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Adulteration of herbal products: Bamboo tea authentication

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Background. Names for ”substances” used in food products are rarely precise. The term bamboo (*Bambusoideae*, *Poaceae*) comprises over 1600 distinct species of which only few are well established sources for food products on the European market (i.e. bamboo sprouts).

Methods. We analysed bamboo species and tea products containing an exotic ingredient (bamboo leaves) using anatomical leaf characters and DNA sequence data. Our primary concern was to determine the taxonomic origin of bamboo leaves to establish a baseline for EU legislation, to introduce a simple PCR based test to distinguish bamboo from other *Poaceae* leaf components and to assess the diagnostic potential of DNA Barcoding markers to resolve taxonomic entities within the bamboo subfamily and tribes.

Results. Based on anatomical and DNA data we can pinpoint the taxonomic origin of genuine bamboo leaves used in commercial products to the genera *Phyllostachys* and *Pseudosasa* from the temperate ”woody” bamboo tribe (*Arundinarieae*). We detected adulteration by carnation in 4 of 8 tea products and, after adapting our objectives, could trace the taxonomic origin of the adulterant to *Dianthus chinensis* (*Caryophyllaceae*), a well known traditional Chinese medicine with counter indications for pregnant women.

Adulteration of Herbal Products: Bamboo Tea Authentication

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ABSTRACT

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Keywords: Food Diagnostics, Food Fraud, ARMS, Bamboo, Bambusoideae, Lemongrass, Carnation, Dianthus, DNA Barcoding, Character Based DNA Diagnostics, Consumer Rights

INTRODUCTION

People are used to acquire and consume food products without having to confront themselves with complex topics like biological systematics and effects of globalisation. The European Union (EU) introduced the Novel Food Regulation (NFR) to protect consumers from products containing unknown potentially dangerous ingredients. To market an exotic food component within the EU, business operators are required to proof that it had been consumed to a considerable amount before 1997. If the component does not comply to this criteria, it has to be considered a novel food and further steps (i.e. safety evaluations) are required before it can be marketed.

Bamboo leaf tea is considered a delicious and healthy drink in Asian countries and has found its way into the European market. The current status of bamboo leaf as food ingredient in the EU, however, is less than clear. The Novel Food Catalogue currently (June 2016) contains entries of several taxa associated with the term ”bamboo”: *Bambusa* spec., *Dendrocalamus latiflorus*, *D. asper*, *Gigantochloa albociliata*, *G. levis*, *Phyllostachys pubescens* and *Sinocalamus oldhamii*. Except for the first entry, all relate to the use of the stem as food source. A note connected to the entry for *Bambusa* spec. states that the use of leaves as food source is not known to any member state and therefore if they were to be used as a food might be subject to the NFR and require a safety assessment.

Bamboo appears to be a very loose term that, particularly in respect of bamboo tea, requires us to ask the question: Which kind of bamboo are the leaves taken from that are used in bamboo tea?

Bamboos

Bamboos are herbaceous or ”woody” plants from the subfamily *Bambusoideae* (*Poaceae*) diversified in temperate and tropical Asia, South America and Africa. They are extensively used by humans (e.g. *Phyllostachys* species in China and neighbouring countries) and cultivated beyond their natural distribution range. Many species are only known from cultivation (e.g. *Bambusa* spec.). Bamboo is industrially

used for construction, furniture and paper production. Domestically it is used as tool (e.g. farming, hunting, fishing, eating, weaving). The leaves of *Gelidocalamus latifolius* and *Indocalamus* species are used to wrap glutinous rice [Wu et al., 2006], those of broad-leaved species (e.g. *Sasa* species) are cut during the first 5 weeks, cleaned, dried, roasted and used for bamboo tea. For ages bamboo tea has been considered a delicious and healthy drink in the bamboo countries and is now spreading to other regions (e.g. Europe). It contains neither theine nor caffeine and is rich in protein, calcium, iron, magnesium and recommended for various pharmaceutical applications, particularly stomach pain [Liese, 2015]. In Japan the leaves of *Sasa* plants (*S. palmata*, *S. senanensis* and rarely *S. yahikoensis* and *S. kurilensis*), which are called "Kuma-zasa", have been used to treat burns or urinary hesitancy [Sasaki et al., 2007]. In China and Indonesia leaves of different species of *Bambusa*, *Phyllostachys*, *Fargesia* and *Indocalamus* are used for medicinal purposes [http://www.bamboocentral.org/pharmacopoeia.html]. According to Subhuti Dharmananda (http://www.itmonline.org/arts/bamboo.htm, Hsu et al. [1986], Zhen [1995]) the most frequently used leaves in Chinese herbal medicine are collected from the grass bamboo (*Lophatherum gracile*). It is also mentioned that the leaf of the black bamboo (*Phyllostachys nigra*) and of the grass bamboo is often confused both in China and the West [Jiao, 2003]. Taxonomically, there are 1'641 bamboo species, 120 genera and 3 tribes [Soreng et al., 2015] making up the subfamily *Bambusoideae* (*Poaceae*). In appearance bamboos are either "woody" (lignified) or herbaceous. The first group can be divided into two distinct lineages - the temperate (*Arundinarieae*) and tropical (*Bambuseae*) "woody" bamboos. Nested between the "woody" tribes are the herbaceous bamboos (*Olyreae*). Strictly speaking, the earlier mentioned grass bamboo (*Lophatherum gracile*) is not a bamboo. Instead it belongs to another *Poaceae* subfamily (*Panicoideae*), which also harbours members from the genus *Cymbopogon* (lemongrass), another common herbal tea ingredient.

Identification of Herbal Product Components

The classic approach to identify herbal product components is based on described anatomical features of involved plant parts. While characteristics may exist for each species, most of the time the features are more general and can be used to distinguish species of a certain genera from other species of different genera. With increased degree of processing, more and more features get lost due to progressing influence of artefacts or the absence of tissue carrying those features.

Even if no tissue is available at all, DNA still is a viable source for taxonomic placement [Ward et al., 2009, Newmaster et al., 2013]. The probably most prominent approach aiming to pinpoint the identity of a specimen is DNA Barcoding [Hebert et al., 2003]. Ideally, a small standardized region of the (plant) genome (the barcode) is used to determine the species name of a specimen by comparing its barcode to records of verified species references. Using the information of the DNA, this approach is not limited to a certain developmental stage or particular tissue characteristics and is also not biased by environmental factors. However, it has been shown to be of limited use for species-level specimen identification in land plants when using the officially proposed [CBOL Plant Working Group, 2009] chloroplast markers (rbcL and matK). Identification success rates increase when using more variable marker regions [Federici et al., 2013, Roy et al., 2010, Seberg and Petersen, 2009, Taberlet et al., 2007]. Most markers, however, have been shown to be unable to resolve closely related taxa as single DNA Barcoding marker.

Besides DNA Barcoding, which is based on sequence information of well established marker regions, there are other approaches [Wang et al., 2001, Lee et al., 2006, Dnyaneshwar et al., 2006, Huh and Bang, 2006, Marieschi et al., 2010, Torelli et al., 2014] using DNA fingerprinting techniques like Random Amplified Polymorphic DNA (RAPD) or Amplified Fragment Length Polymorphism (AFLP) to develop simple PCR tests based on new and fairly unknown marker regions. Achieving a similar goal but relying on well established markers, PCR-RFLP and ARMS have been used to test the identity of specimens by specific PCR fragment patterns [Newton et al., 1989, Yang et al., 2004, Li et al., 2007, Wang et al., 2007, 2010, Horn et al., 2012, 2013].

Aim

Our primary concern was to determine the taxonomic origin of bamboo leaf samples obtained from commercial teas to establish a baseline concerning EU food law (food vs. novel food). We also aimed to establish anatomical and DNA based differentiation methods for bamboo and similar components. Finally our goal was to assess the diagnostic potential of selected DNA Barcoding markers. After discovering the adulteration of corresponding tea products we naturally extended our objectives by including the adulterant in all analyses.

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MATERIAL AND METHODS

Reference Plants and Commercial Samples

Specimens of bamboo (*Bambusoideae*, table 1), lemongrass and Carnation (*Cymbopogon* and *Dianthus*, supplementary table 2) were acquired and cultivated in the botanical garden of the Karlsruhe Institute of Technology. *Bambusoideae* and *Cymbopogon* specimens were identified to genus level [Farrelly, 1984, Wu et al., 2006, 2007]. At least one specimen of each *Dianthus* species was identified to species level using morphological markers [Wu et al., 2001, Jäger et al., 2008]. Specimen details including images are available through the Barcoding of Life Data Systems web site (project BBOCA, `http://boldsystems.org`). Commercial products were acquired from local and internet sources (Table 2).

Table 1. Reference accessions (Acc) B1 - B13 of *Bambusoideae* species, their taxon names and Genbank sequence accessions of three plastid DNA regions (rbcLa, rbcLb and matK-KIM).

