

## BARCODING PLANTS

# DNA barcoding discriminates a new cryptic grass species revealed in an ethnobotany study by the hill tribes of the Western Ghats in southern India

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## Abstract

Our research brought together traditional aboriginal knowledge (TK) and scientific knowledge (SK) to explore the relationship between scientific and aboriginal systems of botanical classification and the corresponding valorization(s) of biological diversity in the Western Ghats of southern India. We worked with two aboriginal cultures namely 'Irulas' and 'Malasars' of the Nilgiri Biosphere Reserve with an objective of evaluating the ability of different knowledge systems (SK and TK) to distinguish grass species belonging to the genus *Tripogon*, and assess the ability of DNA barcoding to discriminate a new cryptic species '*Tripogon cope*' as deciphered by the hill tribes. We discovered that the aboriginal informants identified a common ethnotaxa 'Sunai pul', which is a cryptic species of grass not recognized by the SK classification. 'sunai pul' is very important to both aboriginal cultures with ritualistic and economic utility. Morphometric analysis confirms the cryptic nature of this new species, which was validated using DNA barcoding. DNA barcode regions matK and trnH-psbA showed distinct sequence variations among the closely related ethnotaxa. Given the cryptic nature of ethnotaxa, we propose that a DNA barcode may be a reliable tool to identify ethnotaxa. We have initiated further studies in other cultures to develop theoretically sophisticated insights concerning the encounter between 'local' and 'scientific' approaches to the use of biodiversity knowledge. Furthermore, the research will add to a unifying global effort to speed up the documentation and understanding of the planet's natural diversity, while simultaneously respecting the cultural heterogeneity as a vital component of biological diversity.

**Keywords:** aboriginal classification, anthropology, barcoding, biodiversity, ethnobotany, floristic, grass, molecular taxonomy, new species, plants, traditional knowledge

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## Introduction

By using the concept of 'assemblage' (Watson-Verran & Turnbull 1995) – a coming together of different ways of knowing and valorizing species variation – a novel approach seeks to add value to both aboriginal knowledge and DNA barcoding to understanding diversity as they work together

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to potentially create new knowledge. Exploring the ways in which varied knowledge practices are worked together as 'useful knowledge' (Strathern 2005) will show how such inquiries contribute to the common aim of the protection of cultural and biological diversity (Biber-Klemm & Cottier 2006). This interdisciplinary approach will share increasing urgent global imperatives to conserve both cultural and biological diversity as urged by the Convention of Biological Diversity (CBD 1992), UNESCO's 'Man and Biosphere Programme' and the Declaration on the Rights of Indigenous People (2007).

There is a unifying global effort to speed up the documentation and understanding of the planet's natural diversity and the scientific underpinnings of different biological classification systems (Ellen *et al.* 2000; Sillitoe *et al.* 2002). This includes many studies that have documented aboriginal classification systems for plants and animals (Berlin 1973; Ellen 1993; Atran 1999; Brown 2000). In fact, our understanding of ethnobiological classification has evolved to consider multiple mechanisms of classification (Newmaster *et al.* 2006, 2007) that goes beyond morphology and includes sensory perception, ecology and utilitarian characters taxonomy (Begossi *et al.* 2005; Ellen 2006; Mourão *et al.* 2006; Lampman 2007; Souza & Begossi 2007; Ragupathy *et al.* 2008a). These ethnoclassification systems are very complicated and require a great deal of time to fully comprehend, reconstruct and utilize.

DNA barcoding may provide a quick tool for identifying cryptic ethnotaxa. DNA barcoding is a method of species identification and recognition using specific regions of DNA sequence data (Hebert *et al.* 2003). Barcoding of animals is well documented and can be reviewed online via the Canadian Barcode of Life ([www.bolnet.ca](http://www.bolnet.ca)) and the Consortium for the Barcode of Life (CBOL; [www.barcoding.si.edu](http://www.barcoding.si.edu)). Although the difficulties of plant barcoding have been debated (Chase *et al.* 2005; Kress *et al.* 2005; Cowan *et al.* 2006; Pennisi 2007), detailed research (Newmaster *et al.* 2006, 2008b; Kress & Erickson 2007; Fazekas *et al.* 2008; Lahaye *et al.* 2008) has demonstrated the utility of barcoding as a powerful tool for plant identification. Given the complex nature aboriginal classification systems, we propose that DNA barcoding may be a quick and reliable identification tool for ethnotaxa.

