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Museomics resolve the systematics of an endangered grass lineage endemic to north-western Madagascar

Christian Silva[1],*, Guillaume Besnard[2], Anthony Piot[2], Jacqueline Razanatsoa[3], Reyjane P. Oliveira[1] and Maria S. Vorontsova[4]

[1]*Universidade Estadual de Feira de Santana, Departamento de Ciências Biológicas, Programa de Pós-graduação em Botânica, Av. Transnordestina s.n., Feira de Santana, Bahia 44036-900, Brazil,* [2]*CNRS, Université de Toulouse, ENFA, UMR5174 EDB (Laboratoire Évolution & Diversité Biologique), 118 route de Narbonne, 31062 Toulouse, France,* [3]*Herbier, Département Flore, Parc Botanique et Zoologique de Tsimbazaza, BP 4096, Antananarivo 101, Madagascar and* [4]*Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK*
*For correspondence. E-mail christian_da_silva@hotmail.com

● **Background and Aims** Recent developments in DNA sequencing, so-called next-generation sequencing (NGS) methods, can help the study of rare lineages that are known from museum specimens. Here, the taxonomy and evolution of the Malagasy grass lineage *Chasechloa* was investigated with the aid of NGS.
● **Methods** Full chloroplast genome data and some nuclear sequences were produced by NGS from old herbarium specimens, while some selected markers were generated from recently collected Malagasy grasses. In addition, a scanning electron microscopy analysis of the upper floret and cross-sections of the rachilla appendages followed by staining with Sudan IV were performed on *Chasechloa* to examine the morphology of the upper floret and the presence of oils in the appendages.
● **Key Results** *Chasechloa* was recovered within tribe Paniceae, sub-tribe Boivinellinae, contrary to its previous placement as a member of the New World genus *Echinolaena* (tribe Paspaleae). *Chasechloa* originated in Madagascar between the Upper Miocene and the Pliocene. It comprises two species, one of them collected only once in 1851. The genus is restricted to north-western seasonally dry deciduous forests. The appendages at the base of the upper floret of *Chasechloa* have been confirmed as elaiosomes, an evolutionary adaptation for myrmecochory.
● **Conclusions** *Chasechloa* is reinstated at the generic level and a taxonomic treatment is presented, including conservation assessments of its species. Our study also highlights the power of NGS technology to analyse relictual or probably extinct groups.

Key words: *Chasechloa*, convergent evolution, *Echinolaena*, extinct species, Forest Shade Clade, genome skimming, herbarium, microendemic, myrmecochory, next-generation sequencing, plastome, Poaceae.

INTRODUCTION

Madagascar is home to one of the most diverse, and at the same time one of the most threatened biotas of the world. This island was elected as the hottest hotspot (Myers *et al.*, 2000), and considered to be one of the world's highest biodiversity conservation priorities (Mittermeier *et al.*, 2005). According to Callmander *et al.* (2011), the Malagasy flora is composed of 10 319 native species of angiosperms, of which 8621 (80 %) are endemic. Despite the high levels of diversity and endemism, only approx. 10 % of the natural habitats originally present in the 594 150 km^2 surface area of Madagascar still remain (Goodman and Benstead, 2005). Since the arrival of humans, approx. 4000 years ago (Gommery *et al.*, 2011), many species of animals and plants have seen their populations considerably decline and some have become extinct (e.g. Burney *et al.*, 1997, 2004). Numerous species are only known from sub-fossil or museum specimens collected in the field within the last two centuries. Assessment of this diversity is challenging due to the scarcity of material and its poor quality for molecular analyses, but next-generation

sequencing (NGS) methods have recently revolutionized the analysis of such specimens (e.g. Staats *et al.*, 2013; Bakker *et al.*, 2016). Organelle genomes have been reconstructed from herbarium DNA samples, allowing a reassessment of the taxonomy and biogeography of recently extinct lineages within a phylogenetic framework, for instance in Poaceae, Lamiaceae and Oleaceae (Besnard *et al.*, 2014; Welch *et al.*, 2016; Zedane *et al.*, 2016).

Poaceae is among the ten families with the largest number of endemic species in Madagascar (271 species; Callmander *et al.*, 2011). Its level of endemism (approx. 40 %, Vorontsova *et al.*, 2016) is relatively low compared with approx. 80 % average endemism for other families of angiosperms in Madagascar (Callmander *et al.*, 2011). The grasses are dominant and ecologically important particularly in the grasslands and savannas, which cover up to 65 % of the island (Moat and Smith, 2007). They are usually considered to be weeds and cattle feed, and are frequently overlooked by scientists (Vorontsova and Rakotoarisoa, 2014). Yet, grasses are also diverse in dry forests as well as rainforests (e.g. bamboos and Forest Shade clade; Dransfield, 2000; Vorontsova and Rakotoarisoa, 2014).

The taxonomy and systematics of Poaceae in Madagascar is incomplete and represents a significant gap in the global knowledge of the family (Vorontsova and Rakotoarisoa, 2014). A research programme is currently underway, with recent studies based on systematic and phylogenetic data (e.g. Besnard *et al.*, 2013; Vorontsova, 2013; Vorontsova *et al.,* 2013, 2015, 2016; Vorontsova and Rakotoarisoa, 2014). However, numerous rare taxa remain poorly known, including the likely endemic genus *Chasechloa* A. Camus (Vorontsova and Rakotoarisoa, 2014).

The genus *Chasechloa* includes few species [i.e. *C. egregia* (Mez) A. Camus, *C. humbertiana* A. Camus and *C. madagascariensis* (Baker) A. Camus] (Camus, 1949, 1954) and has been placed in synonymy under *Echinolaena* Desv., a genus particularly diverse in the New World (e.g. Clayton and Renvoize, 1986; Soreng *et al.*, 2015). The South American *Echinolaena* were showed to be polyphyletic and thus the genus was recircumscribed (Silva *et al.*, 2015). A new genus named *Oedochloa* C. Silva & R.P. Oliveira was proposed to accommodate part of its species, and some other species previously placed in *Ichnanthus* P. Beauv., which was also shown to be polyphyletic and was recircumscribed, erecting another new genus named *Hildaea* C. Silva & R.P. Oliveira (Silva *et al.*, 2015). The phylogenetic placement of the Malagasy taxa of *Echinolaena* (hereafter named 'Chasechloa') was not assessed by Silva *et al.* (2015), but the authors examined the morphology of these taxa and stated that *Chasechloa* is possibly not related to the American taxa.

The South American species of *Echinolaena*, *Hildaea*, *Ichnanthus*, *Oedochloa* and some other panicoid genera possess rachilla appendages at the base of the upper floret. These appendages were hypothesized to be elaiosomes following observation of oils in the cells of these structures (Berg, 1985; Davidse, 1987). They represent an evolutionary adaptation to myrmecochorous dispersal of the caryopsis that arose several times independently within the subfamily Panicoideae (Morrone *et al.*, 2012; Silva *et al.*, 2015). The species of *Chasechloa* present similar structures at the base of the upper floret, but to date there is no evidence of oils within them.

