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DNA Barcoding for the Identification of *Phyllanthus* Taxa Used Medicinally in Brazil

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ABSTRACT

Plants of the genus *Phyllanthus*, principally *Phyllanthus amarus*, *Phyllanthus urinaria*, *Phyllanthus niruri*, and *Phyllanthus tenellus*, are used in Brazilian folk medicine to treat kidney stones as well as other ailments, where the latter two species are listed in the Brazilian Pharmacopeia as *quebra-pedra* (*stone-breaker*). However, only *P. niruri* has been shown to be effective in a clinical setting. Nuclear ribosomal internal transcribed spacer (ITS1–5.8S rRNA-ITS2), internal transcribed spacer 2, and chloroplasts *rbc*L, *mat*K, *psb*A-*trn*H, *trn*L, and *trn*L-*trn*F were screened for their potential as DNA barcodes for the identification of 48 *Phyllanthus* taxa in Brazilian medicinal plant germplasm banks and in "living pharmacies". The markers were also tested for their ability to validate four commercial herbal teas labelled as *quebra-pedra*. Using the criterion of high clade posterior probability in Bayesian phylogenetic analysis, the internal transcribed spacer, internal transcribed spacer 2, and chloroplast *mat*K, *psb*A-*trn*H, *trn*L, and *trn*L-*trn*F markers all reliably differentiated the four *Phyllanthus* species, with the internal transcribed spacer and *mat*K possessing the additional advantage that the genus is well represented for these markers in the Genbank database. However, in the case of *rbc*L, posterior probability for some clades was low and while *P. amarus* and *P. tenellus* formed monophyletic groups, *P. niruri* and *P. urinaria* accessions could not be reliably distinguished with this marker. Packaged dried *quebra-pedra* herb from three Brazilian commercial suppliers comprised *P. tenellus*, but one sample was also found to be mixed with alfalfa (*Medicago sativa*). An herb marketed as *quebra-pedra* from a fourth supplier was found to be composed of a mixture of *Desmodium barbatum* and *P. niruri*.

Introduction

Brazil has implemented a regulatory framework for the production, distribution, and use of medicinal plants and herbs, with the purpose of guaranteeing and promoting safety, efficacy, and quality of verifiable raw materials [1]. To attain expected efficacy, herbal products require correct botanical identification and standardization. The Brazilian Ministry of Health therefore prepared a list of recommended medicinal plants in 2009 [2], with the aim of controlling the preparation of phytotherapeutics to be made available for use by the public [3].

The search for medications for the prevention and treatment of diseases of the urinary tract is of great interest to public health. Urolithiasis is a pathology that affects approximately 10% of the Brazilian population [4, 5], where masses of crystals form in the renal papilla, that can fragment and interfere with urinary flow, causing intense pain [6]. In Brazil, tea prepared from plants commonly referred to as *stone-breaker* or *quebra-pedra* is frequently used for the treatment of renal calculus [4], where these plant

names are usually associated with species of *Phyllanthus* (Phyllanthaceae). Although the Brazilian Pharmacopoeia only recommends *Phyllanthus niruri* in cases of urolithiasis, several species of *Phyllanthus* are also used, particularly *Phyllanthus amarus, Phyllanthus tenellus*, and *Phyllanthus urinaria* [7, 8], and these four species are included in the list of medicinal plants recommended by the Brazilian Ministry of Health [2].

Phyllanthus is the largest genus of the Phyllanthaceae and includes approximately 550 to 750 species [9], of which 88 are found in Brazil and 54 are endemic to the country [10]. *Phyllanthus* species are found in all Brazilian vegetation types, but are especially common in rupestrian areas, Cerrado and Caatinga [9, 11]. *P. niruri* is the most well-known and studied species of the genus and is used in herbal medicine as fresh or dry leaves, aerial parts, or as whole plants [12, 13]. *P. niruri* has been shown to possess lytic and preventive effects on the formation of urinary calculi and promotes ureteral relaxation and glomerular filtration, suggesting potential use in hyperuricemic patients and patients with renal insufficiency [13]. Extracts of fresh and dried *P. tenellus* plants have antiviral and antimicrobial activity [14]. Callus culture extracts of this herb have been shown to possess analgesic properties against neurogenic and inflammatory pain in mice [15]. The toxicity of *Phyllanthus* species is currently uncertain. Aqueous extracts of *P. niruri* [16] and *P. amarus* [17] have been reported as being nontoxic in animal models, while *P. tenellus* extracts, although nontoxic, induced agitation in mice, with spasms and increased respiratory frequency as well as signs of depression, such as lethargy, prostration, and dyspnea [18].

There are many brands of *quebra-pedra* teas and capsules on the Brazilian market that are available from pharmacies, herbalists, and via the internet. However, *quebra-pedra* herb is commonly purchased from local markets, without any quality control or certification of the species [19]. Although *P. amarus, P. tenellus, P. urinaria,* and *P. niruri* are taxonomically well delimited [9, 11], they may be difficult for the nonspecialist to identify and distinguish from related *Phyllanthus* species. They are also often treated together as *quebra-pedra*, leading to inconsistencies in efficacy. In addition, it can become difficult to recognize the species used in a formulation using morphological features alone, especially after processing. Adulteration of medicinal plant preparations is also common, which can have serious consequences for the user [20]. For these reasons, a fast and reliable methodology for the evaluation of herbal medications is desirable.