A recent study in the Western Ghats of southern India was designed to utilize both scientific (SK) and traditional knowledge (TK). This study revealed considerable plant diversity (1715 species of angiosperms including 439 endemics) during a series of botanical expeditions within the Nilgiri Biosphere Reserve (NBR). TK from the local hill tribes ('Irula' and 'Malasar') revealed cryptic diversity undetected by SK methodology, which resulted in the discovery of several new species (Newmaster *et al.* 2008a) of plants that have medicinal utility (Ragupathy *et al.* 2008b). The objective of this research study is to evaluate the ability of different knowledge systems (SK and TK) to distinguish grass species belonging to the genus *Tripogon*, and assess the ability of DNA barcoding to discriminate a new cryptic species '*Tripogon cope*' revealed by the Hill Tribes in our biodiversity survey of the NBR in Western Ghats of southern India. Specifically we: (i) evaluated the ability of field taxonomists and Hill Tribe informants to identify species in the genus *Tripogon* and associate TK with each of the ethnotaxa, (ii) explored morphological variability in the genus *Tripogon* in order to expose cryptic taxa, and (iii) assessed the efficacy of DNA barcoding to discriminate a new cryptic species

'*Tripogon cope*' from other morphologically similar species of *Tripogon*.

## Materials and methods

### Study site

The study area (6°40' to 7°10'E, 10°55' to 11°10'N) is located within the Velliangiri Hills, which forms a major range in the Western Ghats and part of the Nilgiri Biosphere Reserve (NBR). The mountain range of the study area consists of seven hills with different aspects, microtopography and altitudes of 520–1840 m. The Palghat District of Kerala forms the western boundary of the Velliangiri hills, with the plain of Coimbatore district to the east, the Nilgiri Mountains to the north, and the Siuvani hills forming the southern boundary (Fig. 1). The annual temperature of the reserve ranges from 0 °C during winter to 41 °C during summer, with rainfall ranging from 500–7000 mm. Seasonal rivers such as the Neelivaikal, Mayar and Andisunai pass through the hill landscape.

### Ethnobotanical exploration

Ethnobotanical explorations with the 'Malasars and Irulas' were made in the study area from April 2003 to January 2007 that included all seasons. Our research team included five aboriginal 'informants' (traditional botanical experts) from the local villages in the NBR and five botanists from the Biodiversity Institute of Ontario (BIO) OAC Herbarium and Kongunad Arts and Science Botanical Institute in India. The survey used Bernard's (1994) methodology to identify local experts in traditional botanical knowledge. Our data were collected in two steps: (i) botanical inventories were completed on five hillsides (each separated by > 2 km) by the aboriginal informants and the botanists. TK was gathered for each voucher sample. Vouchers were collected and labelled for all taxa identified, and (ii) the collected voucher samples were the subject of interviews within the three villages in order to gather additional TK. The data were gathered in a series of structured, semi-structured and unstructured interviews, and participatory approach regarding plant uses, identification and nomenclature. The interview protocols, data confirmation were based on reports of Stepp & Thomas (2005), Vogl *et al.* (2004), Bernard (1994), Etkin (1993) and Pelto & Pelto (1990). To elucidate cultural domains and determine differences in knowledge or taxonomy among aboriginals, a cross-check was made with other aboriginal respondents by using various research protocols such as free recall lists, pile sorts, and consensus analysis (Werner & Fenton 1973; Weller & Romney 1988). Plant samples were collected from the aboriginal community and preserved for both herbaria and DNA barcode analysis. Leaf, stem and flower parts collected *in situ* were fixed in silica gel, FAA

