertain taxon consisted of two haplotypes and so did the three accessions annotated as *C. pilosa* from GenBank. The accession KP873268 represented one haplotype itself and contained more variation in comparison with our uncertain samples than the accessions KP873266 and KP873267 which belonged to another haplotype.

The NCBI BLASTn results showed that all four accessions generated from uncertain samples highly matched the ITS/5.8S sequences of *C. pilosa*, with Maximal percentage identity (MPI) of 99%. Each of them had only 2 mismatched characters among total aligned length of 640 characters (Table 3).

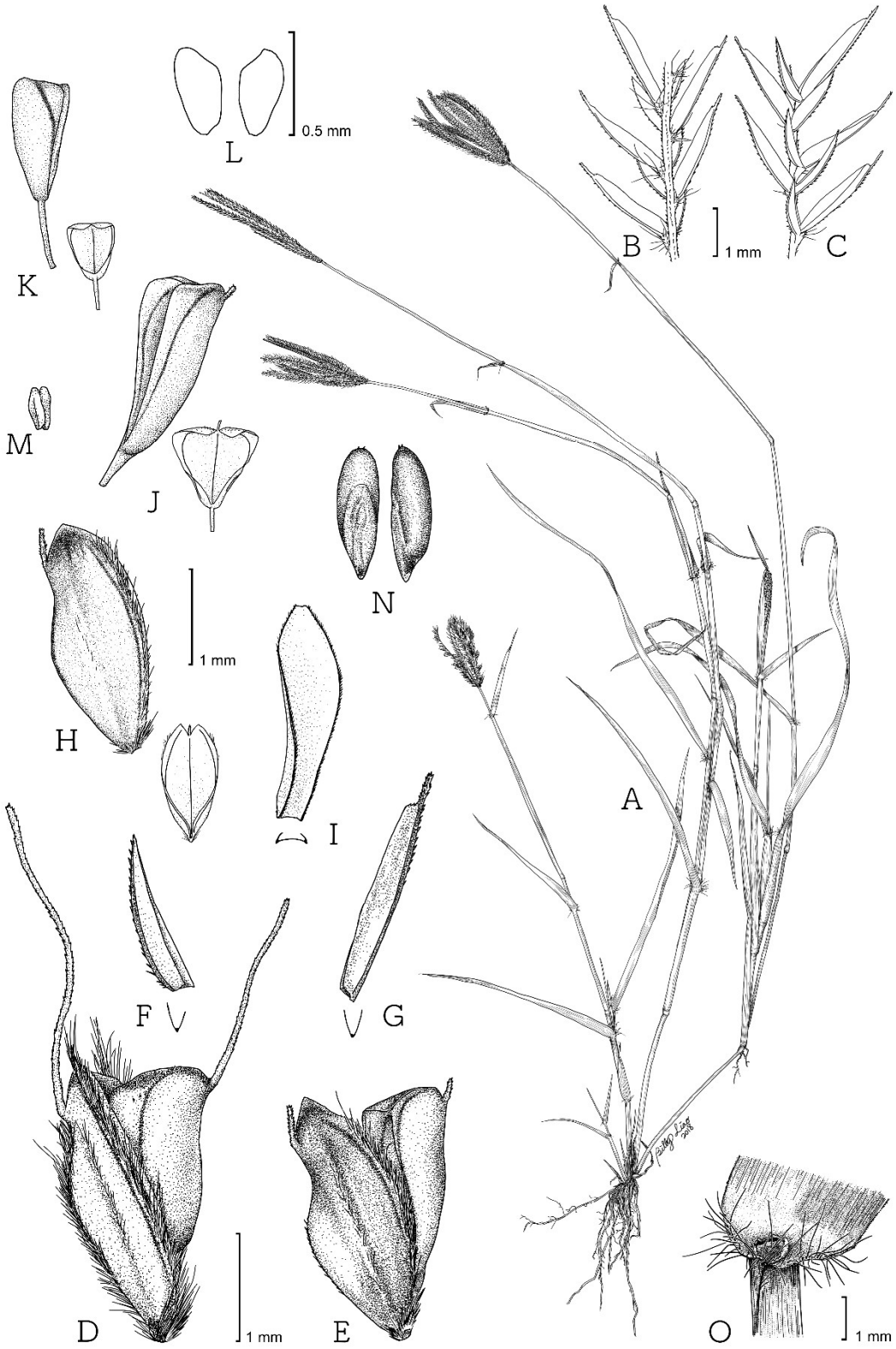

Fig. 1. Illustration of *Chloris pilosa* Schumach. **A**, habit; **B**, **C**: part of rachis with glumes persistent in different view; **D**, **E**: florets with different awn variation; **F**, lower glume; **G**, upper glume; **H**, lemma of first floret (lateral and ventral view); **I**, palea of first floret; **J**, second floret (lateral and ventral view); **K**, third floret (lateral and ventral view); **L**, lodicules; **M**, anther; **N**, caryopsis; **O**, ligule and joint between sheath and blade (based on *Chen 7473* & *7478*). Illustrated by H.-C. Liao.

Table 2. Polymorphic sites of *C. pilosa* and *C. virgata* identified for ITS sequences alignment matrix. Dot (.) means identical to the Hap1 of *C. pilosa* and dash (-) represents indel.

	Position of characters															Voucher or accession number
	15	58	99	112	170	204	221	222	408	442	540	560	575	587	657	
uncertain samples																
Hap1	G	G	C	G	T	T	A	A	-	A	T	T	A	G	A	*Chen 7473, Chen 7476*
Hap2	.	.	.	.	.	.	.	T	-	.	.	C	.	.	.	*Chen 7477, Chen 7478*
C. pilosa																
Hap1	.	.	.	.	.	.	.	T	-	.	C	.	.	.	C	KP873266, KP873267
Hap2	T	.	.	.	.	.	T	T	A	.	.	.	.	.	C	KP873268
C. virgata																
Hap1	.	T	T	T	C	C	.	T	C	G	.	.	.	.	.	*Chen 7572, Chen 7573*
Hap2	.	.	T	.	.	C	.	T	-	.	.	.	.	T	C	KP873294
Hap3	.	T	T	.	C	C	.	T	-	.	.	.	.	.	C	KP873295, KP873297