The objective of the current work was to provide tools to improve discrimination of *quebra-pedra* (*Phyllanthus* taxa) accessions obtained from Brazilian phytotherapy programs and germplasm banks, as well as material collected from the wild. In order to achieve this objective, we evaluated the potential of DNA barcoding technology to differentiate the main medicinal *Phyllanthus* species found in Brazil. We also used the same markers to validate four commercial packaged *quebra-pedra* herb samples obtained from the market.

Results and Discussion

Without resorting to special optimization procedures, nested PCR, amplification of shorter subfragments, or cloning, the proportion of successful PCRs from the vouchered specimens varied from 88% for *rbc*L and *trn*L to 65% for *psb*A-*trn*H (▶ **Table 1**). In the case of the commercial *quebra-pedra* herb samples, PCR success varied from 78% for *trn*L to 0% for the *psb*A-*trn*H spacer region (▶ **Table 2**). We observed no correlation between PCR success and amplicon size, where the smallest, *psb*A-*trn*H, failed most often (▶ **Tables 1** and **2**). Poor and unpredictable DNA integrity was expected from the commercial herb samples, whose treatment prior to and during drying and packaging were unknown to us. Apart from DNA quality issues, PCR success rates may be influenced by stochastic factors, such as primer-template mismatch or inhibition of strand extension, factors which could be manipulated by PCR optimization or use of alternative primers. Sequencing success rates varied from 55% for *mat*K and *psb*A-*trn*H to 83% for *trn*L (▶ **Table 1**), likely related to factors influencing sequence trace quality, such as amplicon length, purity, concentration, base composition, and secondary structure.

One of our goals was to evaluate the performance of single gene regions to reliably separate four *Phyllanthus* species commonly referred to as *quebra-pedra* in Brazil. Using the criterion of high clade posterior probability in Bayesian phylogenetic analysis, the internal transcribed spacer (ITS), ITS2, and chloroplast *mat*K, *psb*A-*trn*H, *trn*L, and *trn*L-*trn*F markers all reliably differentiated *P. amarus, P. tenellus, P. niruri*, and *P. urinaria.* However, for *rbc*L, posterior probability for some clades was low and while *P. amarus* and *P. tenellus* formed monophyletic groups, *P. niruri* and *P. urinaria* accessions could not be reliably distinguished (▶ **Figs. 1** and **2**). Indeed, *rbc*L was accepted as part of the core plant DNA barcode only when used in combination with more discriminatory *mat*K [21]. The *mat*K and ITS markers possess a considerable advantage in that they have already been used to generate a comprehensive molecular phylogeny of Tribe Phyllantheae [22], with sequences from vouchered specimens made available in public databases such as GenBank. Although the genus *Phyllanthus* has been shown to be paraphyletic [22], new ITS or *mat*K sequences can be placed in this phylogenetic framework, facilitating the unambiguous confirmation of the identity of undocumented *quebra-pedra* samples. However, although *P. amarus, P. niruri, P. tenellus*, and *P. urinaria* were all well separated from each other in the ITS and *mat*K trees, only the ITS marker distinguished *P. tenellus* from the closely related *Phyllanthus nummularifolius* (▶ **Fig. 2**). The ITS marker may therefore be considered to be a suitable single DNA barcode for these species. Several large studies have demonstrated the high discriminatory performance of the ITS marker as a plant DNA barcode [21, 23].

The ITS2 region has also been proposed as a supplementary DNA barcode for plants, where the shorter fragment is easier to amplify and sequence than the full ITS region [24]. We therefore ran a Bayesian analysis on just the ITS2 portion of the ITS matrix, where *P. amarus, P. niruri, P. tenellus*, and *P. urinaria* could still be unambiguously distinguished from each other and from related species (▶ **Fig. 3**). While the posterior support for *P. amarus* fell below the level of significance, ITS2 could still effectively be used

▶ **Table 1** *Phyllanthus* spp. samples analyzed by DNA barcoding with Genbank accession numbers of successfully sequenced PCR products.