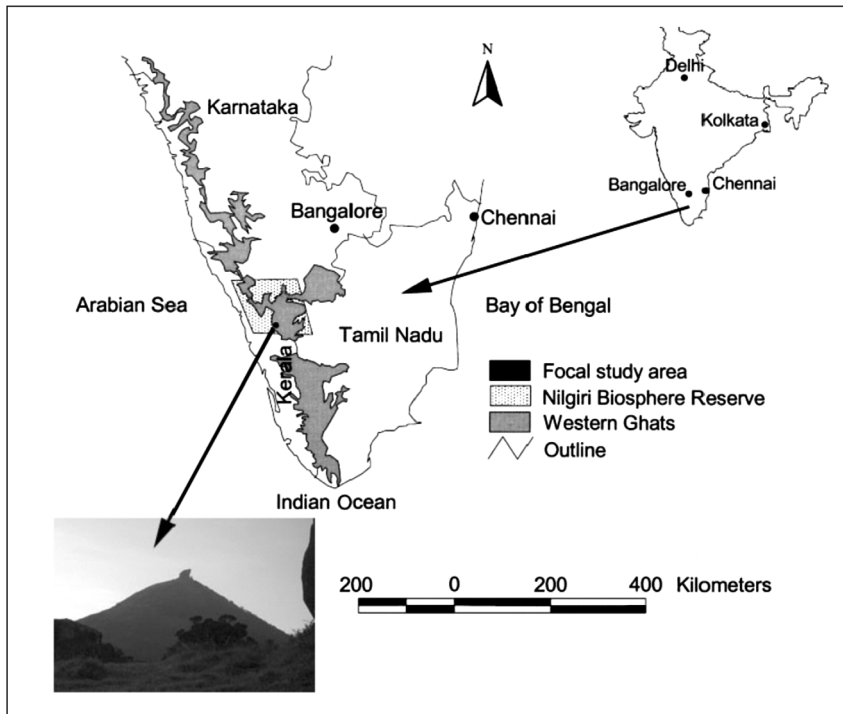


Fig. 1 Location of study site in the Velliangri Hill (arrow, lower left) located on the Nilgiri Biosphere Reserve, Western Ghats, India (Map modified from Newmaster *et al.* 2008).

(50% ethanol, 5% acetic acid, 10% formalin, 35% water) and stored in 70% ethanol for morphological study *ex situ*. These samples were used for measuring the variation in morphological characters and molecular markers. Herbarium voucher specimens were deposited in the herbarium of Kongunadu Arts and Science College, Coimbatore (KASCH). Isotypes of new taxa were deposited at Madras Herbarium (MH), Southern Circle, Botanical Survey of India, Coimbatore and Ontario Agricultural College (OAC) Herbarium at the Biodiversity Institute of Ontario, University of Guelph, Ontario.

#### Identification analysis

Calculation of a Consensus Factor (FIC), and pile sorting relative frequency (RF) was used to test homogeneity of knowledge (SK and TK) in identifying specimens, revealing cryptic taxa or limitations of the classification without the use of molecular data. Voucher samples collected from five collection sites were systematically identified by the taxonomists and aboriginal informants. The relative frequency (RF) of each specimen from the interviews were calculated to determine a quantitative value for choosing a plant name (latin binomial or aboriginal ethnotaxon) from the pool of collected vouchers and placing it in a species concept (Newmaster *et al.* 2007). RF is the simple calculation of the percentage of specimens associated with a taxon when taxonomists or aboriginal informants are presented with a pool of vouchers and asked to perform 'pile sorting' (Weller & Romney 1988). Trotter & Logan (1986) provide the calcu-

lation of a Consensus Factor [ $Fic = N_{ur} - N_t / (N_{ur} - 1)$ ], which is adopted to evaluate the degree of partition into categories (Heinrich 2000). We have adopted this to include 'aboriginal utility' by the aboriginal informants (Heinrich 2000; Ragupathy *et al.* 2008a), where  $N_{ur}$  is the number of use-reports of informants for particular category (TK plant use) factor, where a use-report is a single record for use of a plant mentioned by an individual, and  $N_t$  refers to the number of species used for that particular category for all informants (Ali-Shtayeh *et al.* 2000; Camejo-Rodrigues *et al.* 2003).