Hap4	.	.	T	.	.	C	.	T	-	.	.	.	T	.	C	KP873299, GU359323

Table 3. The most possible identity of the uncertain vouchers comparing ITS sequences using BLASTn.

Voucher	Result of BLASTn	Max score	Maximal percentage identity(MPI)	Mismatch/alignment length
Chen 7473	*Chloris pilosa*	1171	99%	2/640
Chen 7476	*Chloris pilosa*	1171	99%	2/640
Chen 7477	*Chloris pilosa*	1171	99%	2/640
Chen 7478	*Chloris pilosa*	1171	99%	2/640

Reconstructing phylogenetic relationships

Twenty four equally most parsimonious trees with 331 steps were found in MP analysis. The ML analysis found a best tree with log likelihood (Ln*L*) of -2635.21. The best topology sampled by BI analysis had a Ln*L* of -2674.89. Phylogenetic analyses of three different methods showed no conflict in topologies, therefore we showed only BI tree for interpretation (Fig. 2). Bootstrap values of MP and ML, and posterior probabilities of BI for each clade were combined and shown in Fig. 2. The details of BI, MP and ML trees were shown in Suppl. 2, Suppl. 3 and Suppl. 4 as supplementary respectively.

In Fig. 2, four uncertain samples, three accessions annotated as *C. pilosa* from GenBank and seven accessions of *C. virgata* formed a strongly supported monophyletic clade (MP bootstrap: 91, ML bootstrap: 77, posterior probability: 1.000). Under this clade, all four accessions of our uncertain samples were monophyletic but of no strong support (MP bootstrap: 65; ML bootstrap value: 60; posterior probability: 0.991). They got united with KP873266 and KP873267 of *C. pilosa* to form a monophyletic clade of no strong enough support again (MP bootstrap: 42; ML bootstrap: 46; posterior probability: 0.806). In spite of the position of KP873268 placed uncertain, all accessions of *C. virgata* were monophyletic with moderate support (MP bootstrap: 56, ML bootstrap: 50, posterior probability: 0.928). Due to the weak branch support of *C. pilosa* and *C. virgata,* their relationship with our uncertain samples was still unclear.