Species/ Voucher[1]	Collection site/ Institution	Source[2]	ITS	*mat*K	*psb*A-*trn*H	*rbc*L	*trn*L	*trn*L-F	Confirmed species
P. amarus Schumach.									
CPMA 1753	CPMA	GB	MH373406	MH379475	MH379500		MH379594	MH379562	*P. amarus*
CPMA 1754	CPMA	GB	MH373405	MH379478		MH379545			*P. amarus*
CPMA 1755	CPMA	GB	MH373407	MH379483	MH379504			MH379565	*P. amarus*
CPMA 1756	CPMA	GB	MH373412		MH379505		MH379603	MH379558	*P. amarus*
CPMA 1757	CPMA	GB	MH373408		MH379503		MH379605	MH379563	*P. amarus*
CPMA 1760	CPMA	GB	MH373409		MH379501		MH379596		*P. amarus*
CPMA 1761	CPMA	GB	MH373411	MH379480	MH379502		MH379604	MH379567	*P. amarus*
CPMA 1764	CPMA	GB	MH373400	MH379482	MH379508	MH379543	MH379601	MH379564	*P. amarus*
CPMA 1765	CPMA	GB	MH373401	MH379477	MH379507	MH379544		MH379556	*P. amarus*
CPMA 1766	CPMA	GB	MH373413	MH379476	MH379506		MH379598	MH379557	*P. amarus*
DBS 164	CPMA	GB		MH379495		MH379555	MH379631		*P. niruri*
DBS 112	João Pessoa, PB	W	MH373402				MH379600	MH379566	*P. amarus*
RBN 44	EPAGRI	GB	MH373410	MH379479	MH379517		MH379602		*P. amarus*
RFV 2537	Cruz das Almas, BA	W		MH379481		MH379547	MH379597	MH379561	*P. amarus*
RFV 2538	Cruz das Almas, BA	W	MH373423		MH379513	MH379539	MH379608	MH379591	*P. tenellus*
RFV 2610	Fortaleza, CE	W	MH373403	MH379474			MH379599	MH379560	*P. amarus*
P. carolinensis Walter									
DBS 308	Cenargen	GB	MH373429				MH379627	MH379569	*P. carolinensis*
P. niruri L.									
DBS 165	CPMA	GB		MH379497	MH379522	MH379551		MH379568	*P. niruri*
M&G 198	Dourados, MS	W	MH373426						*P. niruri*
RBN 46	EPAGRI	GB	MH373428	MH379494	MH379524	MH379554	MH379632	MH379571	*P. niruri*
RFV 2443	Mateus Leme, MG	W				MH379546	MH379595	MH379559	*P. amarus*
RFV 2445	Belo Horizonte, MG	W				MH379553	MH379628		*P. niruri*
Phyllanthus sp.									
DBS 83	Araxá, MG	PP	MH373427	MH379498	MH379523	MH379552	MH379630		*P. niruri*
DBS 84	Araxá, MG	PP				MH379550	MH379629		*P. niruri*
P. stipulatus (Raf.) G. L.Webster									
RBN 45	EPAGRI	GB	MH373431			MH379548			*P. stipulatus*
P. tenellus Roxb.									
DBS 100	Campos Altos, MG	W	MH373414	MH379484	MH379510	MH379525	MH379607	MH379575	*P. tenellus*
DBS 117	Brasilia, DF	W	MH373415		MH379519		MH379611	MH379577	*P. tenellus*
DBS 170	Brasilia, DF	W	MH373416	MH379485	MH379521	MH379526	MH379619	MH379572	*P. tenellus*
DBS 209	Brasilia, DF	PP	MH373417	MH379486	MH379520	MH379527	MH379625	MH379578	*P. tenellus*
DBS 284	Brasilia, DF	W	MH373418	MH379487		MH379528	MH379606	MH379574	*P. tenellus*
DBS 306	Campos Altos, MG	W	MH373419	MH379488	MH379514		MH379623	MH379573	*P. tenellus*
DBS 307	Cenargen	GB	MH373424		MH379512	MH379529	MH379613	MH379570	*P. tenellus*
RBN 36	Porteirinha, MG	PP			MH379515	MH379530	MH379616	MH379589	*P. tenellus*
RBN 39	Montes Claros, MG	W		MH379489		MH379531	MH379610	MH379579	*P. tenellus*
RBN 47	EPAGRI	GB	MH373420	MH379491	MH379509	MH379532	MH379614	MH379590	*P. tenellus*
RFV 2393	Brasilia, DF	W				MH379542		MH379576	*P. tenellus*
RFV 2430	Goiânia, GO	W	MH373421	MH379492		MH379533	MH379622	MH379581	*P. tenellus*

continued

▶ **Table 1** *Continued*

Species/ Voucher[1]	Collection site/ Institution	Source[2]	ITS	*mat*K	*psb*A-*trn*H	*rbc*L	*trn*L	*trn*L-F	Confirmed species
RFV 2441	Mateus Leme, MG	W			MH379516		MH379620		*P. tenellus*
RFV 2444	Belo Horizonte, MG	W			MH379518	MH379534	MH379609	MH379582	*P. tenellus*
RFV 2461	Rio de Janeiro, RJ	PP			MH379511	MH379535	MH379624	MH379583	*P. tenellus*
RFV 2497	Vitória, ES	W				MH379536	MH379612	MH379584	*P. tenellus*
RFV 2500	Vila Pavão, ES	PP				MH379541	MH379621	MH379585	*P. tenellus*
RFV 2503	Venda Nova do Imigrante, ES	PP					MH379617	MH379586	*P. tenellus*
RFV 2506	Cristalina, GO	PP	MH373422	MH379490		MH379537	MH379618	MH379587	*P. tenellus*
RFV 2535	Ilhéus, BA	W	MH373404	MH379493		MH379538	MH379615	MH379588	*P. tenellus*
P. urinaria L.									
RFV 2536	Ilhéus, BA	W	MH373425	MH379473				MH379592	*P. urinaria*
IAN 189183	Belem, PA	W	MH373430	MH379496	MH379499	MH379549	MH379626	MH379593	*P. urinaria*
PCR success			83%	85%	65%	88%	88%	83%	
Sequencing success			68%	55%	55%	64%	83%	79%	