In this study, we investigated the phylogenetic placement of *Chasechloa* within Poaceae for the first time. Full chloroplast genome data and some nuclear markers were produced by NGS from old herbarium specimens, while some selected markers were generated from recently collected Malagasy grasses. A scanning electron microscopy (SEM) analysis of the upper floret and cross-sections of the rachilla appendages followed by staining with Sudan IV were performed to examine the morphology of the upper floret and the presence of oils in the appendages. We carried out a taxonomic revision in order to provide more detailed descriptions, means for identification and reference data for the Malagasy taxa.

MATERIALS AND METHODS

Taxon sampling

The samples used in this study were either from old herbarium specimens or gathered during field trips in Madagascar between 2011 and 2014. Leaf fragments were obtained from specimens housed in the herbaria K, MO and P (acronyms according to Thiers, 2016), or from silica-dried specimens. The sampling includes three Malagasy accessions of *Chasechloa* [one of *C. egregia* from Nosy Be, and two of *C. madagascariensis* from the Ankarana (north) and Ankarafantsika (north-west)] and other selected endemic and non-endemic Malagasy taxa, which were added to the plastid and nuclear data sets of Besnard *et al.* (2013) and Silva *et al.* (2015). These data sets include representatives of the different lineages within Poaceae, with a broader sampling on Panicoideae for the *ndhF* gene. Lists of taxa, voucher information and GenBank accession numbers for the sequences used in this study are given in Supplementary Data Table S1.

DNA extraction, amplification and sequencing

Fragments of dried tissue (20–50 mg) were ground using mortars and pestles or in a 2 mL tube containing three tungsten balls with a TissueLyser (Qiagen Inc., TX, USA), and then DNA was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) or the BioSprint 15 DNA Plant Kit (Qiagen Inc.).

First, two markers, the plastid gene *ndhF* and the nuclear ribosomal internal transcribed spacer (ITS), were selected because of their broad use in phylogenetic studies in Poaceae (e.g. Hsiao *et al.*, 1999; Grass Phylogeny Working Group II, 2012), and their previous use in phylogenies of related taxa (Silva *et al.*, 2015). *ndhF* and ITS were amplified and sequenced following the procedures described by Silva *et al.* (2015), and others were obtained from GenBank.

Next-generation sequencing and read assembly

The amplification of *ndhF* was not successful for *C. egregia* [*L.H. Boivin s.n.* (LHB), collected in 1851; P00710482], *C. madagascariensis* [*H.* Perrier de la Bâthie *11217* (HPB11217), collected in 1910; P02351581] and another rare Malagasy panicoid of the dry forest understorey with an uncertain phylogenetic placement: *Brachiaria fragrans* A. Camus [*J.M. Bosser 19160* (JMB19160), collected in 1964; P06769493]. We consequently analysed these three old herbarium accessions with the shotgun sequencing method recently reported by Besnard *et al.* (2013, 2014). DNA concentration was estimated using PicoGreen. A 200 ng aliquot of double-stranded DNA was used for HPB11217 and LHB, while only 27 ng were available for JMB19160. The libraries were constructed using the Illumina TruSeq DNA Sample Prep v.2 kit following the instructions of the supplier, but without prior DNA sonication because the DNA was supposedly moderately too highly degraded. Purified fragments were A-tailed and ligated to sequencing indexed adaptors. Fragments with an insert size of approx. 50–300 bp were gel-extracted and enriched with ten cycles of PCR (with a high-fidelity *Taq* polymerase provided in the Illumina TruSeq Nano DNA LT Sample Prep kit) before library quantification and validation. The three libraries were multiplexed with 21 other libraries (generated in other projects). The pool of libraries was then hybridized to the HiSeq 2000 flow cell using the Illumina TruSeq PE Cluster Kit v.3. Bridge amplification was performed to generate clusters, and paired-end reads of 100 nucleotides were collected on the HiSeq 2000 sequencer using the Illumina TruSeq SBS Kit v.3 (200 cycles).

Duplicate paired-end reads were removed before the assembly of genomic regions in each sample using FastUniq (Xu *et al.*, 2012). Partial or complete chloroplast sequences (cpDNA) and nuclear ribosomal units (nrDNA) were assembled using the approach described by Besnard *et al.* (2013). Annotation of the consensus plastid sequence was done with Geneious v6.0.5 (Kearse *et al.*, 2012). In addition to these multiple-copy genomic regions, three low-copy nuclear genes were investigated as in Besnard *et al.* (2013). Gene regions for which sequences were already available for C_3 and C_4 panicoid grasses were targeted. This included two *ppc* genes encoding phosphoenolpyruvate carboxylase (PEPC) and *phyB* (phytochrome B). The *ppc* fragments cover exons 8–10 of two different paralogues (i.e. *ppc-aL2* and *ppc-aR*). Gene sequences from *Cyrtococcum* Stapf (sub-tribe Boivinellinae, tribe Paniceae, subfamily Panicoideae) that were available in GenBank were used as the references. Illumina reads corresponding to *ppc* and *phyB* were assembled as described in Besnard *et al.* (2013).

Data analyses

The data sets were analysed separately because of the different taxon sampling and sequence length variation among the markers. The new sequences for *ndhF*, ITS, *phyB*, *ppc-aL2* and *ppc-aR* were added to the '*ndhF* extended' and ITS data sets from Silva *et al.* (2015) (plus *Lecomtella madagascariensis* A. Camus), and to the *phyB*, *ppc-aL2* and *ppc-aR* data sets from Besnard *et al.* (2013). The matrices (available upon request) were then aligned using MUSCLE (Edgar, 2004) at the EMBL-EBI website (http://www.ebi.ac.uk/Tools/msa/muscle/), and subsequently manually refined using Geneious v6.0.5 (Kearse *et al.*, 2012).

The *ndhF*, ITS, *phyB*, *ppc-aL2* and *ppc-aR* data sets were analysed through Bayesian inference (BI) using MrBayes v.3.2.3 (Ronquist and Huelsenbeck, 2003) under the best-fitting nucleotide substitution model, GTR + G + I, in the CIPRES Science Gateway v.3.3 (Miller *et al.*, 2010). Two parallel analyses were run for 10 000 000 generations using the Metropolis-coupled Markov Chain Monte Carlo (MCMC) algorithm with four random-initiated chains (Huelsenbeck *et al.*, 2001), sampling trees every 1000 generations. The convergence of the runs was monitored using Tracer v.1.6 (Rambaut and Drummond, 2013). Trees generated before the standard deviation of split frequencies reached a value below 0·01 were discarded as burn-in (*ndhF*, 7500 trees; ITS, 2000 trees; *phyB* and *ppc-aL2*, 2500 trees; and *ppc-aR*, 6000 trees). A majority-rule (50 %) consensus tree was computed on the remaining trees with the posterior probabilities as branch support estimates. We also performed maximum likelihood (ML) analyses under a GTR + G + I model and 100 bootstrap replicates using RAxML v.8 (Stamatakis, 2006) in the CIPRES Science Gateway v.3.3 (Miller *et al.*, 2010).