Besides the above, accessions of the other species all form monophyletic clade of very strong bootstrap support respectively except the two species of genus *Cynodon*. In spite of that, all accessions of *Cynodon* formed a monophyletic clade of very strong bootstrap support, too.

Tests of alternative tree topologies

We would like to test the different alternative topologies of ITS phylogeny statistically since the relationship among our uncertain samples, *C. pilosa* and *C. virgata* was still unclear in ITS phylogeny. The three accessions annotated as *C. pilosa* from GenBank were treated into two parts in accordance with their two haplotypes. Meanwhile, we combined four uncertain samples and seven accessions of *C. virgata* as constraint clades respectively. Therefore we got four groups which we would like to figure out their relationship. We fixed the topology of the other species because of their clear positions in ITS phylogeny. Therefore there would be fifteen alternative topologies among the four target groups if we rooted with the other species. The detailed information of the fifteen topologies was shown in Suppl. 1 as supplementary.

The results of the tests of AU, KH, and SH and the relationship between our uncertain samples and *C. pilosa* (KP873266 and KP873267) were shown in Table 4. The rank in Table 4 is in descending order of the p-values of AU test for the fifteen possible topologies. The topologies of #15, #9 and #1 (modified and briefed in Fig. 3) had the obviously higher p-values than the other topologies in all AU, KH and SH tests. They all have p-values of AU test higher than 0.6, of KH test almost 0.5, and of SH test higher than 0.85. In these three topologies, our uncertain samples formed monophyletic clade with the accessions KP873266 and KP873267 although the position of accession KP873268 was still controversial. Below rank 3, the p-values of the other topologies dropped obviously and our uncertain samples and the clade of KP873266 and KP873267 were paraphyletic or polyphyletic in those topologies. Therefore it is most likely that our uncertain samples have closest relationship with the accessions KP873266 and KP873267 which are annotated as *C. pilosa* from GenBank.