Acc	Taxon	rbcLa	rbcLb	matK-KIM
	Bambuseae			
B1	*Bambusa multiplex*	KX146450	KX146413	KX146427
B2	*Dendrocalamus giganteus*	KX146452	KX146415	KX146429
	Arundinarieae			
B3	*Phyllostachys aureosulcata*	KX146453	KX146416	KX146430
B4	*Phyllostachys edulis*	KX146454	KX146417	KX146431
B5	*Phyllostachys nigra*	KX146455	KX146418	KX146432
B6	*Phyllostachys violascens*	KX146456	KX146419	KX146433
B7	*Pseudosasa japonica*	KX146457	KX146420	KX146434
B8	*Sasa borealis*	KX146458	KX146421	KX146435
B9	*Sasa kurilensis*	KX146459	KX146422	KX146436
B10	*Sasa palmata*	KX146460	KX146423	KX146437
B11	*Sasa veitchii*	KX146461	KX146424	KX146438
B12	*Semiarundinaria fastuosa*	KX146462	KX146425	KX146439
B13	*Bergbambos tessellata*	KX146451	KX146414	KX146428

Morphological and Anatomical Evaluations

Small rectangle hand-sections were made in the centre and at the margin of the leaf-blades of the first fully developed dried leaves of reference plants. Leaf fragments were isolated from all commercial products. After visual inspection of specimens using a stereo microscope (Leica S6D) the adaxial and abaxial leaf surfaces were brightened with 60 % chloral hydrate (Carl Roth GmbH) and analysed using a light microscope (Leica DM750). Both instruments are equipped with a digital image system (Leica EC3) that was used to document macroscopic and microscopic leaf structures.

DNA based Evaluations

For DNA based evaluations we chose to retrieve sequence information from the ribulose-bisphosphate carboxylase oxygenase large subunit (rbcL) employing primers for rbcLa [Soltis et al., 1992, Kress et al., 2009] and rbcLb [Dong et al., 2014] , maturase K (matK) employing primers for matK-KIM (Ki-Joong Kim, unpublished) and the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA employing primers ITS5 and ITS4 [White et al., 1990].

DNA Isolation: DNA was isolated from sterilized leaf samples of reference plants and leaf fragments selected from commercial products using the innuPREP Plant DNA Kit (Analytik Jena AG) following the vendor's instructions (SLS protocol). Products containing more than one leaf component (i.e. bamboo and lemongras) were sampled twice, one bamboo sample for sequencing and one mixed sample for PCR diagnostics. Purity and concentration of isolated DNA was determined using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Germany).

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Table 2. Product accessions (Acc) P1 - P8 of fruit (FT) or single component (SC) tea products available in Germany. Fruit teas are mixtures of fruit fragments and one or two leaf components. Genbank accession numbers of rbcLa, rbcLb, matK-KIM and ITS sequences generated in this study are also included.

Acc	Type	Leaf Component(s)	rbcLa	rbcLb	matK-KIM	ITS
P1	FT	bamboo, lemongras	KU722894	KU722852	KU722866	KU722880
P2	FT	bamboo	KU722893	KU722851	KU722865	KU722879
P3	FT	bamboo	KU722891	KU722849	KU722863	KU722877
P4	SC	bamboo whole leaf	KU722892	KU722850	KU722864	KU722878
P5[a]	SC	bamboo	KX233507	KX233494	KX233503	-
P6	FT	bamboo	KX233506	KX233493	KX233502	-
P7	FT	bamboo, lemongras	KX233505	KX233492	KX233501	-
P8[a]	SC	bamboo	KX233508	KX233495	KX233504	-

[a] fine fragmented leaf material is contained in tea bags

Amplification and Sequencing: A 30 µL reaction volume containing 20.5 µL nuclease free water (Lonza, Biozym Scientific GmbH), 1-fold Thermopol Buffer from New England Biolabs GmbH (NEB), 1 mg / ml bovine serum albumin, 200 µmol dm^{-3} dNTPs (NEB), 0.2 µmol dm^{-3} of forward and reverse primer (see supplementary table 1), 100 - 150 ng DNA template and 3 units of Taq polymerase (NEB) was used to amplify marker sequences. The PCR reaction was subsequently evaluated by agarose gel electrophoresis (AGE) using NEEO ultra-quality agarose (Carl Roth GmbH). DNA was visualized using SYBR Safe (Invitrogen, Thermo Fisher Scientific Germany) and subsequent blue light excitation. The fragment size was determined using a 100 bp size standard (NEB). Amplified DNA was purified using a NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH). Sequencing was outsourced to Macrogen Europe (Netherlands).

Evaluation of Sequence Data: Sequencing results were assembled using a perl script. Raw data was converted to fasta (phred 20) and bi-directional reads merged to recover ambiguous characters (N). For additional quality control IUPAC consensus sequences were generated and inspected. Resulting sequences of product samples were used in a BLAST analysis to approximate taxonomic identity.

Phylogenetic Diagnostics

To assess the diagnostic power of the used marker regions in a phylogenetic framework, we combined reference plant sequences with sequences of relevant taxonomic groups retrieved from Genbank (supplementary table 3 and 4). Sequence collections of each marker were aligned (coding regions: Edgar [2004a,b], ITS: Katoh [2002], Katoh and Standley [2013] with L-INS-i), primer sites removed and trimmed using reference plant sequences. Subsequently each dataset was evaluated for its information content (alignment length, variable positions, parsimony information and singleton sites) and phylogenetic trees were computed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA), Maximum Parsimony (MP) and Maximum Likelihood (ML) algorithms implemented in MEGA6 [Tamura et al., 2013]. For UPGMA the evolutionary distances were computed using the p-distance method [Nei and Kumar, 2000] with all ambiguous positions removed for each sequence pair. The MP tree was obtained considering all sites using the Subtree-Pruning-Regrafting (SPR) algorithm [Nei and Kumar, 2000] with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The evolutionary history inferred by using the ML method was based on substitution models in combination with evolutionary rate differences among sites that had the lowest BIC (Bayesian Information Criterion) scores determined by analysing each dataset using MEGA6. Details are summarized in table 3. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining (NJ) method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. All trees were bootstrapped [Felsenstein, 1985] using 500 replicates. Additionally, we computed UPGMA, MP and ML trees using concatenated datasets (rbcL = rbcLa and rbcLb; r+m = rbcLa, rbcLb and matK-KIM). The results were analysed by first collapsing branches corresponding to partitions reproduced in less than 50% bootstrap replicates and recording bootstrap support values for relevant monophyletic groups (sensu Soreng et al. [2015]). All datasets and trees have been deposited

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in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S19113). For representation the dataset and algorithm that provided most support for relevant clades was edited using FigTree V1.4.2 [Rambaut, 2014].

Table 3. Substitution models (K2 = Kimura 2-parameter; T92 = Tamura 3-parameter; GTR = General Time Reversible) and evolutionary rates among sites (+G = discrete gamma distribution) used for ML analysis

Dataset	Model	Rates	BIC
rbcLa	K2	+G	5217
rbcLb	T92	+G	7177
rbcL	T92	+G	9380
matK-KIM	GTR	+G	10829
r+m	GTR	+G	19223

Character based Diagnostics

The PCR diagnostics approach had two objectives. Firstly, we aimed to establish a simple but efficient PCR based differentiation between bamboo (= subfamily = *Bambusoideae*), the common secondary component (lemongrass = genus = *Cymbopogon*) from the same family (*Poaceae*) and the adulterant from the genus *Dianthus* (*Caryophyllaceae*). Secondly, we wanted to assess the diagnostic potential of the used marker regions to resolve entities within the subfamily level and the adulterant genus.

Differentiation of Bamboo Tea components and Adulterant: For the PCR based differentiation protocol we chose rbcLa which had been successfully used before to differentiate above the generic level [Horn et al., 2012, 2013]. Using the rbcLa dataset, we designed primers to detect single nucleotide polymorphisms [Newton et al., 1989, Ward et al., 2009] characteristic for bamboo, lemongrass and carnation. Nucleotide differences between the mentioned components were determined and potential diagnostic primer sequences extracted. One suitable primer for each group was chosen and destabilized according to Newton et al. [1989] (supplementary table 1). The theoretical suitability of a diagnostic primer was determined using primer3 [Untergasser et al., 2007, 2012] with default settings.

The diagnostic primers were evaluated in a multiplex PCR with the universal primer-pair (rbcLa). For each diagnostic primer a separate set of 10 µL PCR reactions containing 6.5 µL nuclease free water (Lonza, Biozym Scientific GmbH), 1-fold Thermopol Buffer (NEB), 1 mg / ml bovine serum albumin, 200 µmol dm^{-3} dNTPs (NEB), 0.3 µmol dm^{-3} of universal forward primer, 0.2 µmol dm^{-3} of universal and diagnostic reverse primer, 25 - 50 ng DNA template and 0.5 units of Taq polymerase (NEB) was used. The PCR products were evaluated by gel electrophoresis using high resolution agarose (Carl Roth GmbH).

Assessment of Diagnostic Potential: To assess the diagnostic potential of DNA markers, we used a character bases DNA Barcoding appraoch - Barcoding with LOGic [Weitschek et al., 2013, Bertolazzi et al., 2009]. We prepared separate single and multi-locus datasets containing only sequences of *Bambusoideae* and *Caryophyllaceae* respectively. Sequences were labelled according to specific taxonomic classes. For the *Bambusoideae* dataset we tested tribe and genus as diagnostic entities. For *Dianthus* we only tested the species as diagnostic entity. Since the general evaluation showed limited variation within rbcL in *Dianthus* we chose to evaluate only matK-KIM as cytoplasmic marker. Additionally we included an ITS dataset that contained all available Genbank *Dianthus* sequences regardless if data also existed for the cytoplasmic markers. The BLOG algorithm was subsequently used with standard settings (except padding=1, percslicing=100 and exclusivefs=1) to find characters or character combinations by which diagnostic entities can be classified.