A dated phylogenetic tree was inferred from the *nhdF* data set using BEAST v1.8.2 (Drummond *et al.*, 2012) in the CIPRES Science Gateway v.3.3 (Miller *et al.*, 2010) under a GTR + G + I substitution model, an uncorrelated lognormal relaxed clock model, a tree prior with a Yule speciation model and a random starting tree. For this analysis, the *ndhF* data set was downsized to Gynerieae and core panicoids (Lecomtelleae,

Andropogoneae, Paniceae, and Paspaleae) to avoid the possibility that under-representation of other subfamilies could affect the outcome of the analysis. Clades were preset according to the results of the ML analysis. Monophyly was enforced only for the core panicoids clade. Four calibration points were used based on the mean ages and standard deviations obtained by Christin *et al.* (2013), one for the crown node of the clade including tribes Gynerieae, Paspaleae, Lecomtelleae, Andropogoneae and Paniceae (normal prior with a mean of 27·6 and s.d. of 4·3), one for the crown node of Andropogoneae (normal prior with a mean of 19·96 and s.d. of 4·5), one for the crown node of Paniceae (normal prior with a mean of 22·41 and s.d. of 4) and one for the crown node of Paspaleae (normal prior with a mean of 21·6 and s.d. of 3·9). Ten independent runs of MCMC chains were run for 15 million generations, sampling trees every 10 000 generations. Convergence of the runs and effective sample size (ESS) values (only ESS >200 were accepted) were assessed using Tracer v.1.6 (Rambaut and Drummond, 2013). The first 1500 trees from each run were discarded as burn-in and tree files were combined using LogCombiner v.1.8.2 (Drummond *et al.*, 2012). Results were summarized in a maximum clade credibility tree using TreeAnnotator v.1.8.2 (Drummond *et al.*, 2012). Mean ages and 95 % confidence intervals of ages are reported on the nodes. The chronogram was edited using FigTree v.1.3.1 (Rambaut, 2009).

Scanning electron microscopy (SEM)

Upper anthecia were gathered for *C. madagascariensis* from the herbarium specimen *Vorontsova et al. 1822* (K), mounted on stubs without pre-treatment and covered with gold. The photomicrographs were taken with the JEOL 6390LV scanning electron microscope. The terminology is in accordance with Shaw and Webster (1983) and the results are compared with data from the literature and our database on upper floret micromorphology (including some unpublished data).

Sudan IV test

The presence of oils in the rachilla appendages was investigated through a histochemical test with Sudan IV (Pearse, 1985). Mature (fertile) florets were gathered for *C. madagascariensis* from the herbarium specimen *Vorontsova et al. 1822* (K), and their appendages were sectioned using a Leica CM1850 cryostat, followed by staining with Sudan IV. Photomicrographs were taken using an Olympus microscope (model BX51).

Taxonomic survey

Based on examination of the specimens housed in the herbaria B, BR, G, K, MA, MO, P, S, TAN, W and WAG, data gathered during the field trip and reassessment of the taxonomy of *Chasechloa*, we provide detailed morphological descriptions, a key to the species, photographs of the living plants, distribution and habitat data, and conservation assessments. All specimens cited were seen by M.S.V.

RESULTS

DNA data sets generated in the present study

Based on NGS data, we assembled a complete chloroplast genome, a nearly complete ribosomal unit and partial gene sequences (*phyB*, *ppc-aR* and *ppc-aL2*) for *Chasechloa egregia*, *C. madagascariensis* and *Brachiaria fragrans*. Gene order on chloroplast genomes was identical to that reported in other panicoid grasses (see EMBL accessions: KX663836–KX663838). The sequencing depth of plastomes and ribosomal units is superior to $140\times$ and $850\times$, respectively, indicative of a high quality sequence (Supplementary Data Table S2). In contrast, the mean sequencing depth of low-copy genes (*phyB*, *ppc-aR* and *ppc-aL2*) was about $3\times$ and thus insufficient for a complete assembly of these regions (Supplementary Data Table S3). New *ndhF* sequences were generated for ten Paniceae accessions, and one additional ITS sequence was obtained for *C. madagascariensis* (Table S1).

Phylogenetic reconstructions and dating analysis

Results of the analyses are shown and discussed based on the *ndhF* marker since it was the most comprehensive sampled marker (Fig. 1; Supplementary Data Fig. S1). Trees resulting from the analyses of the remaining data sets are shown in Supplementary Data Figs S2–S5).

All accessions of *Chasechloa* were recovered in a clade with high support within sub-tribe Boivinellinae, tribe Paniceae (Fig. 1). The *Chasechloa* clade is sister to a clade comprising *Panicum mitopus* K. Schum., *Pseudechinolaena polystachya* (Kunth) Stapf, *Cyrtococcum*, *Brachiaria tsiafajavonensis* A. Camus, *B. comorensis* (Mez) A. Camus, *Acroceras excavatum* (Henrard) Zuloaga & Morrone, *Lasiacis* (Griseb.) Hitchc., *Pseudolasiacis leptolomoides* (A. Camus) A. Camus, *Cyphochlaena madagascariensis* Hack., *Oplismenus* P. Beauv. and *Poecilostachys* Hack. (Fig. 1). This whole group is sister to *Acroceras calcicola* A. Camus (Fig. 1). Analyses of the nuclear ITS and low-copy genes also recovered *Chasechloa* as a monophyletic lineage within Paniceae, consistently associated with other Boivinellinae (Figs S2–S5). *Echinolaena*, *Hildaea*, *Ichnanthus* and *Oedochloa* were also recovered as monophyletic within sub-tribe Paspalinae, tribe Paspaleae (Fig. 1).

The origin of the *Chasechloa* lineage was estimated to have occurred in the transition between the Upper Miocene and the Pliocene, with a mean stem age of 8·9 Mya (with a 95 % confidence interval of 11·7–6·1 Mya) and a mean crown age of 4·8 Mya (7·8–2·1 Mya; Supplementary Data Fig. S6).

Microscopic observations

The upper floret is elliptical, white and somewhat soft when young (Fig. 2A, B). It is slightly obovate, brown, inflated and hardened after full development of the caryopsis, at maturity (Fig. 2E, F). The surface of the lemma and the palea is smooth (Fig. 2A–F) and a little wrinkly when young due to dehydration (Fig. 2A, B). At the base, there is a dorsal germination lid (Fig. 2F, H) and a rachillar stipe subtending the floret, from which the appendages arise (Fig. 2A–H). Both rachilla and appendages also become large and inflated at maturity (Fig. 2G, H). At the apex, there is a dorsal crest and two ventral folds, both covered with stomata (Fig. 2I, J). Staining with Sudan IV demonstrated the presence of oils in the appendages at maturity (Fig. 3A–D).

DISCUSSION

During our recent field work (in north-western and south-western Madagascar in 2015 and 2014, respectively), we were not successful in locating either *Chasechloa egregia* or *Brachiaria fragrans* in areas where they have been previously sampled. Due to the lack of recent collections, the only viable analysis method to evaluate the evolutionary affinities of these taxa was the NGS shotgun approach (so-called 'genome skimming'; Straub *et al*., 2012) from museum specimens. NGS allowed us to retrieve complete plastomes, nrDNA and some selected single-copy genes for these rare Malagasy grass species (Table S1). We in fact generated more data from herbarium samples than from material analysed with PCR and Sanger sequencing, demonstrating the power of NGS to generate genomic data even from poor quality DNA extracts (e.g. Staats *et al*., 2013; Besnard *et al*., 2014; Welch *et al*., 2016; Zedane *et al*., 2016). In particular, we obtained unique data for *C. egregia*, which is probably extinct (see 'Taxonomic Treatment') and has been collected only once in 1851.