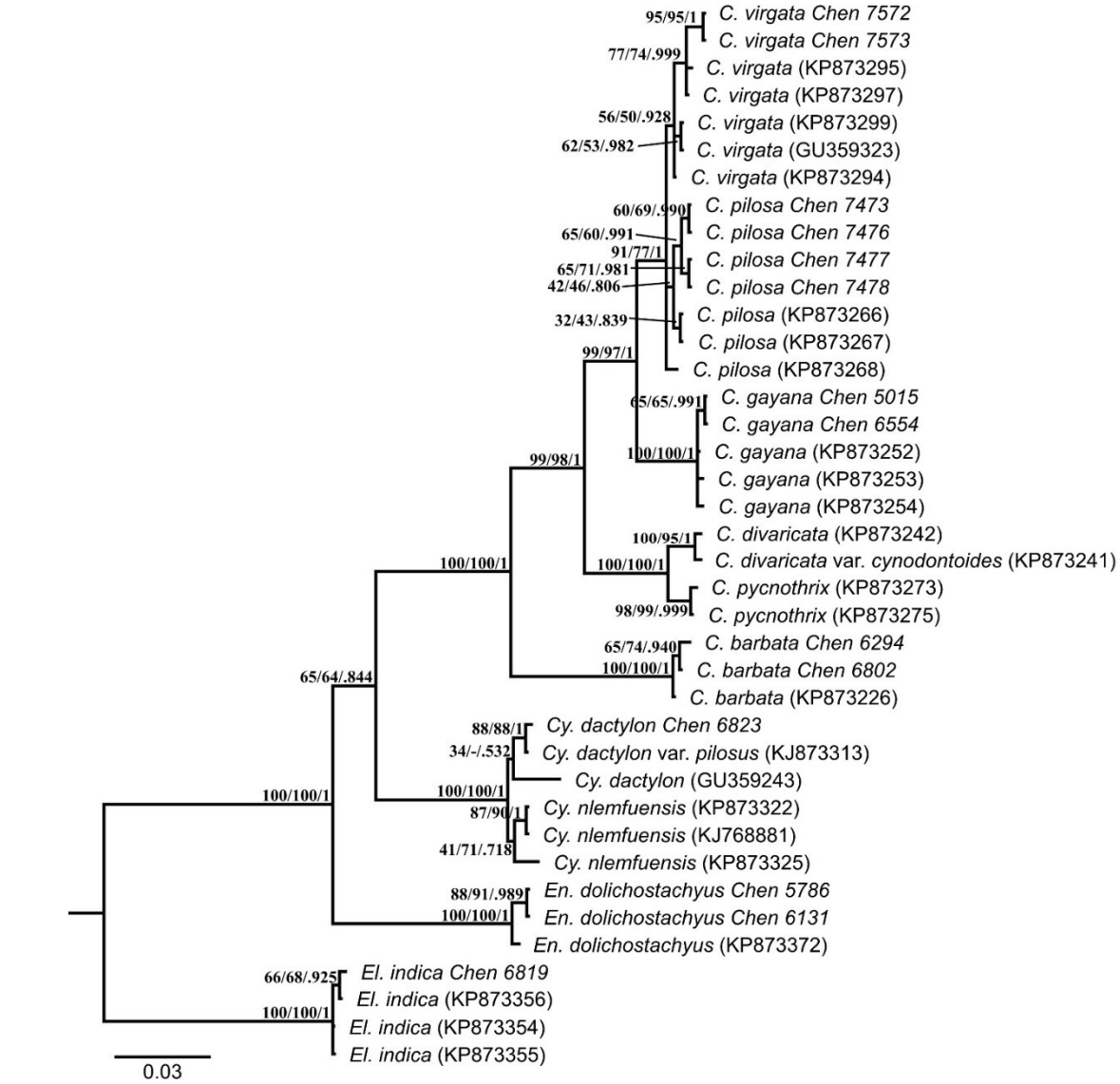

Fig. 2. Bayesian 50% majority-rule consensus tree inferred form the ITS sequences, with >50% clade supports (parsimony bootstrap value/likelihood bootstrap value/posterior probability) shown at each node. Voucher or GenBank number obtained from NCBI database are given following species name.

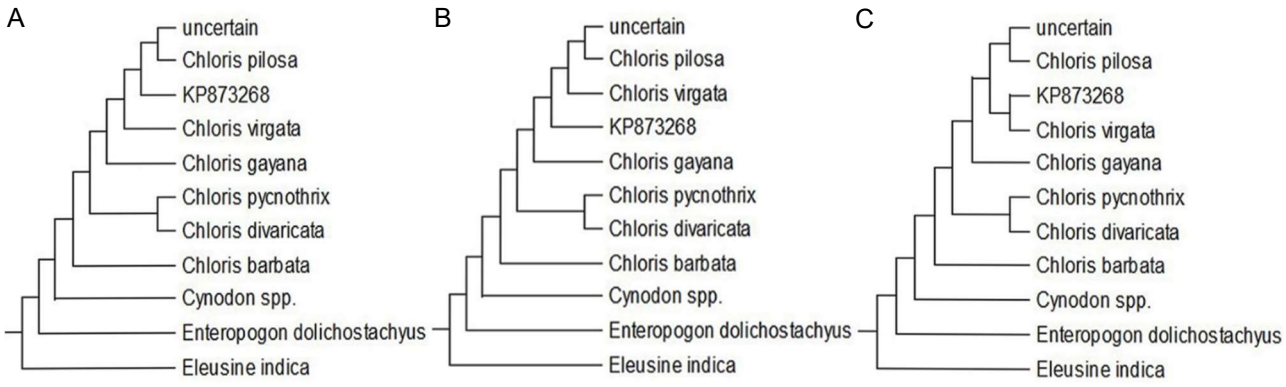

Fig. 3. The three modified and brief tree topologies of the highest p-values of AU, KH, and SH tests. **A**, tree #15; **B**, tree #9; **C**, tree #1.

Table 4. p-values of AU, KH and SH tests for the fifteen alternative tree topologies.