RESULTS

Anatomical Evaluation

Morphology as the study of forms visible to the unaided eye, in food diagnostics is complemented by anatomy, the study of cellular structures. For an intermediate between morphology and anatomy, in this study we used the term ”macroscopic”. The magnification used does not yet allow to observe cellular

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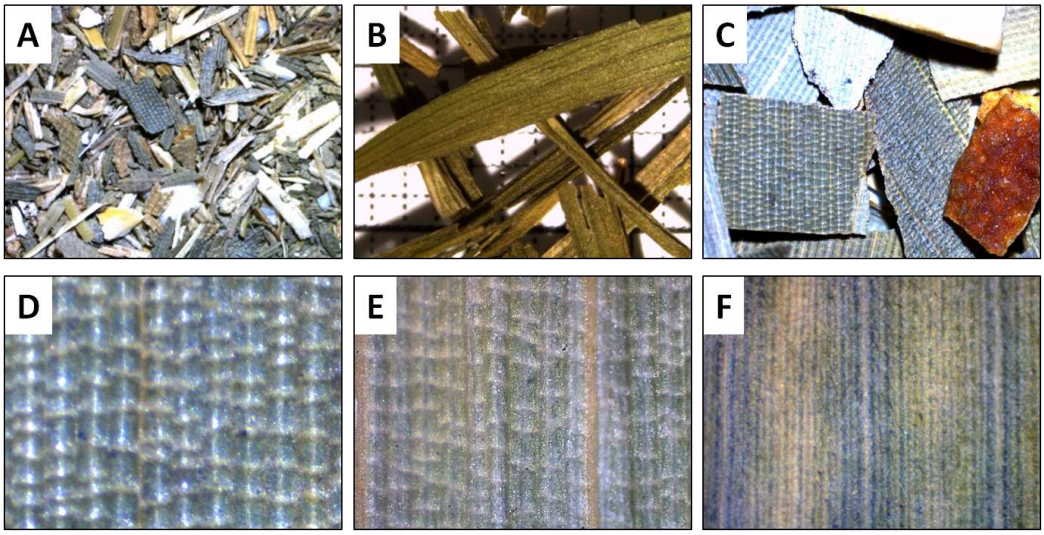
Figure 1. Macroscopic features of bamboo tea products (A-D) and bamboo leaf samples (E and F). Leaf fragments (10 x) of bamboo tea bag product (A), adulterant component (B) and of a bamboo fruit tea (C). Leaf surface (adaxial, 40 x) of bamboo tea component (D) in comparison to Arundinarieae (E, *Sasa palmata*) and Bambuseae (F, *Bambusa multiplex*) dried leaf samples.



structures in detail, but eases the study of their morphological manifestations. Both, microscopic and macroscopic anatomy are common techniques used in food diagnostics [Hohmann and Gassner, 2007].

Macroscopic Features: A characteristic of the bambusoid leaf is a mosaic pattern of longitudinal and transverse veinlets, so called tesselation. Our evaluation of leaf samples from bamboo reference plants supports the description of Farrelly [1984] wherein tesselation of the leaf is a visible characteristic of hardy, monopodial species (*Arundinarieae*, Figure 1E) and is hidden from the unaided eye by tissue in sympodial bamboos whose leaves are often more tough and leathery (*Bambuseae*, Figure 1F).

Evaluating the leaf samples taken from herbal tea products, tesselation was observed in samples P5 - P8 (e.g. Figure 1C and D). While leaf fragments with tesselation always were fragments in longitudinal and transversal respect, leaf components of the remaining products P1 - P4 consisted of thin (approximately 4 mm) linear to lanceolate leaves (Figure 1B), in some instances oppositely arranged at the fragment of a shoot. The observed arrangement of leaves is in direct conflict with the index of contents of corresponding products. *Poaceae* plants always have in two ranks alternately arranged leaves [Wu et al., 2006]. However, since bamboo tea is also available in a form where components are so small, that the arrangement of leaves cannot be determined (tea bags), microscopic features need to be considered.

Microscopic Features: Using light microscopy (100 x), tesselation was observed in all bamboo reference plants. Additionally, characteristic structures of the bambusoid leaf [Wu, 1962, Vieira et al., 2002] were observed: epidermal cells - longitudinal bands composed of long rectangular cells with wavy lateral walls and alternating short rectangular cells, separated by bulliform cells [Beal, 1886, Alvarez et al., 2008] in the upper epidermis (Figure 2 A); and modified epidermal cells - stomata of the *Poaceae* type, microhairs, spines, papillae, bristles and silica cells (Figure 2 B - D).

The microscopic evaluation of commercial samples P5 - P8 was congruent with the results from bamboo reference plants, showing bambusoid features (e.g. tesselation: Figure 2 E). Samples P1 - P4 did not display any bambusoid characteristics but stomata of a different type than *Poaceae* (Figure 2 H and I) and crystal druses (Figure 2 J) along main veins and in intercostal regions. We recognised anomocytic stomata common in *Caryophyllaceae* and *Ranunculaceae* [Rohweder et al., 1971] predominated by the diacytic form. This suggests that samples P1 - P4 probably originated from a *Caryophyllaceae* plant.

DNA based Evaluation

All three cytoplasmic markers were retrieved with great success regarding PCR and sequencing results. ITS however turned out to be particularly problematic with bamboo samples. Preferential and co-amplification of ITS from fungal trace DNA prevented the retrieval of a complete dataset for bamboo

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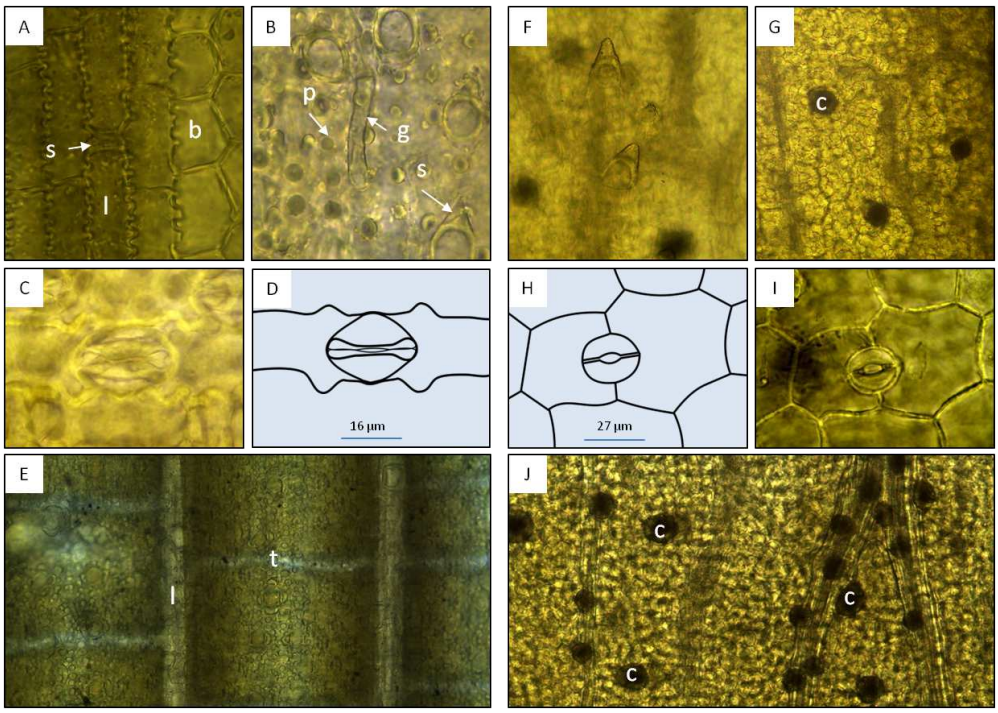
Figure 2. Microscopic features of the bambusoid leaf observed in reference specimens (A - D, 400 x) and product samples (E, 100 x), and microscopic features of *Dianthus chinensis* observed in reference specimens (F and G, 100 x; H and I, 400 x) and adulteration samples (J, 100 x). A: Adaxial epidermis of *Bambusa multiplex* showing longitudinal bands of long rectangular cells (l) with wavy lateral walls and alternating short rectangular cells (s) separated by bulliform cells (b). B: Abaxial modified epidermal structures of *Phyllostachys edulis* (p = papillae, g = geniculate hair, s = spine). C and D: Abaxial epidermis with *Poaceae* type stomata of *Sasa palmata*. E: Epidermis with longitudinal (l) and transverse veinlets (tesselation) observed in product samples. F: Leaf epidermis of *D. chinensis* showing unicellular trichomes. G: Mesophyll of D. chinensis showing crystal druses (c). H and I: Abaxial epidermis of *D. chinensis* with anomocytic stomata (here diacytic). J: Mesophyll with crystal druses (c) along main veins and in intercostal regions observed in product samples.



specimens. Similar problems have been reported by Zhang et al. [1997].

General Assessments

BLAST Analysis of Product DNA Sequences: Single locus markers (rbcLa, rbcLb and matK-KIM) were used in a BLAST analysis. Two groups could be distinguished: P1 - P4 returned hits indicating close relation to *Dianthus* (*Caryophyllaceae*) and P5 - P8 returned hits belonging to genera of *Bambusoideae*.