Phylogenetic placement of Chasechloa and novelties in Boivinellinae

We place the *Chasechloa* lineage within subfamily Panicoideae, tribe Paniceae, sub-tribe Boivinellinae. This was unexpected since *Chasechloa* was previously placed in synonymy under *Echinolaena*, a member of tribe Paspaleae, sub-tribe Paspalinae, to which *Hildaea*, *Ichnanthus* and *Oedochloa* also belong. Our results corroborate the distinction between these genera suggested by Silva *et al.* (2015) based on morphological attributes and geographical distribution.

As pointed out by Silva *et al.* (2015), *Chasechloa* is similar to the species of *Oedochloa* that were previously placed in *Echinolaena*, but it can be easily distinguished from both *Echinolaena* and *Oedochloa* by the morphology of its inflorescences, which are composed of 1–6 one-sided racemes, with the proximal racemes shorter than the terminal raceme, which is erect and continuous with the main axis. In *Echinolaena*, inflorescences are composed of a single one-sided raceme and main axis ending in a sterile prolongation, whereas in *Oedochloa* they are composed of 3-7 one-sided racemes, with the proximal racemes longer than the others, which become shorter towards the apex of the inflorescence, ending in a single spikelet (Silva *et al*., 2015). The comparison with other genera of Panicoideae with rachilla appendages is addressed in Silva *et al.* (2015).