Rank	Tree topology tested	Relationship between uncertain taxon and *C. pilosa*	AU	KH	SH
1	#15	monophyletic	0.685	0.499	0.861
2	#9	monophyletic	0.660	0.501	0.904
3	#1	monophyletic	0.616	0.490	0.852
4	#10	paraphyletic	0.319	0.276	0.346
5	#6	paraphyletic	0.183	0.228	0.229
6	#8	paraphyletic	0.181	0.228	0.228
7	#7	paraphyletic	0.077	0.202	0.217
8	#3	polyphyletic	0.075	0.202	0.217
9	#2	polyphyletic	0.075	0.202	0.217
10	#12	paraphyletic	0.074	0.202	0.217
11	#13	paraphyletic	0.073	0.202	0.217
12	#11	paraphyletic	0.073	0.202	0.217
13	#14	paraphyletic	0.073	0.202	0.217
14	#5	paraphyletic	0.071	0.202	0.218
15	#4	paraphyletic	0.037	0.196	0.248

DISCUSSION

According to the result of BLASTn, ITS phylogeny reconstruction, and statistical tests for alternative topologies, we were confident to confirm the conclusion of our identification based on morphological characters. The uncertain taxon belongs to *C. pilosa* actually (Fig. 4). This species is native to equatorial Africa and known as a weed or forage grass (Anderson, 1974; Clayton *et al.*, 1974; Zon, 1992). It also has been reported to be naturalized in Australia by uncertain means (Nightingale *et al.*, 2005). In addition, Barkworth (2003) speculated that it might escape from experimental forage planting occasionally in North America.

In Taiwan, based on the specimen records, *C. pliosa* might appear in 2009 or so (voucher: *Hsu 15363*). It tends to occupy open arable field, and has spread to several districts in central Taiwan. According to our investigation, *C. pilosa* has colonized several reproducible populations along County Highway 139 in Nantou County of Taiwan although it has not been long since the species appeared in Taiwan. We suppose that *C. pilosa* has tendency to expand its colonizing area and become an invasive weed. This is the first report about its occurrence in Asia not because of intentional human activities. We don't have any idea about how it can cross such a far distance, including land and sea, to reach Taiwan. Nevertheless, we need to be cautious and pay attention to the risk that *C. pilosa* invades nearby regions of Taiwan potentially.

We could divide the individuals of *C. pilosa* from Taiwan into two types based on the variation of awns in spikelets. These two types are sympatric in Taiwan (Vouchers: *Chen 7476* and *7477*). This is the same as the species in its original habitats in equatorial Africa. Anderson (1974) mentioned that variation of awn length in spikelets of *C. pilosa* was recorded in Africa, while there seems no correlation between geographic distribution and morphological variation. On the other hand, only populations with short mucros were recorded in Australia (Nightingale *et al.*, 2005). Clayton *et al.* (1974) and Anderson (1974) both regarded two types of variation as a species. Different from the opinion of previous studies, Vanden Berghen (1987) differentiated the plants into two varieties on the basis of the particular description of Hackel (Hackel, 1906). He treated the individuals with short mucros as a variety, *C. pilosa* var. *nigra.* Vanden Berghen (1987) also pointed out that two varieties required similar habitat but had certain segregation in distribution. In his opinion, *C. pilosa* var. *nigra* was mostly distributed in western Africa including Mauritania through Cameroun, whereas *C. pilosa* var. *pilosa* was distributed along tropical Africa including Central African Republic, Democratic Republic of the Congo, Angola and Tanzania. Sympatry of the two varieties were also found in Senegal, Mali, Nigeria, and Cameroon. However, this treatment was neglected in several following publications (Zon, 1992; Phillips, 1995; Nightingale *et al.*, 2005).

Of the sequences of ITS, our four uncertain samples consisted of two haplotypes. It is in accordance with the two types of morphological characteristics mentioned above. The best tree of ITS phylogeny showed that four uncertain samples of *C. pilosa* from Taiwan formed a monophyletic clade with moderate support which was sister to another clade of African samples, the accessions of *C. pilosa* from GenBank. Interestingly, two subclades diverged further and they were formed by the individuals of long awn and short mucros in spikelets respectively. However, the two subclades had only moderate support. It might be just a coincidence and it is not appropriate to deduce too much arbitrarily based on so few samples of Taiwan.