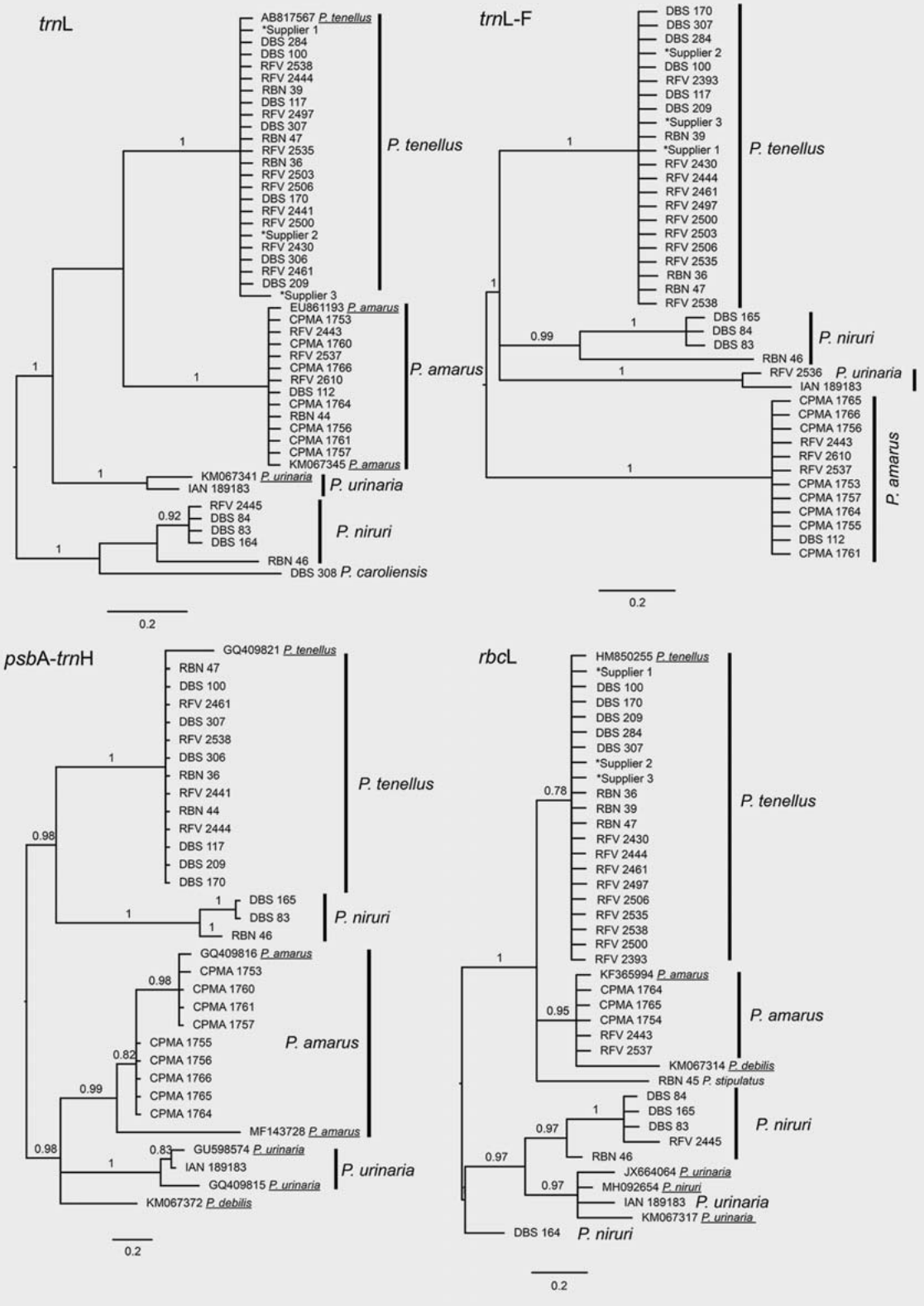
[1] Collection/sample number acronyms: DBS – Dijalma Barbosa da Silva; RBN – Rosa de Belém das Neves; RFV – Roberto Fontes Vieira; CPMA – Coleção de Plantas Medicinais e Aromáticas, Campinas State University-Unicamp; EPAGRI – Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina, Itajaí, SC; IAN – Herbário Embrapa Amazônia Oriental; M&G – Martins and Goçalves. [2] Source codes: GB – Germplasm Bank; PP – Phytotherapy program (Brazilian "Farmácias vivas" project); W – Wild collected samples.

▶ **Table 2** DNA barcoding of commercial *quebra-pedra* teas.