Information Content: Final single marker dataset alignments contained 553, 814 and 837 nucleotides for rbcLa, rbcLb and matK-KIM respectively. Combining rbcLa and rbcLb (rbcL) excluding redundant data, the alignment had 1'126 positions. The combination of rbcLa, rbcLb and matK-KIM had 1'963 positions respectively. The *Dianthus* ITS dataset of reference plant accessions contained 611 nulceotides. Including Genbank accessions (supplementary table 4) the datset was comprised of 85 sequences with 618 positions. Information content (i.e. number and proportion of variable sites and parsimony informative positions) within *Bambusoideae* and *Dianthus* datasets is shown in table 4.

In both taxonomic groups most variation among single locus cytoplasmic markers was detected in the matK-KIM region. Considering parsimony information, rbcLa in bamboo and rbcLb in *Dianthus* show the highest proportion (57 and 100 % respectively). The combination of single locus data obviously contains all variation and informative sites but reduces the proportion in combined datasets. Among the *Dianthus* datasets the nuclear marker (ITS) contains the highest variation and thus delivers most information.

Sequence data of adulterated (P1 - P4) and genuine (P5 - P8) bamboo products as well as all reference plants are deposited in Genbank. Sequence accessions from other studies that were included in this study

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Table 4. Information content of bamboo and *Dianthus* genetic marker datasets comprised of 43 and 14 DNA sequences respectively. Sequences were obtained from references (plant and Genbank sequence accessions) and product samples. Length (Len), conserved (Con), variable (Var), parsimony informative (PaI) and singleton (Sin) characters as well as the number of haplotypes (Hap) are listed for cytoplasmic markers (rbcLa, rbcLb and matK-KIM) and combinations of those (rbcL = rbcLa + rbcLb and r+m = rbcLa + rbcLb + matK-KIM). For *Dianthus* the same information is listed for a nuclear (ITS) marker, one simple dataset for comparison and one extended (^{e}) dataset consisting of 85 *Dianthus* sequences.

Bamboo

Marker	Len	Con	Var	%	PaI	%	Sin	%	Hap
rbcLa	553	523	30	5.4	17	56.7	13	43.3	17
rbcLb	814	761	53	6.5	26	49.1	27	50.9	13
rbcL	1126	1057	69	6.1	35	50.7	34	49.3	22
matK	837	748	89	10.6	40	44.9	49	55.1	25
r+m	1963	1805	158	8.0	75	47.5	83	52.5	31

Dianthus

Marker	Len	Con	Var	%	PaI	%	Sin	%	Hap
rbcLa	553	553	0	0.0	0	0.0	0	0.0	1
rbcLb	814	808	6	0.7	6	100.0	0	0.0	3
rbcL	1126	1120	6	0.5	6	100.0	0	0.0	7
matK	837	826	11	1.3	8	72.7	3	27.3	3
r+m	1963	1946	17	0.9	14	82.4	3	17.6	7
ITS	611	566	45	7.4	39	86.7	6	13.3	12
ITS^e	618	498	120	19.4	72	60.0	48	40.0	87

, ^{e} extended dataset

are contained within supplementary table 3 and 4.

Phylogenetic Analysis

Clade Support: Comparing the support for relevant clades using different phylogenetic methods with single and multi-locus datasets reveals several interesting aspects (Figure 3).

Sequence accessions of *Borinda* (*Arundinarieae*) and *Chusquea* (*Bambuseae*) cluster in the *Bambuseae* and *Arundinarieae* clade respectively. We therefore introduced additional evaluation classes: *Arundinarieae* modified (mod.) and *Bambuseae* modified (mod.). For these classes the position of both mentioned sequence accessions was ignored when assessing monophyly.

The bamboo subfamily (*Bambusoideae*, Figure 3-1) is supported with more than 50 % of replicates by all marker regions except rbcLa, using MP and ML methodology. When using rbcLa the *Oryzoideae* clade resides among the bamboo members making *Bambusoideae* a non-monophyletic clade. Support for the bambusoid subfamily is constantly equal or above 70% except when using the combined rbcL-sub-regions and the MP approach. With support between 50 and 60 % of two of five tested datasets (matK and r+m), UPGMA only gives weak and inconsistent support for the subfamily.

Focusing on the three bambusoid tribes (*Arundinarieae*, figure 3-4; *Bambuseae*, figure 3-2; *Olyreae*, figure 3-3) none of the markers and methods strongly support all corresponding clades at the same time. Applying MP with matK or combined cytoplasmic data yields high support (>70 %) for *Arundinarieae* and *Olyreae*. Both clades are also supported according to ML, using combined rbcL (>63 %), matK (>84 %) and combined cytoplasmic (>98 %) datasets. The *Olyreae* clade (Figure 3-3) receives consistent support using any dataset with the MP approach (rbcLb 58 % - r+m 98 %). Similarly, except when using the rbcLb dataset, the ML approach offers high support (rbcLa 61 % - r+m 98 %). The *Arundinarieae* clade (Figure 3-4) also is consistently supported by all three phylogenetic approaches, particularly when using matK (UPGMA 93 % - MP 98 %) or the combined cytoplasmic dataset (99 %). Considering an alternative taxonomic configuration (*Arundinarieae* mod., line in figure 3-4) some of the single datasets offer support for the corresponding clade. However, a significant difference between the support for the *Arundinarieae* clade (99 %) and the modified clade (57 %) can be observed when using the combined

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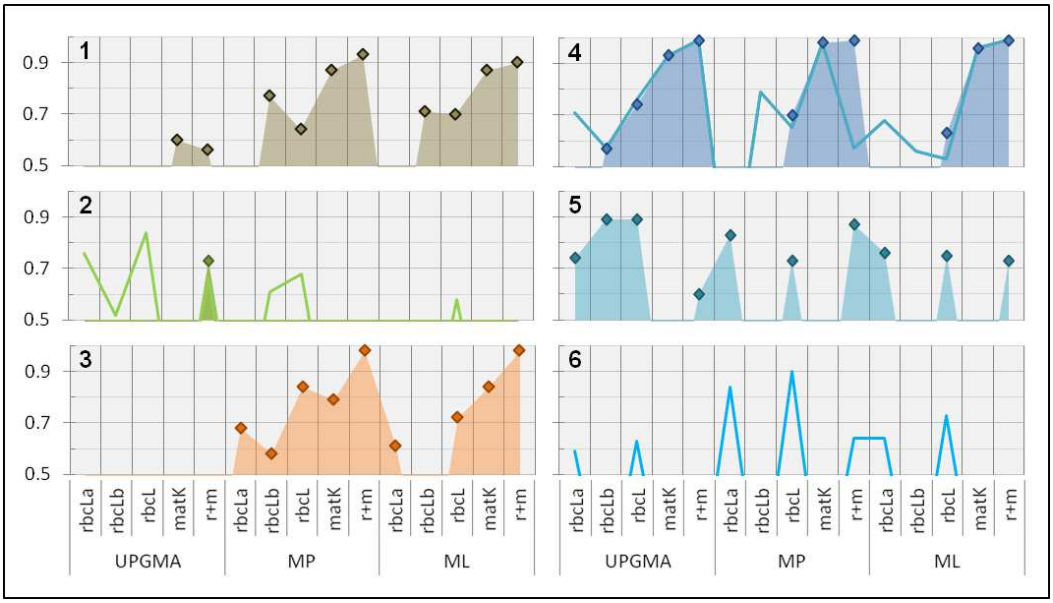
Figure 3. Phylogenetic evaluation. The phylogenetic evaluation shows bootstrap support (y-axis) for relevant clades (1: *Bambusoideae*, 2: *Bambuseae* s.str. and mod., 3: *Olyreae*, 4: *Arundinarieae* s.str. and mod., 5: *Sasa*, 6: *Phyllostachys* mod.) using UPGMA, MP and ML methodology with single locus (rbcLa, rbcLb and matK-KIM) and multi-locus (rbcL: rbcLa + rbcLb; r+m: rbcL + matK-KIM) data. Lines indicate support for an alternative composition of the corresponding clade or taxonomic group. Please refer to the discussion for further information.



cytoplasmic dataset in a MP analysis. The *Bambuseae* clade (Figure 3-2) only once is supported above 50 % (UPGMA: r+m) unless considering an alternative taxonomic configuration (*Bambuseae* mod., line in figure 3-2). In all cases where a *Bambuseae* mod. clade is supported, the *Chusquea* sequence accession fails to cluster (support >50 %) with other *Bambuseae* sequences. In every other instance where general support for *Bambuseae* is missing, the *Chusquea* sequence clusters with *Sasa* (MP, ML: rbcLb) and only some of the *Bambusaea* sequences form supported clusters. A sister clade consisting of *Otatea* and *Olmeca* is consistenly formed (UPGMA: matK; MP: rbcLa, matK, r+m; ML: rbcLa, rbcLb, r+m) along other *Bambuseae* sequences. In the ML analysis using matK the *Olyrae* clade resides within the *Bambuseae* clade resulting in the non-monophyly of the clade.

Support on the genus level is rare. Only *Sasa* (Figure 3-5) and *Thamnocalamus* form monophyletic clades. The *Sasa* clade can be observed in 10 of 15 cases, all based on rbcL data. A monophyletic *Thamnocalamus* clade can only be observed when using rbcLa data. Since product samples frequently clustered within a clade containing *Phyllostachys* we introduced another evaluation class: *Phyllostachys* modified (mod.). This class consists of all *Phyllostachys*, *Fargesia*, *Indocalamus* and *Drepanostachyum* sequence accessions. This clade can be observed using rbcLa and the combined rbcL dataset (UPGMA, MP and ML) as well as when using the combined cytoplasmic dataset (MP). Also in this case, support appears to be solely derived from rbcL data. Although rbcLb data does not offer direct support, its contribution to the combined dataset can clearly be observed by increased support values (e.g. up to almost 10 % in ML analysis).