We used ITS phylogeny in the taxonomic studies of grasses in our previous studies very successfully (Chen *et al.*, 2009; Chen *et al.*, 2011b) and it was quite efficient to use ITS as barcode to discriminate taxa in many other previous studies. However, it was frustrating that ITS phylogeny couldn't provide reliable information about the relationship among our uncertain taxon, *C. pilosa*, and *C. virgata* because of the weak branch support. Besides *C. pilosa* and *C. virgata*, the relationship of the two species of genus *Cynodon, Cynodon dactylon* and

Fig. 4. Photographs of *Chloris pilosa* Schumach. **A**, habit; **B**, Inflorescences with prominently awned spikelets (voucher: *Chen 7478*); C, Inflorescences with mucronate spikelets (voucher: *Chen 7476*).

Cy. nlemfuensis was not well resolved either. In such a situation, to involve more different DNA sequences such as *mat*K, *rbc*L and the other core barcodes of plants might be helpful. Alternatively we chose another approach of statistic tests to test whether our uncertain samples would be grouped with *C. pilosa* instead of utilizing more DNA barcodes.

According to the results of AU, KH, and SH tests shown in Table 4, the three topologies which had obviously highest p-values all revealed that our uncertain taxon and *C. pilosa* (KP873266 and KP873267) were monophyletic although the position of accession KP873268 was still controversial (Fig. 3). It means our uncertain taxon is closest to KP873266 and KP873267 which were annotated as *C. pilosa* in GenBank. Another haplotype of *C. pilosa*, KP873268, has an extraordinary ITS sequences in comparison with KP873266 and KP873267. More study to elucidate its taxonomic status might be necessary. However, we are not going to discuss it further because it is not the purpose of this study and we have little information about this accession.

Now that we have confirmed the identity of the uncertain taxon as *C. pilosa*, we provide an identification key for the species of genus *Chloris* in Taiwan, also its nomenclature history and description below.

Key to the species of *Chloris* in Taiwan

1a. Spikelet outline lanceolate or oblong; apex of 2nd lemma bifid. . 2
1b. Spikelet outline obovate; apex of 2nd lemma obtuse, truncate, or obscurely lobed …….. 3
2a. Digitate racemes flexible ……. ***C. divaricata*** var. ***divaricata***
2b. Digitate racemes straight ……... ***C. divaricata*** var. ***cynodontoides***
3a. Spikelet with 2 awns or short mucros on florets …….. 4
3b. Spikelet with 3 prominent awns on florets …… 6
4a. Lowest lemma with 1.5–4 mm spreading hairs on upper margin …. …….. ***C. virgata***
4b. Lowest lemma with 0.5–1.5 mm hairs on upper margin ……. 5
5a. Perennial, usually with prominent stolon; second lemma lanceolate . ……. ***C. gayana***
5b. Annual, occasionally rooting at the lower nodes; second lemma clavate ……... ***C. pilosa***
6a. Inflorescence expanded; second lemma 1–1.5 mm, nearly as long as width ……... ***C. barbata***
6b. Inflorescence contracted; second lemma 1.6–2 mm, longer than width ……..……. ***C. formosana***

Nomenclature history and description

Chloris pilosa Schumach., Beskr. Guin. Pl. 55. 1827; Anderson, Brigham Young Univ. Sci. Bull., Biol. Ser. 19(2): 58. 1974; Clayton *et al.*, Fl. Trop. East Afr. Gramineae part 2: 345, 1974; Barkworth, Fl. North America north of Mexico 25: 210. 2003; Nightingale *et al.*, Fl. Aust. 44B: 281. 2005.

毛虎尾草

Chloris breviseta Bentham, *in* Hooker f., Niger Flora 566. 1849.
Chloris nigra Hackel, Bol. Soc. Broteriana 21:179. 1906.
Chloris virgata Sw. var. *breviseta* (Bentham) Pilger, *ex* Peter, Beih. Repert. Sp. Nov. 40:262. 1931.
Chloris pilosa var. *nigra* (Hackel) Vanden Berghen, Bull. Jard. Bot. Belg. 57(3–4): 455. 1987.