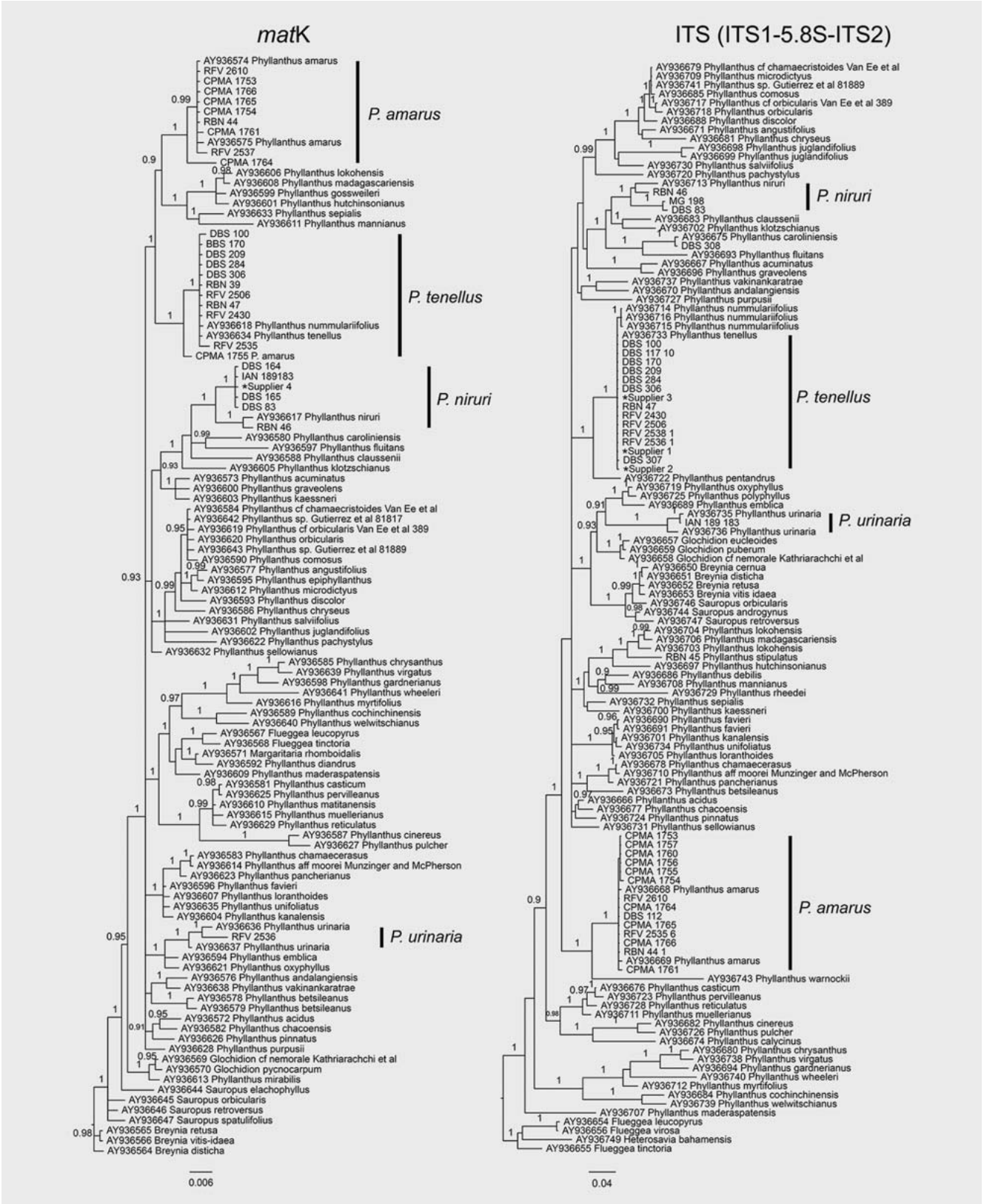
Supplier	Botanical name given	DNA Barcode						Identification (BLAST top hit)
		ITS	matK	psbA-trnH	rbcL	trnL (CD)	trnL-F (EF)	
1		Yes	–	–	Yes	Yes	Yes	*P. tenellus*
2	*P. niruri*	Yes	–	–	Yes	Yes	Yes	*P. tenellus*
2	*P. niruri*	Yes	–	–	–	–	Yes	*M. sativa* (alfalfa)
3		Yes	–	–	Yes	Yes	Yes	*P. tenellus*
4		–	–	–	–	Yes	–	*Desmodium barbatum*
4		–	Yes	–	–	Yes	–	*D. barbatum*
4		–	Yes	–	–	Yes	–	*P. niruri*
4		–	Yes	–	–	–	–	*D. barbatum*
4		–	Yes	–	–	Yes	–	*D. barbatum*
Approx amplicon size		750 bp	750 bp	350 bp	550 bp	600 bp	450 bp	
PCR Success		44%	44%	0%	33%	78%	44%	

as a DNA barcode for *quebra-pedra* samples, particularly in lower quality DNA preparations, such as those expected from commercial herb samples.