All other *Poaceae* groups (i.e. *Bambusoideae* outgroups *Oryzoideae* and *Triticum*, and secondary component groups *Panicoideae* and *Cymbopogon*) receive consistent and strong (>85 %) support. One exception worth mentioning is the low (MP: 52 %) and missing support (UPGMA and ML) for *Panicoideae* (represented by *Cymbopogon* and *Lophaterum*) when using rbcLb data.

Support for the genus of particular interest (*Dianthus*, >= 72 %) as well as the corresponding family (*Caryophyllaceae*, 100 %) and outgroup (*Silene*, >= 64 %) are consistent and strong with rare low points, i.e. using matK data with ML (*Silene*) and using rbcLb data with ML (*Dianthus*).

Phylogenetic Representation: Using the combined cytoplasmic dataset with sequences recovered from product components and building a MP tree, basically visualizes the BLAST results within an

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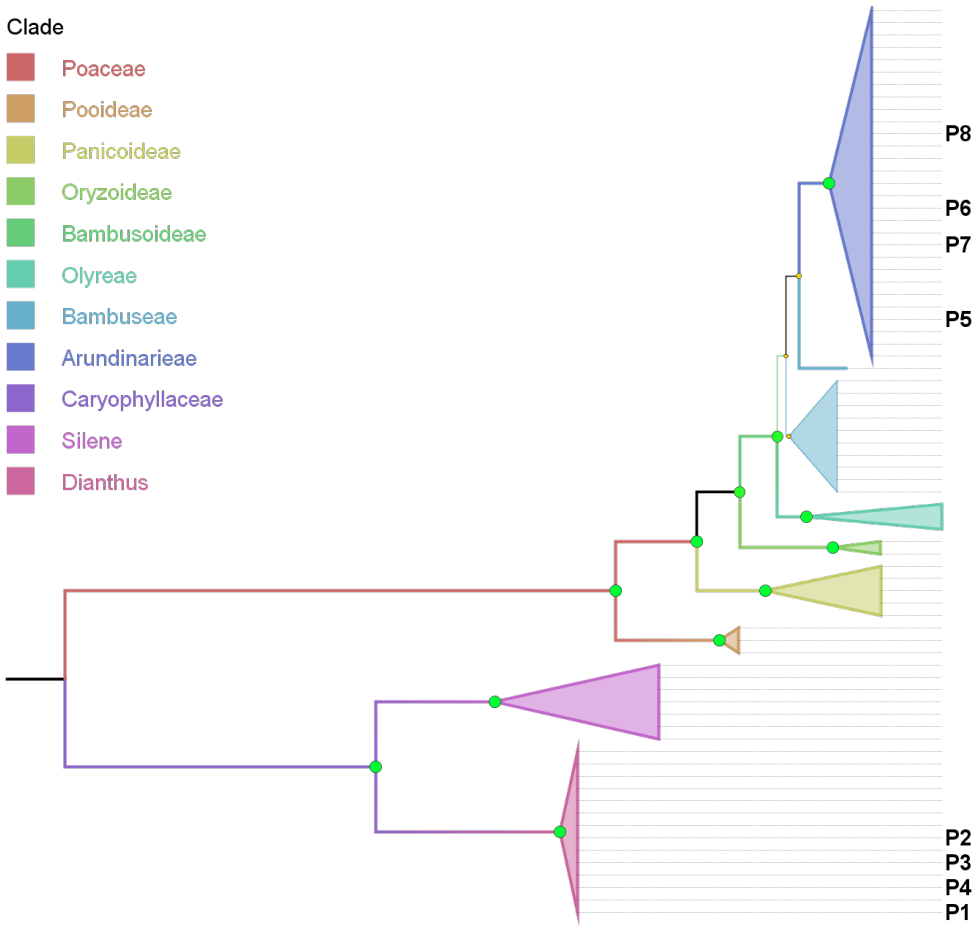
Figure 4. Phylogenetic tree based on combined cytoplasmic sequence data using Maximum Parsimony (MP). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are indicated by the size and colour of the nodes. The analysis involved 74 nucleotide sequences and 1999 positions in the final dataset.



evaluated phylogenetic framework (Figure 4). Product samples P1 - P4 clearly are located within the *Dianthus* (*Caryophyllaceae*) clade and product samples P5 - P8 are located within the *Arundinarieae* (*Bambusoideae*, *Poaceae*) clade.

Diagnostic Analysis

Differentiation of Tea Components and Adulterant: Based on a rbcLa dataset containing bamboo, lemongrass and *Dianthus* sequences we designed three reverse ARMS primer (supplementary table 1) with diagnostic nucleotides located at position 407, 254 and 223 respectively. The evaluation of multiplex PCRs, applying these specific primers in separate reactions together with rbcLa universal primers (Figure 5), shows sufficient specificity and amplification of diagnostic fragments (bamboo, 457 bp; lemongrass 306 bp; *Dianthus* 268 bp) to differentiate the three leaf components present in commercial tea products. Products P1 - P4 show diagnostic fragments of size 268 bp indicating the presence of *Dianthus* (figure 5-D) and are lacking bamboo diagnostic fragments (figure 5-B). Products P5 - P8 show the exact opposite pattern, no diagnostic fragments specific for *Dianthus* but for bamboo. Additionally, the presence of lemongrass in products P1 and P7 is shown by diagnostic fragments of the corresponding size (figure 5-L, 306 bp). All reference plants of the corresponding groups have been tested for positiv reaction using the diagnostic primer and negativ (null) reaction using any diagnostic primer of different groups.

Assessment of Diagnostic Potential: The evaluation of bambusoid tribe classification using BLOG shows consistency among markers. Only the Arundinarieae tribe shows 4 % false negative classifications when using rbcLa data.

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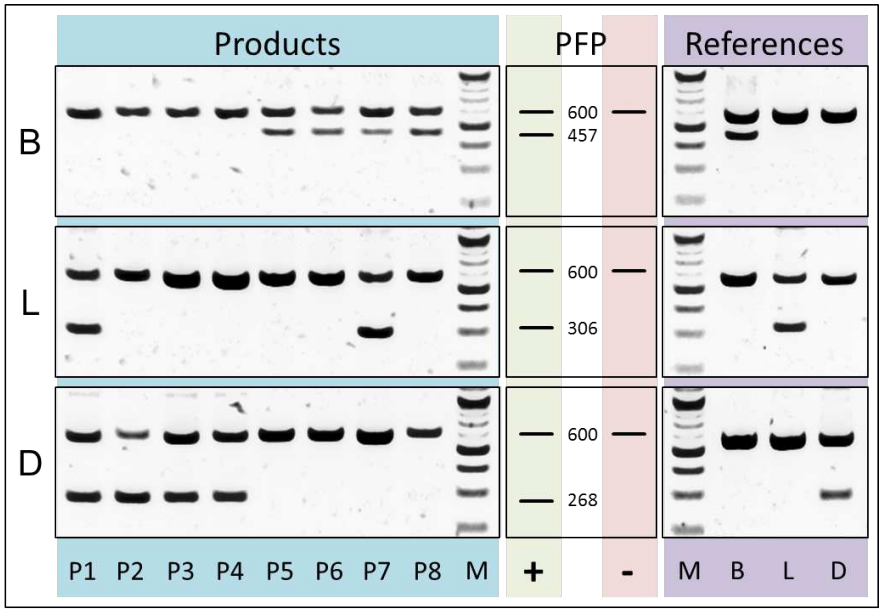
Figure 5. ARMS Diagnostics: Comparison of multiplex PCR results using rbcLa universal primers and diagnostic primers for Bamboo (B), lemongrass (L) and *Dianthus* (D) tea components. On the left are the results using DNA templates derived from products P1 to P8. Based on rbcLa sequence data fragment patterns were predicted (PFP). The rbcLa fragment with a size of around 600 bp represents the positive reaction control. Smaller fragments are called diagnostic fragment and indicate (+) the presence of a particular component (e.g. 306 bp fragment for lemongrass). On the right are representative results using DNA templates derived from reference plants. For the approximation of fragment size a 100 bp (NEB) size standard (M) was used.



Comparing bambusoid genus classification (supplementary figure 1), the combined cytoplasmic dataset provides the highest diagnostic coverage of bambusoid genera. Only 14 of 23 bambusoid genera are at least partially diagnostically covered using single locus rbcLa. Using rbcLb, 20 of 23 genera are classified with 3 genera only partially (<50 %) covered. The combination of rbcLa and rbcLb reflects the result of rbcLb with full coverage of two of these genera (*Fargesia* and *Pseudosasa*) and an a slightly increased coverage of the third (*Phyllostachys*). Additionally, using provided LOGic formulas, the sequence of product sample P8 provides consistent characters (i.e. pos234=T AND pos490=T AND pos878=G) with that of *Pseudosasa*. The diagnostic value of the matK-KIM region is similar to that of rbcLa with 13 of 23 genera at least partially covered. The combined dataset of rbcL and matK-KIM only leaves two genera without diagnostic markers (i.e. *Semiarundinaria* and *Dendrocalamus*) and no false positives are detected (figure 6-A). Using provided LOGic formulas, sequences of product samples P5 - P7 provide consistent characters (i.e. pos12=T AND pos263=T AND pos701!=A AND pos738!=C AND pos1434=G) with that of *Phyllostachys*.