#### Morphometric classification analysis

Thirty-six morphological variables were recorded from the 40 *Tripogon* specimens used in the multivariate phenetic analysis. A matrix of 40 specimens and 36 morphological characters were used in a multivariate analysis. Canonical ordination was used to detect groups of specimens and to estimate the contribution of each variable to the ordination. A principal component analysis (PCA) (ter Braak 1998) was used to identify the length of the ordination axis. Unimodal, indirect ordination detrended correspondence analysis (DCA) was used to explore variation in species scores. A cluster analysis was used to classify the specimens, as it is better in representing distances among similar specimens, whereas DCA is a handy tool in representing distances among groups of specimens (Sneath & Sokal 1973). Cluster analysis was performed with NTSYS (Rohlf 2000). A distance matrix was generated using an arithmetic average (UPGMA) clustering algorithm and standardized data based on average

taxonomic distance subjected to the unweighted pair-group method. A discriminant function analysis (DFA; SPSS 1999) was used to rigorously test the classification of specimens provided in the cluster analysis. The object of DFA is to predict multivariate responses that best discriminate subjects among different groups (Ramsey & Schafer 1997). A total of 36 morphological characters for each of the 40 specimens were used as input for a DFA. The 40 specimens used as input for a DFA were each coded as belonging to one group (designated as *a priori* groups), which: (i) determined if the classification was accurate, (ii) provided distinct functions for the classification of the taxa and, (iii) indicated if there are important morphological characters for each of the canonical discriminate functions.

### DNA barcoding

Three DNA regions (*rbcL*, *matK* and *trnL-F*) were selected based on the previous plant barcoding studies (Newmaster *et al.* 2006, 2008b; Kress & Erickson 2007; Fazekas *et al.* 2008; Lahaye *et al.* 2008). We isolated total genomic DNA from approximately 10 mg of dried leaf material from each sample using the kit, NucleoSpin 96 Plant II (Macherey-Nagel). Extracted DNA was stored in sterile microcentrifuge tubes at  $-20^{\circ}\text{C}$ . The selected loci were amplified by polymerase chain reaction (PCR) (see primers in Table 1) on a PTC-100 thermocycler (Bio-Rad). DNA was amplified in 20- $\mu\text{L}$  reaction mixtures containing 1 U AmpliTaq Gold Polymerase with GeneAmp 106 PCR buffer II (100 mM Tris-HCl pH 8.3, 500 mM KCl) and 2.5 mM  $\text{MgCl}_2$  (Applied Biosystems), 0.2 mM dNTPs, 0.1 mM of each primer (0.5 mM for *matK*), and 20 ng template DNA. Amplified products were sequenced in both directions with the primers used for amplification, following the protocols of the University of Guelph Genomics facility ([www.uoguelph.ca/~genomics](http://www.uoguelph.ca/~genomics)). Products from each specimen were cleaned using Sephadex columns and run on an ABI 3730 sequencer (Applied Biosystems). Bidirectional sequence reads were obtained for all the PCR products. Sequences were assembled using Sequencher 4.5 (Gene Codes Corp), and aligned manually using Bioedit version 7.0.9 (37). The sequences were submitted to BOLD and GenBank.

## Results

### Identification analysis

Although the ability of field taxonomists and Hill Tribe informants to identify species in the genus *Tripogon* was high, the respective classifications of SK and TK are not homogeneous. Taxonomists identified seven taxa from the 40 specimens with 96% (RF) accuracy among individuals. Aboriginal informants identified eight taxa from the same 40 specimens with 98% RF among the informants. A com-