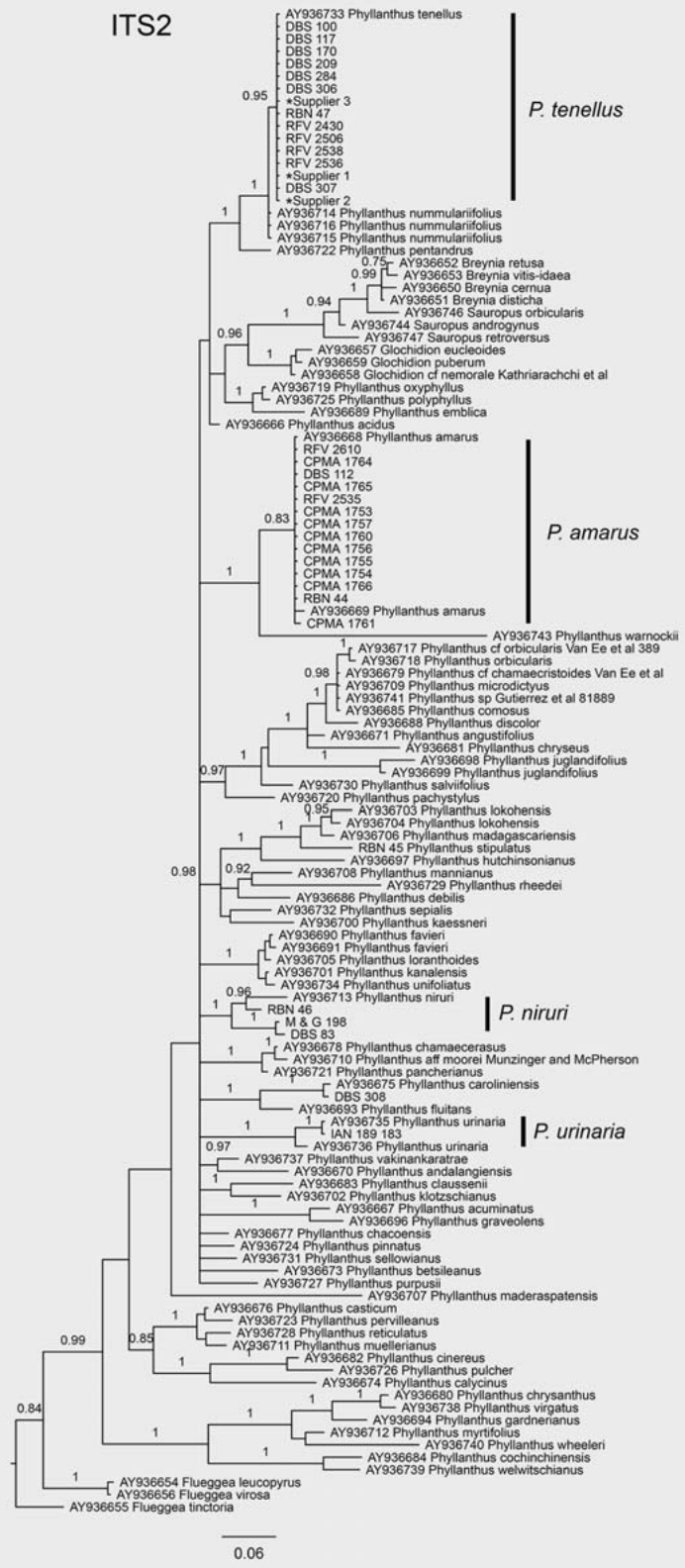
In our examination of packaged commercial *quebra-pedra* herbs from four different Brazilian suppliers, samples from suppliers one and three were found to contain only *P. tenellus,* while the product from supplier two contained not only *P. tenellus*, but also *Medicago sativa* (alfalfa), which was confirmed by perfect BLAST scores [25]. Four out of five samples from supplier four returned top BLAST hits as *Desmodium* sp. (Fabaceae), a plant genus containing several species of common weeds, botanically unrelated to, and easily distinguishable from, *Phyllanthus* spp. A fifth repeat sample from supplier four was identified as *P. niruri*. However, in the Brazilian city of Porto Alegre, *Desmodium incanum,*



▶ **Fig. 1** Midpoint rooted Bayesian consensus trees for *trn*L, *trn*L-F, *psb*A-*trn*H, and *rbc*L markers. Reference sequences obtained from Genbank, where available, are indicated by underlined species names, and sequences derived from commercial *quebra-pedra* herb samples are indicated with an asterisk. The scale bars represent the number of expected substitutions per site, and numbers above branches represent posterior probabilities.



▶ **Fig. 2** Bayesian consensus trees for *mat*K and ITS (ITS1–5.8S-ITS2) markers. The scale bars represent the number of expected substitutions per site, and numbers above branches represent posterior probabilities > 0.9. Genbank accession numbers (Prefix AY******) precede species names for previously published reference sequences [19], and sequences derived from commercial *quebra-pedra* herb samples are indicated with an asterisk. Groups clustering with references *P. tenellus*, *P. amarus*, *P. niuri,* and *P. urinaria* are indicated. The matK tree was rooted using the *Breynia disticha* sequence and the ITS tree was rooted using the *Flueggea tinctoria* sequence.



▶ **Fig. 3** Bayesian consensus tree for ITS2. The scale bars represent the number of expected substitutions per site, and numbers above branches represent posterior probabilities > 0.8. Genbank accession numbers (Prefix AY******) precede species names for previously published reference sequences [19], and sequences derived from commercial *quebra-pedra* herb samples are indicated with an asterisk. Groups clustering with references *P. tenellus, P. amarus, P. niuri*, and *P. urinaria* are indicated. The tree was rooted using the *Flueggea tinctoria* sequence.

Euphorbia prostrata, Euphorbia serpens (Euphorbiaceae), *Cunila microcephala* (Lamiaceae), and *Heimia salicifolia* (Lythraceae) have all been reported to be known and sold as *quebra-pedras*, along with *P. niruri* and *P. tenellus* [26]. In Brazilian folk medicine, all of these species are considered remedies for a diverse range of conditions, including renal problems and colic [26]. However, among these, only the *Phyllanthus* species were included in the 2009 list of recommended medicinal plants by the Brazilian Ministry of Health [2].

Although more scientific evidence regarding the medicinal efficacy of *P. niruri* exists, several *Phyllanthus* species are also popularly known as *quebra-pedra* in Brazil. As well as this nomenclatural confusion due to the common name being broadly used, *P. niruri, P. tenellus*, and *P. amarus* are morphologically very similar, particularly in the vegetative state, exacerbating problems in their identification [27]. In the case of supplier two, the packaging declares the contents to be *P. niruri*. However, as well as including alfalfa as either filler or contaminant, the *Phyllanthus* component was identified as *P. tenellus*, which was the species also used by suppliers one and three. These other suppliers do not give the scientific name of the contents in the herb packaging, referring instead only to *quebra-pedra*. It is therefore unclear whether three out of four of the sampled commercial suppliers are using *P. tenellus* in good faith as *quebra-pedra* or are using misidentified plant material. *P. tenellus* is commonly used in the treatment of liver disorders [28] and in Brazilian folk medicine, it is used in the treatment of renal calculi, diabetes, hepatitis, and asthma [18]. Only one or the four commercial teas was found to contain *P. niruri*, but the herb was also mixed with unrelated plant material that may have unexpected consequences for the consumer.