The sub-tribe Boivinellinae, also informally known as the 'Forest Shade Clade' (Giussani *et al*., 2001), is a poorly known pantropical group, particularly diverse in Madagascar, that until now included 13–15 genera and 113–135 species of largely sprawling or trailing broad-leaved C_3 grasses usually found in shady or damp conditions, also comprising C_3–C_4 intermediates
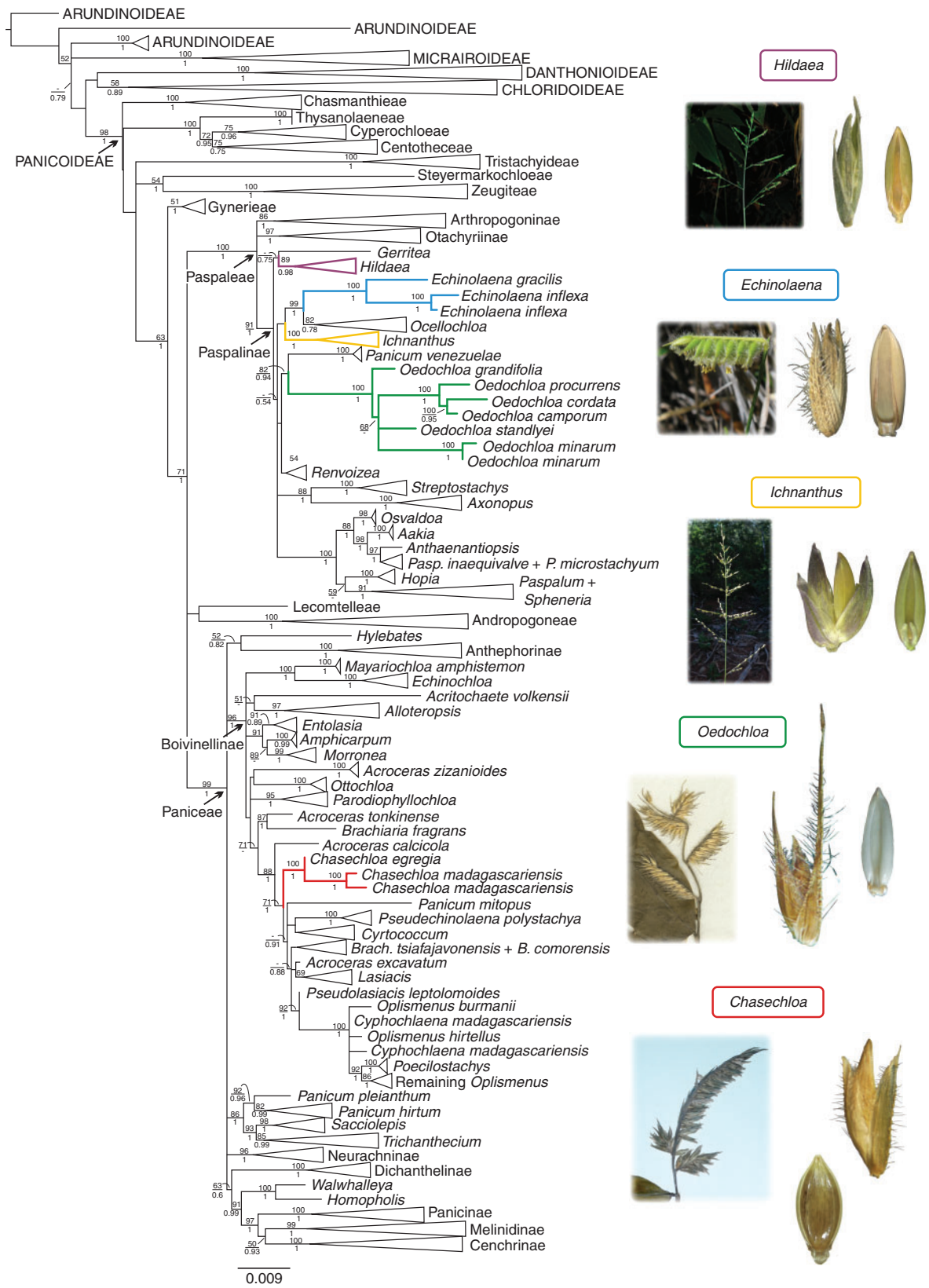
FIG. 1. Maximum likelihood (ML) phylogram from the analysis of the plastid marker *ndhF* showing the phylogenetic placement of *Chasechloa*. ML bootstrap support values are indicated above branches and Bayesian support values below the branches. Some branches were collapsed to reduce the length of the tree. The full tree is available in Fig. S1.
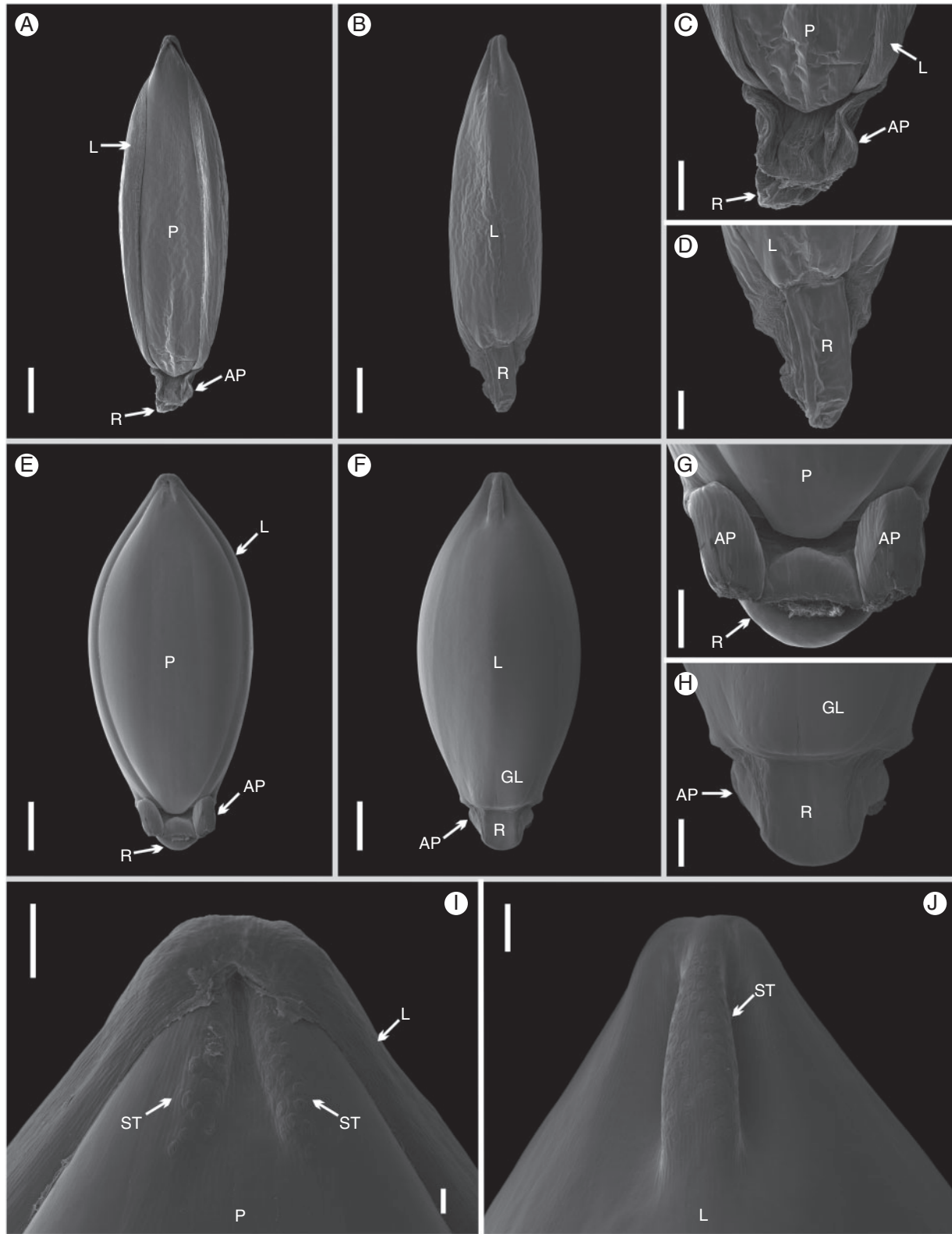
FIG. 2. Scanning electron microscopy (SEM) images of the upper floret of *Chasechloa madagascariensis* (*Vorontsova et al. 1822*). (A–D) Young floret; (A) ventral view; (B) dorsal view; (C) base, ventral view; (D) base, dorsal view. (E–J) Mature floret; (E) ventral view; (F) dorsal view; (G) base, ventral view; (H) base, dorsal view; (I) apex, ventral view; (J) apex, dorsal view. Scale bars = 500 μm (A, B, E, F); 200 μm (C, D, G, H); 100 μm (I, J). AP, appendages; GL, germination lid; L, lemma; P, palea; R, rachilla; ST, stomata.
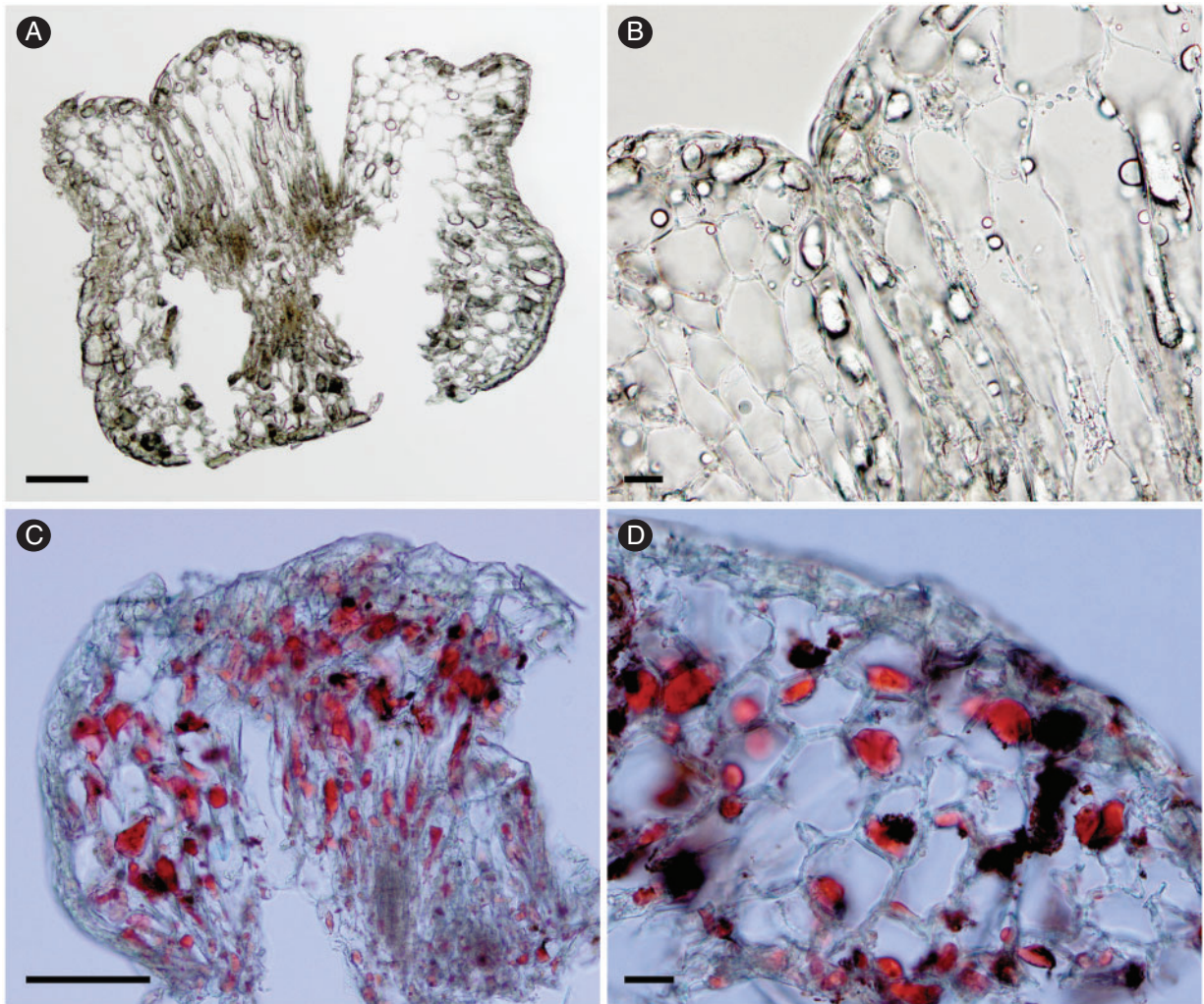
Fɪɢ. 3. Cross-sections of the rachilla appendages at the base of the mature (fertile, i.e. with caryopsis) upper floret of *Chasechloa madagascariensis* (*Vorontsova et al. 1822*). (A and B) without staining; (C and D) staining with Sudan IV. Scale bars = 100 µm (A, C); 20 µm (B, D).