**Table 1** PCR primers used for amplification of plastid DNA sequences in this study

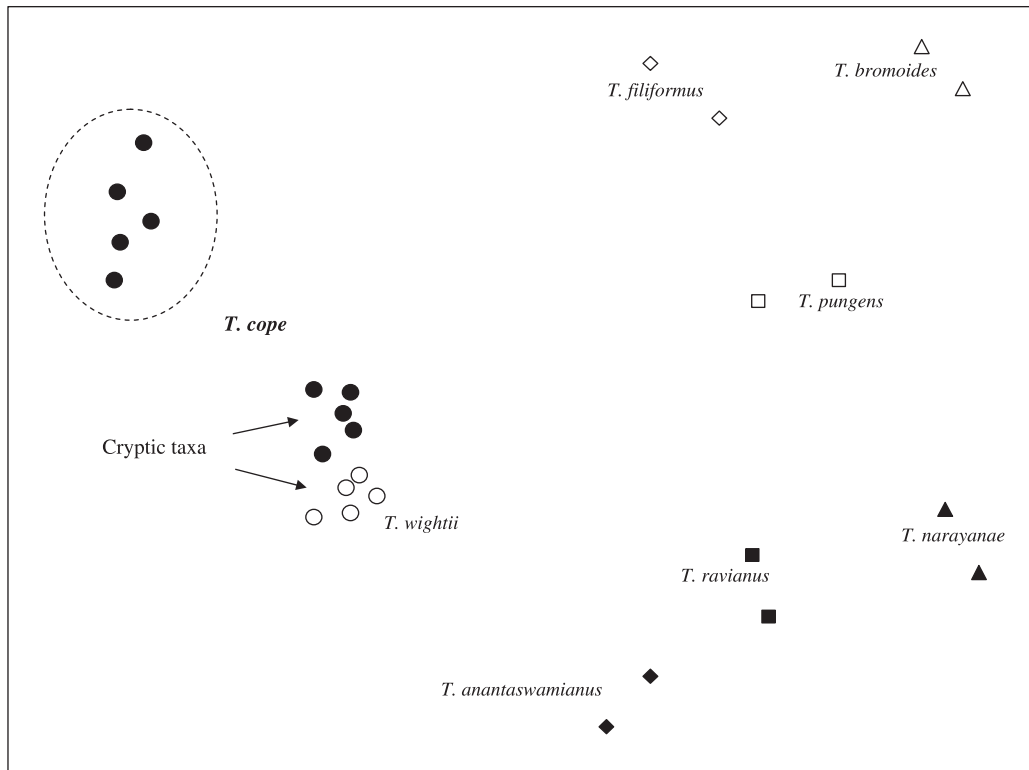
Plastid locus	Primer name	Sequences
<i>matK</i>	<i>matK</i> X F	TAATTTACGATCAATTCATTCTC
	<i>matK</i> 5r	GTTCTAGCACAAAGAAAGTCG
	3F_KIM	CGTACAGTACTTTTGTGTTTACGAG
	1R_KIM	ACCCAGTCCATCTGGAAATCTTGTTTC
<i>trnH-psbA</i>	<i>trnH-F</i>	CGCGCATGGTGGATTACAAATCC
	<i>psbA-R</i>	GTTATGCATGAACGTAATGCTC
<i>rbcL</i>	<i>rbcL-aF</i> ajf 634R	ATGTCACCACAACAGAGACTAAAGC-GAAACGGTCTCTCCAACGCAT

parison of the vouchers revealed that both classification systems grouped 87.5% of the samples into seven taxa. One of the groups in the SK classification contained 10 vouchers. These 10 vouchers were split into two distinct ethnotaxa; 'Sunai pul' and 'Kattai pul'.

Surveys of traditional knowledge revealed various utility among the eight ethnotaxa. There was no partition of Fic among the 'Malasars and Irulas'. High consensus factors (0.95–0.99) revealed that seven of the ethnotaxa are commonly used for a variety of purpose: snake hunting, fodder for domesticated animals and thatching. One of the ethnotaxa 'Sunai pul' is a unique grass which is very important to both cultures with ritualistic and economic utility. 'Sunai pul' was not distinguished by the SK classification with vouchers lumped within the taxonomy of *Tripogon wightii*, which was labelled as 'Kattai pul' within the TK classification.

### Morphometric classification analysis

A discriminant function analysis (DFA) used 36 morphological quantitative characters to classify heterogeneity in 40 specimens into seven known taxa of *Tripogon* (*T. anantawamianus* Sreeekumar *et al.* *T. bromoides* Roth, *T. filiformis* Nees ex Steud., *T. narayanae* Sreeekumar, Nair & Nair, *T. pungens* C. E. C. Fischer, *T. ravianus* Sunil & Pradeep, *T. wightii* Hook.f.). The canonical correlation from the discriminant functions is the ratio of sums of squares to the total sums of squares between groups. Thus, the first discriminative function is responsible for 68% of the differences between groups (variability in the discriminative scores). The second function is responsible for an additional 12% of the variance between groups and the third function is responsible for an additional 9% of the variance. Wilk's Lambda was used to test the hypothesis that there are no differences in variance ( $P < 0.001$ ) between the groups of taxa which represent different species (SPSS 1999). There were significant differences ( $P < 0.001$ ) for first two canonical functions. One hundred per cent of the groups (representing eight species) were correctly classified using the DFA into eight distinct groups of taxa including the new species of *Tripogon cope* as