Annual; culms erect or geniculately ascending, 30–70 cm tall, usually rooting at the lower nodes, glabrous. Culms node glabrous. Leaf sheaths laterally compressed, keeled, glabrous to pilose, outer margins glabrous, joint between sheath and blade pilose. Ligule ciliolate membrane, about 0.5 mm long. Leaf-blades linear, flat or folded, 10–35 cm long, 2–10 mm wide, margins scabrous, surface scaberulous on both sides. Inflorescence digitate racemes, open or contracted, base exserted from uppermost sheath. Base of racemes pubescent. Racemes 3–12, erect or ascending, straight, 3–5 cm long. Rachis tenacious, scabrous. Pedicels short, scabrous, with spreading long hair on margins. Spikelets bisexual, solitary, imbricate, laterally compressed, 2–3 mm long, pale to black; spikelet with one fertile floret and two sterile floret. Lower glume lanceolate, 1–1.5 mm long, membranous, 1-nerved, midvein keeled scabrous, apex acute. Upper glume lanceolate, 2–2.5 mm long, membranous, 1-nerved, midvein keeled scabrous, apex mucronate, mucro about 0.3 mm long. First floret fertile; lemma broadly ovate or elliptic on side view, 2.5–3 mm long, callus beared, 3-nerved, midvein keeled, keel strongly gibbous, sides with a glabrous or pubescent groove, sparsely to densely cillate on the margins and keel, apex 2-toothed, awned or with a short murco, subapical, awn up to 6 mm long; palea oblong, 2–3 mm long, 2-keeled, ciliolate along the keels. Second floret sterile, lemma 1.5–2.2 mm long, widened and inflated distally, surface glabrous below and becoming scabrous at the apex, apex truncate, awned or with a short murco, awn up to 3 mm long. Third floret reduced to a small clavate scale, less than 1 mm long, awnless. Anther 3, about 0.5 mm long. Caryopsis obovoid, trigonous, 1.3–1.5 mm long.

Distribution: Native to tropical Africa. It is introduced into North America as forage for experimental purpose. It was also reported to be naturalized in Australia occasionally.

Habitat: In grassy place along road side, arable and waste lands.

Specimens examined in Taiwan: NANTOU: Caotun Township, Caotun Commercial and Industrial High School, *Hsu 15363* (TAIE); Nantou city, County Highway #139, @32.9K, *Chen 7473* (TAIE), *Chen 7474* (TAIE); County Highway #139, @36K, *Chen 7476*; Fengming village, *Chen 7477* (TAIE); Zhongliao Township, Tazihwan, *Chen 7478* (TAIE); Mingjian Township, Xinguang villige, *Chen 7485* (TAIE); Jiji Township, Chenggong Road, *Chen 7493* (TAIE); Wuchang Temple, *Chen 7508* (TAIE); YUNLIN: Douliu city, *Hsu 17638* (TAIE).

Taxonomic remark: *C. pilosa* closely resembles *C. virgata* and *C. gayana*. It can be distinguished from those two species by shorter length of spikelets and much broader lemmas and, also, spikelets of *C. pilosa* usually become black in color when mature (Table 5). In the field of Taiwan, *C. pilosa* is usually found associating with other *Chloris* species, such as *C. barbata* and *C. divaricata* var. *divarcata*, in one habitat. It can be easily distinguished from *C. barbata* with clearly three awns in spikelets and purplish red in color, and from *C. divaricata* var. *divarcata* with horizontal racemes.

Table 5. Comparisons of morphological characters among *C. pilosa*, *C. virgata* and *C. gayana*.

	C. pilosa	*C. virgata*	*C. gayana*
Habit	Annual, usually rooting at the lower nodes.	Annual.	Perennial, usually stoloniferous.
Number of floret on a spikelet	3, with 1 fertile floret and 2 sterile florets.	Usually 2, with 1 fertile floret and 1 sterile floret.	Usually more than 3, with 1 fertile floret and 2–4 sterile florets.
Lemma of the first floret (fertile floret)	2.5 to 3.0 mm long, sparsely to densely cillate on the margins.	2.5 to 3.5 mm long, long-cillate on the margins, with spreading hairs up to 4 mm long near the apex.	3.0 to 4.0 mm long, cillate on the margins, with tuft of hairs near the apex.
Lemma of the second floret (sterile floret)	Clavate, 1.5 to 2.2 mm long.	Clavate, 1.5 to 2.5 mm long.	Lanceolate, 2.0 to 3.0 mm long.
Length of awn	2 short macros or up to 6 mm long.	5 to 15 mm long.	2 to 6 mm long

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