and C₄ NADP-ME taxa (i.e. *Alloteropsis* J. Presl and *Echinochloa* P. Beauv.; Grass Phylogeny Working Group II, 2012; Morrone *et al.*, 2012; Kellogg, 2015; Soreng *et al.*, 2015; Lundgren *et al.*, 2016). *Chasechloa* and *Pseudolasiacis* (A. Camus) A. Camus were analysed here for the first time and placed within the Boivinellinae, as well as previously unplaced members of Malagasy *Brachiaria* (Trin.) Griseb. *sensu lato* (*B. comorensis*, *B. fragrans* and *B. tsiafajavonensis*) and *Panicum* L. *sensu lato* (*Panicum mitopus*), raising the number of genera in the sub-tribe to 19 (including *Microcalamus*, not sampled by us, and excluding *Brachiaria* and *Panicum sensu lato*).

Origin and diversification of Chasechloa, *current distribution and implications for conservation*

Most taxa belonging to the *Chasechloa* sister group (except for the New World *Acroceras excavatum* and *Lasiacis*) are endemic or partly endemic to Madagascar, as well as *Acroceras calcicola*, sister to the whole clade. All species of this lineage

occur in forest shade environments. As Madagascar has been a separate land mass for >80 Mya (Scotese, 2000; Yoder and Novak, 2006), the high endemicity of this clade suggests that *Chasechloa* diversified on the island. The age of *Chasechloa*, dated to the transition between the Miocene and the Pliocene, is temporally congruent with the origin of some angiosperm genera endemic to north and west Madagascar (e.g. *Chadsia* Bojer, *Colvillea* Bojer, *Pongamiopsis* R. Vig., *Humbertioturraea* J.-F. Leroy; Buerki *et al.*, 2013). Its species richness also fits in with patterns typical for Madagascar, where most endemic genera are monotypic or have only two or three species (Callmander *et al.*, 2011).

Our inferred divergence time estimates also indicate that the origin of *Chasechloa* could be linked to environmental changes associated with a worldwide aridification (Zachos *et al.*, 2001), the establishment of the Indian monsoons (e.g. Yoder amd Novak, 2006; Buerki *et al.*, 2013) and/or the uplift of eastern Malagasy mountains (e.g. Vences *et al.*, 2009; Roberts *et al.*, 2012). In particular, *Chasechloa* is present in the Sambirano region (north-western Madagascar), which is characterized by

heavy seasonal rains. Sub-humid forests have expanded in this area during the Late Miocene (e.g. Yoder and Novak, 2006), and a few lineages have diversified in this area during this period (e.g. *Tina* Schult.; Buerki *et al.*, 2011). The genus currently inhabits a restricted area of the island, in north-western wet and seasonally dry forests. It is likely that *C. egregia* is now extinct at its only known location on Nosy Be (see 'Taxonomic Treatment'). Madagascar is largely covered by grasslands and the forests represent around 20 % of the area (Moat and Smith, 2007). These forested areas are severely impacted by human activity (Mittermeier *et al.*, 2005) and global climate change (Ingram and Dawson, 2005), threatening this lineage among many others. Thus, we re-emphasize the importance of increasing the protected area coverage on the island pointed out by Mittermeier *et al.* (2005).

Morphology of the upper floret and convergent evolution for myrmecochory

Overall morphology of the upper floret of *Chasechloa* resembles most Paniceae and Paspaleae genera. Change in shape, colour and consistency after fertilization was also observed for *Ichnanthus* by Silva *et al.* (2013), and we also have seen it in *Echinolaena*, *Hildaea*, *Oedochloa* and other genera within Poaceae, including outside Panicoideae. It is probably related to protection of the seeds and attraction of dispersal agents.

The dorsal crested apex is a feature of unknown function also found in other genera of Boivinellinae: *Acroceras*, *Cyrtococcum*, *Entolasia* Stapf, *Mayariochloa* Salariato, Morrone & Zuloaga, *Microcalamus* Franch., *Oplismenus* (Kellogg, 2015) and *Ottochloa* Dandy (C. Silva, pers. obs.). In *Chasechloa*, the crest surface is fully covered with stomata, but we do not know whether stomata also occur in these other genera. This apex may have been inherited by some genera from the common ancestor of the sub-tribe, or may have originated independently several times. The possibility of multiple independent origins is made more likely by the presence of this character outside Boivinellinae, in *Homopholis* C.E. Hubb., *Rupichloa* Salariato & Morrone and *Urochloa* P. Beauv. (Kellogg, 2015). Further research is required to understand its function and evolution within Paniceae. The structures we describe as ventral folds also have stomata on the surface. We found no reports about similar features or their proposed functions in other genera of Boivinellinae.

The presence of oils in the appendages at the base of the upper floret is here established for *Chasechloa*. Although we have no direct evidence of dispersal by ants, the presence of elaiosomes is a sign of adaptation for myrmecochory (Lengyel *et al.*, 2009). Myrmecochory, i.e. seed dispersal mediated by ants, is a globally important plant–animal mutualism consistently associated with higher rates of diversification in angiosperms, and considered to be one of the best examples of convergent evolution (Lengyel *et al.*, 2009, 2010). This seed dispersal syndrome is estimated to occur in at least 11 000 species of angiosperms and to have independently evolved 101–147 times, mostly from the Eocene when ants became numerically dominant (Dunn *et al.*, 2007; Lengyel *et al.*, 2010).

The studies mentioned above may have underestimated the origins of myrmecochory in grasses because elaiosomes are also known to be present in a series of other genera such as *Chasechloa* (this study), *Echinolaena*, *Hildaea*, *Ichnanthus*, *Oedochloa*, *Yakirra* Lazarides & R.D. Webster, among others (Berg, 1985; Davidse, 1987; Morrone *et al.*, 2012; Silva *et al.*, 2015). In Boivinellinae, only *Chasechloa* possesses elaiosomes in its upper floret. The outer bracts of the spikelets of *Lasiacis* accumulate oils and become shiny and black when they are pollinated and fertilized, but fruit dispersal is bird mediated in this genus (Davidse and Morton, 1973). Myrmecochory and its evolutionary consequences are still poorly known within Poaceae and deserve further attention.

In Madagascar, only one species of ant has been recorded as attracted to seeds: *Aphaenogaster swammerdami* Forel, which disperses the seeds of *Commiphora guillauminii* H. Perrier (Böhning-Gaese *et al.*, 1996). These ants carry the seeds into their colony, remove the arils and discard the seeds undamaged on the refuse (Böhning-Gaese *et al.*, 1996). A similar ant behaviour was observed in Australia for *Yakirra* (Berg, 1985), a grass genus similar to *Chasechloa* in its upper floret with oil-accumulating rachilla appendages. Ants of the genera *Aphaenogaster* Mayr, *Pheidole* Westwood or *Tetramorium* Mayr could be those attracted to the elaiosomes of *Chasechloa* (B. Fisher, pers. comm.); no data are currently available.