**Fig. 2** Scatter plot of the first two axes from a detrended correspondence analysis (DCA) for 35 quantitative variables (morphological taxonomic characters) of 22 specimens (classification of 8 *Tripogon* species within the NBR). A second scatter plot from a DCA analysis that included the variable 'life cycle' is overlaid changing placement of the ethnosppecies *Tripogon cope* (black dots) within the dotted circle.

documented by Newmaster and colleagues (Newmaster *et al.* 2008a).

The ordination analyses utilized DCA in the separation of eight species from the 40 specimens that were analysed and provided a measure of the important morphological variables in the classification. DCA was used to classify the 40 specimens into distinct groups representing seven species. High eigenvalues for the X-axis (0.912) and the Y-axis (0.687) indicated that the gradient axes were of considerable length and justified the use of DCA. The X-axis (axis 1) is strongly correlated with several floral characters; these include the length of the raceme, spikelets, glumes and fertile lemmas. The Y-axis (axis 2) is strongly correlated with several other characters; these include position and number of awns on the lemma, shape of lemma, leaf blade length and venation. The DCA ordination of the first two canonical functions identifies seven distinct species and a cryptic cluster of 10 specimens that represent *Tripogon cope* in the proximity of the respective allied species, *Tripogon wightii* (Fig. 2). In fact, they are only separated by a cryptic floral character i.e. a slight variation in the awns at the apex of the lemma. If a new variable 'duration of life cycle' is added to DCA analysis, the resulting ordination indicates clear separation among the five samples of *Tripogon cope* (annual) from the five samples of *Tripogon wightii* (perennial).

**Table 2** Summary statistics for coding and noncoding DNA (*rbcL*, *matK* and *trnH-psbA*) ethnotypes examined from all 10 populations *Tripogon* (intraspecific *p*-distance was 0.00 for all regions)

Parameters	<i>rbcL</i>	<i>matK</i>	<i>trnH-psbA</i>
Size (in bp)	600	800	615
No. of variable sites ( <i>S</i> )	0	2	1
Mean interspecific <i>p</i> -distance	0	0.003	0.002

#### DNA barcoding

DNA barcoding discriminates the cryptic species *Tripogon cope* ('Sunai pul') from the morphologically similar species of *Tripogon wightii* ('Kattai pul'). Amplifications were highly specific with a clear background in the agarose gel. Although there were no differences in the *rbcL* sequences for these two cryptic species, the *matK* and *trnH-psbA* sequences were consistently different (Table 2). Several segregating sites in the *matK* and *trnH-psbA* sequences are found consistently among the five distant populations. If these regions are considered in a two-gene approach, there is a gross interspecific variation (*p*-distance 0.00234) and no intraspecific variation. Interspecific variation among all eight species

ranged from ( $p$ -distance 0.002–0.003). Intraspecific  $p$ -distance was 0.00 for all regions within all eight species.