These worrisome results are unfortunately not unusual in the literature. In a study using a combination of *rbc*L and ITS2 as DNA barcodes to validate 44 herbal products representing 12 companies and 30 different species of herbs in the North American market, 59% of tested products contained DNA barcodes from plant species not listed on the labels [20]. Almost half of the products were authenticated, but one-third of these also contained contaminants and/or fillers not listed on the label. Substitution occurred in 30 out of 44 of the products and only 2 in 12 companies had products without any substitution, contamination, or fillers. Some of the contaminants were found to pose serious health risks to consumers [20]. In a survey of 257 dried leaf, flower, and root samples from 8 distinct species approved by the World Health Organization for the production of medicinal herbs and sold in Brazilian markets, levels of substitution as high as 71% were found, with only 42% of the samples belonging to the correct genus [29]. Using qualitative and quantitative chemical analyses, this study also identified cases in which the correct species was sold, but the expected chemical compounds were absent. In a study of *Phyllanthus* using the *psbA-trnH* marker, 76% of the market samples from southern India contained *P. amarus* as the predominant species and lacked admixtures. The remaining 24% of shops sold five different species of *Phyllanthus*, namely, *Phyllanthus debilis, Phyllanthus fraternus, P. urinaria, Phyllanthus maderaspatensis*, and *Phyllanthus kozhikodianus* [28].

Adulteration and taxonomic confusion may be exacerbated when the biological material used in herbal remedies possesses few easily recognizable morphological features, such as isolated roots and barks or finely fragmented leaves and flowers. In a study of medicinal roots sold by herbalists in Marrakech, Morocco, 18% of the samples were misnamed. Much of these discrepancies, however, have been explained by the lack of one-to-one correspondence between the vernacular names of plants and biological species [30, 31]. For example, a survey of medicinal herbs from markets and shops in Iran found that 27% of samples belonged to different genera than those expected [32]. A survey of Brazilian bitter barks, sold as antimalarial quinas, identified species belonging to six different families, many of which are endangered or without use in traditional medicine, indicating an almost complete lack of standardization or efficacy of the sold materials [33].

In the informal market, supplies of *quebra-pedra* are often limited, since the plants are usually sourced from the wild. In Brazil, *Phyllanthus* species are often ruderal, growing on waste ground and roadside verges, where the use of wild-collected plants can lead to the obvious risk of using the wrong species. In our survey, two samples were found to have been misidentified among wild-collected material (▶ **Table 1**). Among samples collected from local phytotherapy programs (PP) (▶ **Table 1**), six were confirmed by DNA barcoding as *P. tenellus*, in agreement with the morphological identification, and another as *P. niruri*. Two further PP samples recorded only as *Phyllanthus* sp. were also confirmed as *P. niruri*. Among the germplasm bank samples, DNA barcode identifications agreed with the expected *Phyllanthus* species names in all cases except voucher DBS 164, which was listed as *P. amarus*, but confirmed as *P. niruri*.

We are not aware of any large-scale commercial cultivation of *Phyllanthus* spp. to supply the herbal medicine industry in Brazil. Rather, the material is sourced from unregulated and informally cultivated stocks of uncertain taxonomic provenance or is collected from the wild, risking poor product quality and an unpredictable therapeutic outcome. Greater regulation, dissemination of information, and improved access of the market to accurately identify germplasm would help to alleviate these problems.

Our results indicate that the different *Phyllanthus* species usually associated with the Brazilian colloquial name *quebra-pedra,* which may otherwise be difficult to identify by a nonspecialist, can be confidently distinguished from each other by a choice of commonly used DNA barcoding markers. Furthermore, since they are well represented in *Phyllanthus* species in the public DNA sequence databases, the ITS and *mat*K markers enable differentiation of *quebra-pedra* samples from closely related species, facilitating the organization and distribution of correctly identified germplasm. Further sequencing efforts including a wider sample of species will be required to determine whether the *trn*L, *trn*L-F, and *psb*A-*trn*H markers are similarly discriminatory, while the *rbc*L marker is unlikely to be able to resolve closely related *Phyllanthus* species when used alone. Our brief survey of commercial packaged *quebra-pedra* herbs has clarified the need for botanical standardization of these products, where their composition and adulteration is now easily exposed, contributing to improved quality and value for the consumer.

▶ **Table 3** Primers and annealing conditions used for DNA barcode marker PCR.