All markers, either as single or in combination, offer diagnostic solutions for the genera of *Olyreae*. While *Arundinarieae* genera are moderately covered using rbcLa data and are almost completely void of diagnostic solutions considering matK-KIM data, in *Bambuseae* the situation is reversed, matK-KIM being more informative. In regard of single locus diagnostics rbcLb is superior in the bambusoid group.

Comparing matK-KIM and ITS datasets for *Dianthus* (Figure 6-B) shows the inability to distinguish *D. chinensis* and *D. longicalyx* based on matK-KIM data. Using ITS, information content increases enough to diagnose *D. chinensis* with a unique LOGic formula (pos181=g AND pos595=c) that also applies to product samples P1 - P4.

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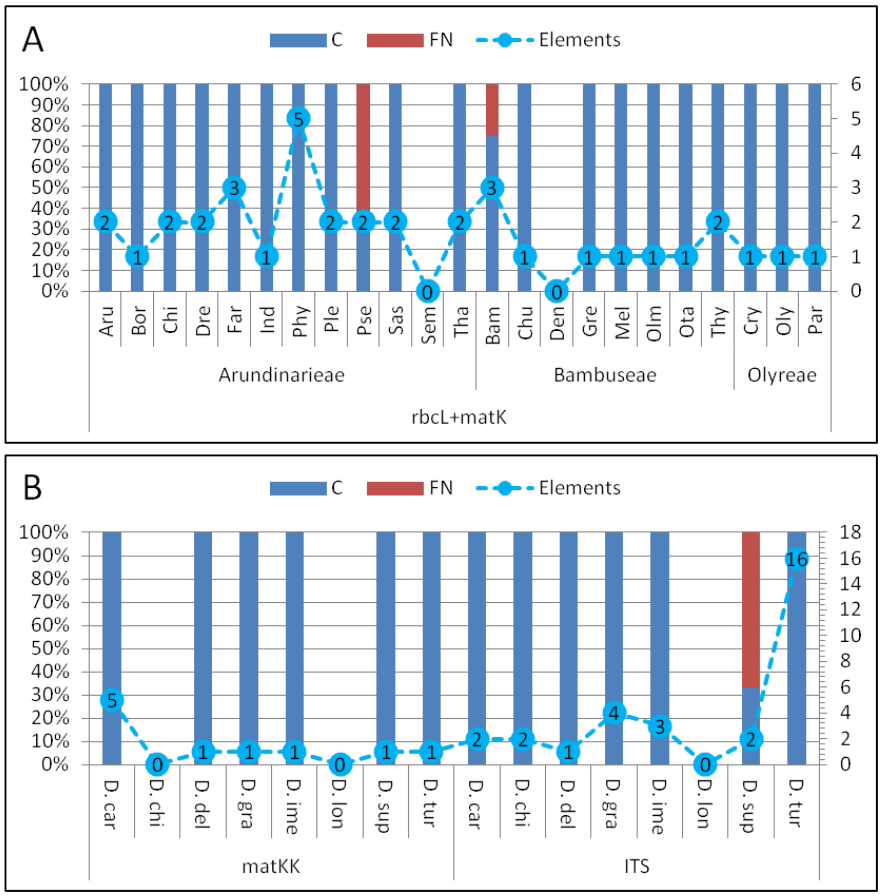
Figure 6. Barcoding with LOGic formulas (BLOG) analysis of bamboo (A) using the combined cytoplasmic dataset and Dianthus (B) using matK-KIM and ITS (extended) single marker datasets. Results for species that only were present in the extended ITS dataset are not shown. The proportion (in %, primary y-axis) of coverage (C, blue) and false negatives (FN, red) using logic formulas is shown as bars. The number of elements (nucleotide positions) within the LOGic formula are represented in a line graph (secondary y-axis) .



DISCUSSION

Anatomical Evaluations

Due to the absence of bambusoid leaf characteristics in samples P1 - P4, we can exclude a *Poaceae* and *Bambusoideae* origin of the leaves used in corresponding tea products. Stomata type and pattern of epidermal cells in comparison to reference plants from the genus *Dianthus* suggest the origin of leaves to be found within this group.

In contrast, observation of bambusoid leaf characteristics in samples P5 - P8 leads to the conclusion that genuine bamboo leaves have been used in corresponding tea products. Investigating the possibility to differentiate between bambusoid tribes, the most promising feature appears to be tesselation. The ability to observe this pattern without or only limited magnification (<= 10 x) in members of the *Arundinarieae* and the necessity of higher magnification (>= 40 x) in members of the *Bambuseae* can be used to separate both woody bamboo tribes [**?**]. Tesselation has also been observed with low magnification in samples P5 - P8. This suggests that the source species for bamboo tea leaves are likely to be from the *Arundinarieae* tribe.

Particular characteristics to differentiate between the bamboo genera were suggested by Wu [1962]. The wavyness of the walls of upper and lower epidermal cells in some species is different, while in other species the wavyness is constant. However, no quantification methodology nor any standard was suggested. Modifications of epidermal cells (i.e. uni- and bicellular hairs, spines, bristles and silica cells)

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also can contribute to a diagnostic evaluation but appear not to be exclusively distributed in one particular genus. Further studies are necessary to establish standards for potential diagnostic characters and to evaluate their phenotypic plasticity.

One of the most challenging aspects of microscopic studies of dried bambusoid leaf samples are abundant papillae, often overarching the stomates [Zhang and Clark, 2000], and achieving sufficient clearing of the tissue samples.

Tesselation is also a usefull diagnostic marker in separating bamboo from other *Poaceae* groups (e.g. lemongrass). Additional anatomical markers for this purpose are fusoid cells [Motomura et al., 2004, **?**, **?**] and invaginated arm cells in the chlorenchyma [Zhang and Clark, 2000]. Both cell types, however, only can be observed in cross sections. Due to the processed nature (i.e. drying) of product samples, a more laborious sample preparation method is required (embedding) and results are likely to be biased by artefacts introduced by the drying process (e.g. collapsed parenchymatic cells). Based on our analyses, we compiled an anatomic diagnostics key for the differentiation of bamboo, lemongrass and carnation (supplementary table 5).

DNA based Evaluations

Morphological traits used to determine the genus of bamboo specimens were shown to be highly congruent with plastid RFLP data and the plastid genome has been extensively evaluated for its phylogenetic and phylogenomic potential to elucidate relationships that have been intractable [Watanabe et al., 1994]. The analysis of six bamboo chloroplast genomes, however, revealed low levels of variation in *Bambusoideae* and difficulties in resolving diversification among temperate woody clades (*Arundinarieae*) even with complete chloroplast genome sequences Zhang et al. [2011].

With DNA Barcoding, ideally, one is able to determine the species of a specimen by comparing sequence information of a standardized DNA region to a database of species barcodes. Since there is no single universal locus in plants available with whom one could determine the identity of specimens with high success rates, using more than one locus is the most promising choice. Beside the official plant barcode markers (rbcL and matK) other complementary markers can be used. Lack of taxonomic universality (ycf1) and sequencing universality (psbA-trnH) as well as co-amplification of fungal DNA or interference of paralogs in downstream analysis (ITS) are common issues associated with alternative markers. While psbA-trnH has been shown to outperform rbcL and matK in some cases [Kress and Erickson, 2007, Wong et al., 2013], in temperate bamboos has much lower divergence rates and showed even less discrimination power than rbcL [Cai et al., 2012].

Combining rbcL and matK barcoding marker data in a phylogenetic analysis, we were able to limit the possible taxonomic origin of bamboo leaves used in tea products to the bambusoid tribe *Arundinarieae* and 3 of 4 product samples could be further traced to a *Phyllostachys* clade. Using the combined cytoplasmic dataset in a character based DNA Barcoding approach (i.e. BLOG) further improved our results and we were able to connect characteristic patterns (LOGic formulas) of two bambusoid genera (i.e. *Phyllostachys* and *Pseudosasa*) to the genuine bamboo product samples (P5 - P7 and P8 respectively). In general our evaluation of diagnostic potential demonstrated the diagnostic value of rbcL and matK on the generic level in bamboos and provides solutions to diagnose most (19 of 23) of the bamboo genera for which rbcL and matK sequence information is currently available in Genbank.

Using the highly universal DNA Barcoding marker rbcLa, we introduced a PCR based diagnostic solution for the detection of an adulterant of bamboo tea (carnation). Using 85 ITS sequences retrieved from reference plants and Genbank in a charcter based DNA Barcoding approach, the classification pattern of *D. chinensis* was also found in sequences obtained from adulterant samples P1 - P4. The diagnostic solution also includes the differentiation of the two *Poaceae* tea components (bamboo and lemongrass). To improve the significance (i.e. taxonomic depth) of the genetic test, other markers need to be evaluated. While ITS has been used in bamboo [Cai et al., 2012], fungal contamination and ITS paralogs decrease the applicability of this marker considerably. Other available DNA markers are for example GBSSI [Zhang et al., 2012, Yang et al., 2008, 2010, Peng et al., 2008] and COS [Li et al., 2008, Liu et al., 2013]. To improve the robustness of the test, sampling within the temperate bamboo genera needs to be increased. Furthermore, the genetic test could be improved by optimizing reaction conditions for the combined use of more than one diagnostic primer.