Reinstatement of Chasechloa

We accept *Chasechloa* at the generic level, and present a taxonomic revision of this genus endemic to north-western Madagascar. An analysis of the type collections and herbarium specimens indicated that *C. humbertiana* is within the range of morphological variation of *C. madagascariensis*. Thus we place *C. humbertiana* in synonymy under *C. madagascariensis*. The condensed racemose inflorescence units resembling toothbrushes are quite unique within the Boivinellinae. The pollination biology and dispersal of *Chasechloa* are in need of further study.

TAXONOMIC TREATMENT

Chasechloa A. Camus, *Bull. Soc. Bot. France* **95**: 330 (1949).

Type: *C. madagascariensis* A. Camus

Erect, loosely tufted, rhizomatous perennial 0·2–1 m tall. Culms branching near the base or not branching. Leaf blades membranous to chartaceous, ovate to lanceolate or linear–lanceolate. Flowering culms with a terminal one-sided raceme, continuous with the main axis, and 2–5 lateral one-sided racemes. Rachis narrowly winged, finely pubescent or more rarely glabrous, with a tendency to develop a covering of tubercle-based hairs, apex ending in a single spikelet. Spikelets paired, two-flowered, laterally compressed. Glumes keeled, two-thirds to as long as the florets or slightly exceeding the florets, sub-equal with the upper somewhat longer or shorter, with 5–9 partly visible veins. Lower floret male, with a fully developed palea. Lower lemma with 9–11 veins, apically dentate. Upper floret with a basal swollen stipe (rachilla) provided with appendages, and a dorsal crest and two ventral folds at the apex. Upper lemma and upper palea smooth, shiny, white becoming brown at maturity.

Key to species

Culm internodes 1–10 cm long; 1–3 racemes per flowering culm..***Chasechloa madagascariensis***

Culm internodes 10–15 cm long; 4–6 racemes per flowering culm...***Chasechloa egregia***

Chasechloa madagascariensis (Baker) A. Camus, *Bull. Soc. Bot. France* **95**: 331 (1949). *Echinolaena madagascariensis* Baker, *J. Linn. Soc. Bot.* **21**: 452 (1885).

Type: Madagascar, Diego Suarez (Antsiranana), bois sablonneux derrière le village d'Ambanihala, 1846, *Bernier 2278* (holotype K, isotype P02441420); Fig. 4.

Chasechloa humbertiana A. Camus, *Mém. Inst. Sci. Madagascar, Sér. B, Biol. Vég.* **5**: 203 (1954), **synon. nov.**

Type: Madagascar, Antsiranana, plateau calcaire de l'Ankarana du Nord entre Ambilobe et Anivorano, 200–350 m, 4–9 March 1951, *H.M. Humbert and R. Capuron 25536* (Lectotype selected here, P02441421; isolectotypes, B100162303, BR0000009428614, G00007816, G00074080, K000979165, MA729055, MO2160599, P02441422, P02441423, P02441424, TAN, WAG0102738).

Rhizomatous perennial 20–50 cm tall, the rhizomes short, knotty, woody, 1·5–2·5 mm in diameter. Culms wiry, 0·4–1·8 mm in diameter, sometimes rooting at the lower nodes, the nodes finely pubescent, sometimes glabrescent or villous, the internodes 1–10 cm long, finely pubescent, sometimes glabrescent or villous. Leaf sheaths glabrous to pubescent or villous, usually with cilia along the margin. Ligule a truncate ciliate membrane 0·2–0·5 mm long. Leaf blades membranous to chartaceous, ovate to lanceolate, 5–15 × 0·8–4 cm, yellow-green to green or red-brown, discolorous or concolorous, with cross-veins visible when dry, basally unequal and usually clasping the culm, apically acute to acuminate, glabrous to pubescent or villous, the margin often ciliate with tubercle-based hairs at the base. Racemes 1–5 cm long, 1–3 per flowering culm, with 0·2–1(–3) cm between the racemes, the rachis 0·6–1·2 mm wide, finely pubescent, often with tubercle-based hairs along the margins. Spikelets 5–13 mm long, green to white at the apex or purple when fresh, yellowish green to yellow-brown when dry, glabrous to pubescent or villous with tubercle-based hairs. Glumes 4–13 mm long, two-thirds to as long as the florets or slightly exceeding the florets, acute to caudate. Lower lemma 6–12 mm long. Upper lemma 4–7 mm long.

Distribution: Northern and north-western Madagascar (Fig. 5).

Habitat: Understorey of seasonally dry deciduous forest on limestone or sand, 30–350 m elevation.

IUCN conservation assessment: We assess this species as Vulnerable (VU) as the extent of occurrence is estimated to be < 20 000 km^2, the species is known from fewer than ten locations and the populations have been observed to be in decline (IUCN, 2012). A field study carried out in February 2015 searched for *Chasechloa* in several locations outside protected areas where it has been previously recorded, noted reduction in forest canopy, and failed to locate *Chasechloa*. The only location where a successful collection was made was the Réserve Spéciale d'Ankarana which is under threat from sapphire mining in spite of its successful management programme (Cardiff and Befourouack, 2003).

Specimens examined: Madagascar. Antsiranana: Ambondromifehy, Réserve Spéciale d'Ankarana, Mahoro, sentier botanique partie sud, forêt sur calcaire, 12°52'19"S, 49°13'52"E, 300 m, 23 January 2003, *M. Bardot-Vaucoulon, O. Andrianantoanina, A. Toly and J. Manesy 1281* (K, MO5966882, P00455583, TAN); Anivorano du Nord, PK 88, May 1970, *J.M. Bosser 20230* (P02351590); collines et plateaux calcaires de l'Ankarana du nord, Ouest de la grande route, Mt Ambatopiraka, 24 January 1959, *H.M. Humbert and M.G. Cours 32670* (P02329354); Ankarana National Park, path to Ambohimalaza viewpoint, 1 km from the road leading to 'Campement des Princes', 28 February 2015, *M.S. Vorontsova, F. Randriatsara and J. Razanatsoa 1822* (K, MO, P, TAN); Ankarana du Nord, PK 103, May 1970, *J.M. Bosser 20216* (P02351586); Daraina, forêt de Bekaraoka, partie sud, ca. 1 km au Nord Ouest, à 250 m à l'E du point coté 329, 15 March 2003, *L. Gautier, S. Wohlhauser and L. Nusbaumer 4387* (G00007173, MO5966423); Montagne d'Andavkafanihy, calcaires de l'Ankarana, KM 105 de la route d'Ambilobe, 3 February 1960, *M.G. Cours and H.M. Humbert 5600* (P02329355); dans un bois derrière le village d'Ambanihala à Diego Suarez, *Bernier 48* (P02351584, P02351585, P02351587, P02351588); Orangea, 16 April 1970, *J.M. Bosser 20199* (MO4844119, P02351577); commune rurale de Daraina, forêt de Bobankora, partie sud, 2 February 2005, *L. Nusbaumer and P. Ranirison 1452* (G00019257, MO5966420, P06770648). Mahajanga: Ankarafantsika près de Marovoay, June 1910, *H. Perrier de la Bâthie 11217* (P02351581); Belambo, près de Maevatanana, April 1922, *H. Perrier de la Bâthie 14649* (P02351580); Haut Bemarivo, Boina, February 1907, *H. Perrier de la Bâthie 11305* (P02351578); bois, Morataiha, Rive gauche de la Betsiboka, March 1899, *H. Perrier de la Bâthie 909 bis* (P02351579); environs de Majunga, March 1926, *H. Perrier de la Bâthie 17623* (P02351583); près de Majunga, February 1926, *H. Perrier de la Bâthie 17624* (P02351582). Province unknown: *J. Goudot s.n.* (P02351576).