Marker	Primer	Sequence 5′-3′	Tm °C	Reference
ITS	Nnc18S10	AGGAGAAGTCGTAACAAG	58	[36]
	C26A	GTTTCTTTTCCTCCGCT		
trnL	c	CGAAATCGGTAGACGCTACG	54	[37]
	d	GGGGATAGAGGGACTTGAAC		
trnL-trnF	e	GGTTCAAGTCCCTCTATCCC	54	[37]
	f	ATTTGAACTGGTGACACGAG		
psbA-trnH	trnH-GUG	CGCGCATGGTGGATTCACAATCC	52	[38]
	psbA	GTTATGCATGAACGTAATGCTC		[39]
matK	3F_KIMf	CGTACAGTACTTTTGTGTTTACGAG	48	[40]
	1R_KIMr	ACCCAGTCCATCTGGAAATCTTGGTTC		
rbcL	rbcL*a*_F	ATGTCACCACAAACAGAGACTAAAGC	52	[40]
	rbcL*a*_R	GTAAAATCAAGTCCACCRCG		

Materials and Methods

Plant material

Leaf samples of *Phyllanthus* species currently used phytotherapeutically by the population were collected from several different sources in Brazil; predominantly from “living pharmacies” (farmácias vivas or phytotherapy programs) [34] and germplasm banks. In addition, some samples of wild plant material, locally used or recognized as *quebra pedra*, were also collected (▶ **Table 1**) under the authorization of the Ministry of Environment (process IBAMA 02001.00663/2012-91). The institutional germplasm bank samples were from CPMA-UNICAMP-CPQBA, Campinas, São Paulo State, from Embrapa Genetic Resources and Biotechnology, Brasília, Distrito Federal, and from EPAGRI, Itajaí, Santa Catarina State. Voucher specimens were deposited in the Embrapa herbarium (CEN) and identified by Marcos José da Silva, an expert in the taxonomy of the genus *Phyllanthus*. All specimens were originally collected from Brazil, except the CPMA *P. amarus* accessions, which were originally collected in India. Four different commercial packaged herbs/teas, labelled as *quebra-pedra*, were also obtained from local natural product pharmacies. Five separate samples of discrete plant fragments of about 20 mg were randomly taken from each herb package. DNA was then separately extracted from each fragment, facilitating a survey of the botanical purity of each package.

Molecular methods

Genomic DNA was purified from about 20 mg of silica gel dried leaves using a cetyl trimethyl ammonium bromide (CTAB) extraction method [35], modified by the use of 3% CTAB and 1.5% β-mercaptoethanol in the extraction buffer. The same method was used to extract DNA from the commercial *quebra-pedra* herb samples. Vouchers are given in ▶ **Table 1**.

The nuclear ribosomal ITS1–5.8S rRNA-ITS2 region (ITS) was amplified by PCR reactions comprising approximately 2 ng genomic DNA and 1× PCR buffer (Phoneutria Biotecnologia e Serviços Ltda) containing 2.0 mM MgCl2, 0.2 mM dNTP’s, 0.1 mg/mL bovine serum albumin (BSA), 1 U Taq polymerase (Phoneutria), 1.3 M betaine, and 0.25 µM of each primer. Thermal cycling consisted of 2 min at 95 °C then 35 cycles of 20 s at 95 °C, 30 s at 58 °C, and 90 s at 72 °C, followed by 7 min at 72 °C. For the chloroplast loci, PCR mixes were adopted as for ITS, but without betaine. Thermal cycling for all chloroplast markers was standardized as 2 min at 94 °C then 35 cycles of 20 s at 94 °C, 40 s at the appropriate annealing temperature for the primers used, and 1 min at 72 °C, followed by 7 min at 72 °C. Primer sequences and annealing temperatures are given in ▶ **Table 3**.

PCR products were verified by agarose gel electrophoresis and were then prepared for sequencing using ExoSAP (Applied Biosystems). Both DNA strands were sequenced with the Big Dye v.3.1 kit (Applied Biosystems) using the amplification primers. Sequence reactions were resolved on an ABI3730 automatic sequencer (Applied Biosystems). Raw sequence reads were trimmed for quality, contigs assembled, and any strand base calling mismatches resolved, if possible, using Chromas Pro (v. 1.5, Technelysium Pty Ltd.). Trimmed reads failing to automatically assemble were excluded. ITS and *mat*K data sets from a previously published phylogeny of Tribe Phyllanthae [21] were included in our analysis to confirm discrimination of *P. amarus, P. niruri, P. tenellus*, and *P. urinaria* from closely related *Phyllanthus* species. Sequences were organized into matrices in Bioedit (v. 7.2.6; [41]) and aligned using MAFFT v. 7 with the G-INS-i option [42], which uses iterative refinement and assumes global homology. The ends of the aligned data matrices were trimmed to the mean length of the newly sequenced samples, excluding primer sequences. Sequences derived from vouchered specimens (excluding commercial tea samples) were deposited in Genbank and accession numbers are given in ▶ **Table 1**.

Data analysis

Trees based on data from each marker were constructed using the Bayesian Markov Chain Monte Carlo (MCMC) method as implemented in MrBayes 3.2.1 [43], chosen for reasons of analytical

robustness. Reversible-jump MCMC [44] was invoked to account for uncertainty among components of the GTR model family (nst = mixed rates = invgamma). Four runs, comprising one cold and three heated MCMC chains, were conducted in parallel for two to five million generations, with sampling every 1000 generations. This runtime was sufficient for the convergence diagnostic, the standard deviation of split frequencies, to fall below 0.01 in all repeated analyses. The first 25 % of the trees were discarded (burn-in) prior to calculation of the 50 % majority rule consensus trees.

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Conflict of Interest

The authors declare no conflict of interest.

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