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Conclusion

What is Bamboo Tea? According to the NCBI Taxonomy the common name for the tribe *Bambuseae* is bamboo. This reflects an old systematic opinion [Zhang and Clark, 2000] when *Bambuseae* still contained most *Arundinarieae* species (e.g. *Sasa* and *Phyllostachys*). However, the most recent scientific usage of the term bamboo is found in Soreng et al. [2015] where bamboo is the common name for the subfamily *Bambusoideae* (*Poaceae*). This group is characterized by high morphological diversity that appears not to be discretely associated with subordinate taxonomic entities. The reasons are believed to be related to morphological inter-gradation interpreted in various ways and the presence of hybrids that have been stabilized through clonal propagation [Triplett and Clark, 2010]. The taxonomic confusion within the group also is related to a peculiarity of the reproduction mode of bamboo. While most flowering plants are flowering regularly each year, bamboo is one of the groups where dramatically extended intervals exist - some as long as 120 years [Veller et al., 2015, Liese, 2015].

Although DNA based approaches to classification of bamboos are characterized by limited information of genetic markers, the subfamily has been well established and the temperate woody clade (*Arundinarieae*) was resolved to an acceptable degree, delivering additional information about associations of particular genera and biogeographic hypotheses [Triplett and Clark, 2010]. All commercial samples of genuine bamboo tea analysed in the present study could be placed within the *Arundinarieae* tribe using macroscopic leaf characteristics. Furthermore, they could be traced to internal groups by phylogenetic methodology (*Phyllostachys* clade) and a character based DNA Barcoding approach (*Phyllostachys* and *Pseudosasa* genera).

Carnation = Bamboo tea? From an evolutionary perspective, bamboo and carnation are fairly different groups of plants with more than a hundred million years of independent development between them [Chaw et al., 2004]. How is it possible to confuse such distinct groups?

Scientific names exist because they allow us to communicate precisely. However, it is also common for humans to label things by its appearance instead of its true identity. So it is not surprising to find a simple explanation for a potentially severe adulteration of teas supposedly containing bamboo leaves: A product description (retrieved in July 2014 from `http://www.happyluckys.com/bamboo-tea-carnation`) of so called Bamboo Tea Carnation is advertised by the following sentence:

”There are well over a hundred varieties of bamboo growing in China. This is not one of them, actually belonging to the genus of Carnations (*Dianthus*), but the young shoots closely resemble bamboo in appearance...”

Communication using the term bamboo in conjunction with tea obviously is ambiguous and may have caused the declaration error on corresponding products. Since these products had been on the marked for at least 1.5 years before they were discontinued, we must ask what consequences this may have had for consumers?

Several species of carnation are mentioned in an ethno-medicinal context [Chandra and Rawat, 2015]. Particularly in traditional Chinese medicine two species - *D. chinensis* and *D. superbus* - are widely used as Dianthi herba for the treatment of diuresis and strangury [Committee, 2010]. Chemical constituents are saponins [Oshima et al., 1984, Hong-Yu et al., 1994], flavonoids, sterol, glycosides and cyclopeptides [Han et al., 2015, 2014, Hsieh et al., 2004]. Studies on bioactivity have shown various effects. Cyclopeptides for example showed anti-bacterial, anti-fungal, estrogen-like, uterotonic, haemolytic and cardio-toxic effects. The uterotonic effect is the reason why Qu mai (Dianthi herba) should not be prescribed to pregnant women [Wu, 2005]. By selling bamboo tea that actually contains *Dianthus* species, consumers are betrayed. Additionally, if the *Dianthus* species is known to have an effect on the dynamics of the uterus, pregnant women are put in harms way. Our data strongly suggests that leaves found in adulterated bamboo tea are from *D. chinensis* and measures to prevent this kind of misdirections have to be implemented immediately.

Legal Scientific Framework: Article 2 of the European General Food Law Regulation [European Commission, 2002] specifies “food” as any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans. Tea products analysed in this study either consist of different ”substances”, one of which is ”bamboo leaf”, or only contain the latter. Consulting the List of Substances of the Competent Federal Government and Federal State Authorities (german version) for the category ”plants and plant parts”, common names (e.g. apple, lemon and orange) used in ingredient lists of teas are found and mapped to the scientific name of the

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corresponding plant the ”substance” (e.g. fruit) is derived from. The common name bamboo can be mapped to two species of *Dendrocalamus* (*D. asper* and *D. latiflorus*) which are the source for bamboo sprouts. No other entries for bamboo are present. The english version of the mentioned list does not provide associations of common names with scientific names, representing one common unnecessary obstacle consumers and food business operators are confronted with. Since bamboo is an exotic group, we have to assume that corresponding substances used in products fall under the novel food legislation and might be listed in the novel food catalogue.

Foods or food ingredients which have not been used for human consumption to a significant degree in the European Union (EU) before 15 May 1997 are governed by the provisions of the Novel Food Regulation (NFR) [European Commission, 1997]. The Novel Food Catalogue (NFC) (`http://ec.europa.eu/food/safety/novel_food/catalogue/index_en.htm`) lists products of animal and plant origin that are subject to the NFR or are being evaluated in that regard. The information is based on data provided by the EU Member States. It is stated to be a non-exhaustive list and should serve as orientation on whether a product will need an authorisation under the NFR. Analysing the content of the NFC, there are currently (Jun.2016) 6 species of 4 genera mentioned: *Bambusa oldhamii* (listed with the synonym: *Sinocalamus oldhamii*), *Dendrocalamus latiflorus*, *D. asper*, *Gigantochloa albociliata*, *G. levis* and *Phyllostachys edulis*. The immature shoot of these species is used as food substance and according to the NFC none of them are subject to the NFR. Additionally there exists an entry for *Bambusa* species with a status indicating that history of use as a food of bamboo leaves is not known to any Member State and thus, bamboo leaves, if they were to be used as a food might be subject to the NFR and require a safety assessment before they may be placed on the market. According to this statement, based on current scientific data, the leaves of over 1600 species of the *Bambusoideae* (*Poaceae*), if used as ”substance” in tea, put corresponding products in violation of the NFR.

The same is most likely true for leaves of *Dianthus* species, particularly of the species *D. chinensis* which we found in tea products in place of genuine bamboo leaves. Due to their application in traditional Chinese medicine and contraindications for pregnant women, the admissibility as food has to be questioned.

Concluding, the use of the term bamboo for product components has several disadvantages. Firstly, a false impression of identity is promoted. Although the corresponding taxonomic entity has been shown to be monophyletic and offers unique characteristics, the contained morphological diversity deserves recognition beyond the subfamily rank. Secondly, the systematically broad range of the term may be perceived as ignorance and promote intentional adulteration or may lead to additional accidental confusions caused by lack of clarity. Any scientific approach for the safety assessment of botanicals and botanical preparations needs precision in regard of the corresponding taxonomy. Using a too broad approach always will proof to be negligent and impede precise diagnostics. Experience tells us, that we cannot identify all natural units with little effort. To be able to differentiate on a level where genetic markers show coherence between the unit and its inherited chemical profiles - which ultimately is the empirical dimension used to assess safety - systematic knowledge is of primary importance.

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REFERENCES

Alvarez, J. M., Rocha, J. F., and Machado, S. R. (2008). Bulliform cells in Loudetiopsis chrysothrix (Nees) Conert and Tristachya leiostachya Nees (Poaceae): Structure in relation to function. *Brazilian Archives of Biology and Technology*, 51(1):113–119.

Beal, W. J. (1886). The Bulliform or Hygroscopic Cells of Grasses and Sedges Compared. *Botanical Gazette*, 11(12):321–326.

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Bertolazzi, P., Felici, G., and Weitschek, E. (2009). Learning to classify species with barcodes. *BMC bioinformatics*, 10 Suppl 1:S7.

Cai, Z. M., Zhang, Y. X., Zhang, L. N., Gao, L. M., and Li, D. Z. (2012). Testing four candidate barcoding markers in temperate woody bamboos (Poaceae: Bambusoideae). *Journal of Systematics and Evolution*, 50(6):527–539.

CBOL Plant Working Group (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences*, 106(31):12794–12797.

Chandra, S. and Rawat, D. (2015). Medicinal plants of the family Caryophyllaceae: a review of ethnomedicinal uses and pharmacological properties. *Integrative Medicine Research*, 4(3):123–131.

Chaw, S. M., Chang, C. C., Chen, H. L., and Li, W. H. (2004). Dating the monocot-dicot divergence and the origin of core eudicots using whole chloroplast genomes. *Journal of Molecular Evolution*, 58(4):424–441.

Committee, C. P. (2010). Chinese pharmacopoeia. *China Medica Science Press: Beijing, China*.

Dnyaneshwar, W., Preeti, C., Kalpana, J., and Bhushan, P. (2006). Development and application of RAPD-SCAR marker for identification of Phyllanthus emblica LINN. *Biological & pharmaceutical bulletin*, 29(November):2313–2316.

Dong, W., Cheng, T., Li, C., Xu, C., Long, P., Chen, C., and Zhou, S. (2014). Discriminating plants using the DNA barcode rbcLb: An appraisal based on a large data set. *Molecular Ecology Resources*, 14(2):336–343.

Edgar, R. C. (2004a). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC bioinformatics*, 5(1):113.

Edgar, R. C. (2004b). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research*, 32(5):1792–7.

European Commission, T. (1997). Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. *Official Journal of the European Communities*, L43(14/02/97):1–6.

European Commission, T. (2002). Regulation (EC) No 178/2002 of the European Parliament and the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities*, L31(01/02/2002):1–24.

Farrelly, D. (1984). *The book of bamboo: a comprehensive guide to this remarkable plant, its uses, and its history.* Sierra Club Books.