## Discussion

The genus *Tripogon* Roem. & Schult. is comprised of nearly 40 species in tropical and subtropical regions (Watson & Dallwitz 1992; Peterson *et al.* 1997; Clayton *et al.* 2006). Significant taxonomic contributions to the understanding of *Tripogon* are presented in the catalogue of world grasses by Peterson *et al.* (2001), a revision of African species of *Tripogon* (Phillips & Launert 1971; Phillips 1974), new species of *Tripogon* from Africa (Cope 1992), a summary of grass genera worldwide (Watson & Dallwitz 1992), an online world grass flora by Clayton *et al.* (2006), and nomenclature changes by Veldkamp (1996). Rúgolo de Agrasar & Vega (2004) reported that Asia constitutes the centre of diversity for this genus, with 23 species of which 16 species are native to China (Clayton *et al.* 2006) and 21 species including eight endemics are native to India (Newmaster *et al.* 2008a). Most of what has been published comes from the Indian flora and includes three new species of *Tripogon* (Naik & Patunkar 1973; Sree-kumar *et al.* 1983; Sunil & Pradeep 2001; Newmaster *et al.* 2008a). Most Indian species of *Tripogon* are halophytic to xerophytic, glycophytic and occur in open habitats. Of the 21 species in India, only eight occur in our study area (NBR). All Indian *Tripogon* have oblong spikelets, unkeeled fertile lemmas, two lateral apical lemmatal teeth, and a terminal lemmatal awn. The close resemblance of *T. cope* to *T. wightii* has resulted in misidentifications by taxonomists during field study. These cryptic species are only differentiated by minor floral characters; slight variation (1 mm) in the rachilla internodes and the number (1–3) of awns at the lemma apex. However, these species are clearly discriminated by different life cycles (annual vs. perennial), DNA barcodes (*matK* and *trnH-psbA*) and local aboriginal classification systems. We are currently working on a systematic study of the *Tripogon* of India-Asia of which our preliminary results support the results in this study.

Although a taxonomist could not differentiate cryptic *Tripogon* species, they were differentiated by aboriginal informants. This was consistent both in the field and when specimens were displayed within local villages. This is not the first account where TK classification systems recognize more taxa than the SK classification system. In a review of aboriginal biological classification systems (Newmaster *et al.* 2006c, 2007), we found a handful of studies where aboriginal informants recorded more taxa in a particular area than taxonomists challenging the SK classification system (Conklin 1954; Balakrishnan *et al.* 2003; Rengalakshmi 2005). None of these studies allowed a direct comparison within an experimental design. We designed such an experiment with the Irulas in a remote area of Tamil Nadu and discovered that their TK classification system is more robust than

the SK classification system used to survey a diverse group of plants in the exactly the same area (Newmaster *et al.* 2007). The TK classification system is based on a multi-mechanistic model that includes broader categories than the SK classification of the same specimens. Although there are differences in SK and TK classifications, we found high congruity in the two classifications when focusing on a group of grasses. Further research is needed to explore biodiversity using both SK and TK simultaneously. This study used the concept of 'assemblage' of biodiversity knowledge, which includes a coming together of different ways of knowing and valorizing species variation in a novel approach seeking to add value to both TK and SK. The benefits to society-at-large include a greater understanding of the utility of particular 'taxa' after variation among taxa has been explored.

DNA barcoding may provide an important tool for identifying cryptic species and validating ethnotaxa. One of the greatest utilities of barcoding is its use in overcoming taxonomic impediments; identifying cryptic materials such as unknown leaves, roots, etc. Barcoding was used in the study of nutmeg (Newmaster *et al.* 2008a) to identify species in the Myristicaceae that are primarily separated by androecium characters in small, short-lived flowers. This study identified several cryptic taxa including population level differences in *C. sprucei* associated with ecotypic differences and in *C. mexicana* associated with vicariance, suggesting several new cryptic species. DNA barcoding is a tool for ethnobiology and TK in validating ethnotaxa, helping in overcoming hurdles of ambiguity, to gain credibility in science, stimulating new theory on understanding, preserving and valuing biological and cultural diversity. Given the cryptic nature of ethnotaxa, we propose that DNA barcoding may be a quick and reliable tool to identify these taxa. We have initiated further studies in other cultures to develop theoretically sophisticated insights concerning the encounter between 'local' and 'scientific' approaches to biodiversity knowledge. These will further contribute to a rich body of scholarship on the social, cultural and political underpinnings of classification systems more widely. Furthermore, the research will add to a unifying global effort to speed up the documentation (via DNA barcoding) and understanding of the planet's natural diversity, while simultaneously respecting cultural heterogeneity as a vital component of biological diversity itself. This is aligned with the Convention on Biological Diversity (CBD 1992) that was signed by over 150 nations, and thus the world's complex array of human–natural–technological relationships has effectively been re-organized.

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### Conflict of interest statement

The authors have no conflict of interest to declare and note that the funders of this research had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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