Notes: This species encompasses a range of variation in indumentum, lower glume length, and shape of the lower glume apex; variabilities in these characters do not correlate with each other or with geographical location. The number of racemes per flowering culm, the length of racemes, the length of the spikelets, and the size of the leaves appear to vary with the local environmental conditions. This species was observed and collected in the Réserve Spéciale de l'Ankarana in February 2015.

The lectotype for *Chasechloa humbertiana* was selected from the original material annotated by Aimée Camus at P and constitutes the best quality flowering material.

Chasechloa egregia (Mez) A. Camus, *Bull. Soc. Bot. France* **95**: 331 (1949). *Panicum egregium* Mez, *Bot. Jahrb. Syst.* **56**: 5 (1921). *Echinolaena boiviniana* A. Camus, *Bull. Soc. Bot. France* **75**: 912 (1928 publ. 1929), **nom. illeg. superfl.**

Type: Madagascar. Antsiranana, Nosy Be, crête de Loucoubé, près de la vigie, March 1851, *L.H. Boivin s.n.* (holotype P00710482, isotypes B100394106, G00007815, G00386796, K000979166, P00710482, P00710483, S11-6499, W0027576, W0027577).

FIG. 4. *Chasechloa madagascariensis* in the Réserve Spéciale de l'Ankarana, Madagascar, *Vorontsova et al. 1822*. (A) Habitat in the understorey of semi-deciduous forest. (B) Perennial shrubby habit. (C) Flowering habit.
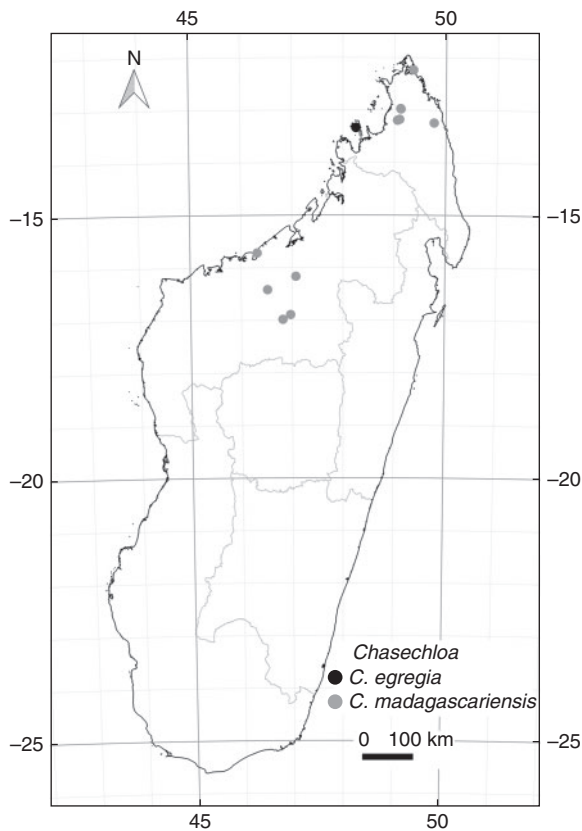
F_{IG}. 5. Geographical distribution of the two species of *Chasechloa* in Madagascar (see records in 'Taxonomic Treatment').

A likely rhizomatous perennial 1 m tall; the rhizome morphology is not known. Culms wiry, 2–3 mm in diameter, sometimes rooting at the lower nodes, the nodes finely pubescent, the internodes 10–15 cm long, glabrous. Leaf sheaths glabrous or with some tubercle-based hairs, often with long cilia along the margin. Ligule a truncate membrane approx. 0·5 mm long. Leaf blades chartaceous, linear–lanceolate to lanceolate, 11–18 × 1–1·5 cm, green, concolorous, with cross-veins visible when dry, basally equal and never clasping the culm, apically acuminate, glabrous to pubescent, the margin often ciliate with tubercle-based hairs at the base. Racemes 1–8 cm long, 4–6 per flowering culm, 0·5–3 cm between the racemes, the rachis approx. 1 mm wide, finely pubescent. Spikelets 5–6 mm long, yellow-brown when dry, glabrous to finely pubescent, often with some tubercle-based hairs. Glumes 3·5–5 mm long, two-thirds to three-quarters, to as long as the florets, apiculate. Lower lemma 5–6 mm long. Upper lemma approx. 3·5 mm long.

Distribution: Known only from the highest point of the Réserve Naturelle Intégrale de Lokobe, Nosy Be, Madagascar (Fig. 5).

Habitat: Under wet forest canopy at 300 m elevation.

IUCN conservation assessment: The only collection of this species was made in 1851. *Chasechloa egregia* is assessed here as probably 'Extinct in the Wild' [EW] following IUCN categories and criteria (IUCN, 2012). The island has been subject to extensive development and environmental degradation. An expedition by M. S. Vorontsova and colleagues to Nosy Be in February 2015 visited the Réserve Naturelle Intégrale de Lokobe, the locality cited in the isotype herbarium sheet P00710483. The search did not reveal any populations of *Chasechloa*. Clear conclusions may not be drawn at this stage but we would like to highlight its extreme rarity and the recent lack of records for this species.

Specimens examined: Known from the type collection only.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: voucher information and GenBank accession numbers for the sequences used in this study. Table S2: sequence characteristics of nuclear ribosomal DNA units and complete chloroplast genomes generated in the present study by NGS. Table S3: number of paired-end reads, size of the assembled fragments and mean sequencing depth for the three low-copy genes analysed in this study. Figure S1: full maximum likelihood phylogram from the analysis of the plastid marker *ndhF* showing the phylogenetic placement of *Chasechloa*. Figure S2: maximum likelihood phylogram from the analysis of the nuclear marker ITS showing the phylogenetic placement of *Chasechloa*. Figure S3: maximum likelihood phylogram from the analysis of the nuclear marker *phyB* showing the phylogenetic placement of *Chasechloa*. Figure S4: maximum likelihood phylogram from the analysis of the nuclear marker *ppc-aL2* showing the phylogenetic placement of *Chasechloa*. Figure S5: maximum likelihood phylogram from the analysis of the nuclear marker *ppc-aR* showing the phylogenetic placement of *Chasechloa*. Figure S6: maximum clade credibility chronogram of the core Panicoideae resulting from the BEAST analysis based on the *ndhF* data set.

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