Federici, S., Galimberti, A., Bartolucci, F., Bruni, I., De mattia, F., Cortis, P., and Labra, M. (2013). DNA barcoding to analyse taxonomically complex groups in plants: The case of Thymus (Lamiaceae). *Botanical Journal of the Linnean Society*, 171:687–699.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39(4):783–791.

Han, J., Huang, M., Wang, Z., Zheng, Y., Zeng, G., He, W., and Tan, N. (2015). Cyclopentapeptides from Dianthus chinensis. *Journal of peptide science : an official publication of the European Peptide Society*, 21(7):550–3.

Han, J., Wang, Z., Zheng, Y.-Q., Zeng, G.-Z., He, W.-J., and Tan, N.-H. (2014). New dicyclopeptides from Dianthus chinensis. *Yao xue xue bao = Acta pharmaceutica Sinica*, 49(5):656–60.

Hebert, P. D. N., Cywinska, A., Ball, S. L., and DeWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings. Biological sciences / The Royal Society*, 270(February 2003):313–321.

Hohmann, B. and Gassner, G. (2007). *Mikroskopische Untersuchung pflanzlicher Lebensmittel und Futtermittel : der Gassner*. Behr's Verlag, Hamburg, 6. aufl. edition.

Hong-Yu, L., Koike, K., and Ohmoto, T. (1994). Triterpenoid saponins from Dianthus chinensis. *Phytochemistry*, 35(3):751–756.

Horn, T., Barth, A., Rühle, M., Häser, A., Jürges, G., and Nick, P. (2012). Molecular diagnostics of Lemon Myrtle (Backhousia citriodora versus Leptospermum citratum). *European Food Research and Technology*, 234(5):853–861.

Horn, T., Völker, J., Rühle, M., Häser, A., Jürges, G., and Nick, P. (2013). Genetic authentication by RFLP versus ARMS? The case of Moldavian dragonhead (Dracocephalum moldavica L.). *European Food Research and Technology*, 238(1):93–104.

Hsieh, P.-W., Chang, F.-R., Wu, C.-C., Wu, K.-Y., Li, C.-M., Chen, S.-L., and Wu, Y.-C. (2004). New

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2198v1 | CC BY 4.0 Open Access | rec: 1 Jul 2016, publ: 1 Jul 2016

cytotoxic cyclic peptides and dianthramide from Dianthus superbus. *Journal of natural products*, 67(9):1522–7.

Hsu, H., Chen, Y., Sheu, S., and Hsu, C. (1986). *Oriental material medica - a concise guide*. Oriental Healing Arts Institute.

Huber, E. (2014). Molekulare Authentifizierung von Bambus als Novel Food. Technical report.

Huh, M. K. and Bang, K. H. (2006). Identification of Atractylodes japonica and A. macrocephala by RAPD analysis and SCAR markers. *Silvae Genetica*, 55(1983):101–105.

Jäger, E., Ebel, F., Hanelt, P., and Müller, G. (2008). *Rothmaler Band 5. Exkursionsflora von Deutschland. Krautige Zier-und Nutzpflanzen*. Spektrum Akademischer Verlag.

Jiao, S.-D. (2003). *Ten Lectures on the Use of Medicinals from the Personal Experience of Jiao Shu De*. Paradigm Publications.

Katoh, K. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, 30(14):3059–3066.

Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution*, 30(4):772–80.

Kress, W. J. and Erickson, D. L. (2007). A Two-Locus Global DNA Barcode for Land Plants: The Coding rbcL Gene Complements the Non-Coding trnH-psbA Spacer Region. *PLoS ONE*, 2(6):e508.

Kress, W. J., Erickson, D. L., Jones, F. A., Swenson, N. G., Perez, R., Sanjur, O., and Bermingham, E. (2009). Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. *Proceedings of the National Academy of Sciences of the United States of America*, 106(44):18621–18626.

Lee, M. Y., Doh, E. J., Park, C. H., Kim, Y. H., Kim, E. S., Ko, B. S., and Oh, S.-E. (2006). Development of SCAR marker for discrimination of Artemisia princeps and A. argyi from other Artemisia herbs. *Biological & pharmaceutical bulletin*, 29(April):629–633.

Li, M., Wunder, J., Bissoli, G., Scarponi, E., Gazzani, S., Barbaro, E., Saedler, H., and Varotto, C. (2008). Development of COS genes as universally amplifiable markers for phylogenetic reconstructions of closely related plant species. *Cladistics*, 24(5):727–745.

Li, X., Ding, X., Chu, B., Ding, C., Gu, S., Qian, L., Wang, Y., and Zhou, Q. (2007). Molecular authentication of Alisma orientale by PCR-RFLP and ARMS. *Planta Medica*, 73:67–70.

Liese, W. (2015). *Bamboo : The Plant and its Uses*. Tropical Forestry. Springer, Cham.

Liu, H., Guo, X., Wu, J., Chen, G. B., and Ying, Y. (2013). Development of universal genetic markers based on single-copy orthologous (COSII) genes in Poaceae. *Plant Cell Reports*, 32(3):379–388.

Marieschi, M., Torelli, A., Poli, F., Bianchi, A., and Bruni, R. (2010). Quality control of commercial Mediterranean oregano: Development of SCAR markers for the detection of the adulterants Cistus incanus L., Rubus caesius L. and Rhus coriaria L. *Food Control*, 21(7):998–1003.

Motomura, H., Fujii, T., and Suzuki, M. (2004). Silica deposition in relation to ageing of leaf tissues in Sasa veitchii (Carrière) Rehder (Poaceae: Bambusoideae). *Annals of Botany*, 93(3):235–248.

Nei, M. and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press.

Newmaster, S. G., Grguric, M., Shanmughanandhan, D., Ramalingam, S., and Ragupathy, S. (2013). DNA barcoding detects contamination and substitution in North American herbal products. *BMC medicine*, 11(1):222.

Newton, C. R., Graham, a., Heptinstall, L. E., Powell, S. J., Summers, C., Kalsheker, N., Smith, J. C., and Markham, a. F. (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic acids research*, 17(7):2503–2516.

Oshima, Y., Ohsawa, T., and Hikino, H. (1984). Structures of Dianosides G, H and I, Triterpenoid Saponins of Dianthus superbus var. longicalycinus Herbs1. *Planta medica*, 50(3):254–258.

Peng, S., Yang, H. Q., and Li, D. Z. (2008). Highly heterogeneous generic delimitation within the temperate bamboo clade (Poaceae: Bambusoideae): Evidence from GBSSI and ITS sequences. *Taxon*, 57(3):799–810.

Rambaut, A. (2014). Tree Figure Drawing Tool.

Rohweder, O., Schlumpf, R., and Krattinger, K. (1971). Anmerkungen zum diacytischen Spaltöffnungstyp und zur taxonomischen Bedeutung der Spaltöffnungen im allgemeinen. *Mitteilungen aus dem Botanischen Museum der Universität Zürich*, 5(255):275–285.

Roy, S., Tyagi, A., Shukla, V., Kumar, A., Singh, U. M., Chaudhary, L. B., Datt, B., Bag, S. K., Singh, P. K., Nair, N. K., Husain, T., and Tuli, R. (2010). Universal plant DNA barcode loci may not work in

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2198v1 | CC BY 4.0 Open Access | rec: 1 Jul 2016, publ: 1 Jul 2016

complex groups: A case study with Indian berberis species. *PLoS ONE*, 5(10):e13674.

Sasaki, Y., Komatsu, K., Takido, M., Takeshita, K., Kashiwagi, H., and Nagumo, S. (2007). Genetic profiling of Sasa species by analysis of chloroplast intron between rbcL and ORF106 and partial ORF106 regions. *Biological & pharmaceutical bulletin*, 30(8):1511–1515.

Seberg, O. and Petersen, G. (2009). How many loci does it take to DNA barcode a crocus? *PLoS ONE*, 4(2):2–7.

Soltis, P. S., Soltis, D. E., and Smiley, C. J. (1992). An rbcL sequence from a Miocene Taxodium (bald cypress). *Proceedings of the National Academy of Sciences of the United States of America*, 89(January):449–451.

Soreng, R. J., Peterson, P. M., Romaschenko, K., Davidse, G., Zuloaga, F. O., Judziewicz, E. J., Filgueiras, T. S., Davis, J. I., and Morrone, O. (2015). A worldwide phylogenetic classification of the Poaceae (Gramineae). *Journal of Systematics and Evolution*, 53(2):117–137.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C., and Willerslev, E. (2007). Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Research*, 35(3):e14.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution*, 30(12):2725–9.

Torelli, A., Marieschi, M., and Bruni, R. (2014). Authentication of saffron (Crocus sativus L.) in different processed, retail products by means of SCAR markers. *Food Control*, 36(1):126–131.

Triplett, J. K. and Clark, L. G. (2010). Phylogeny of the Temperate Bamboos (Poaceae: Bambusoideae: Bambuseae) with an Emphasis on Arundinaria and Allies. *Systematic Botany*, 35(1):102–120.

Tscharntke, T., Hochberg, M. E., Rand, T. A., Resh, V. H., and Krauss, J. (2007). Author sequence and credit for contributions in multiauthored publications. *PLoS biology*, 5(1):e18.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., and Rozen, S. G. (2012). Primer3 - new capabilities and interfaces. *Nucleic acids research*, 40(15):e115.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J. A. M. (2007). Primer3Plus - an enhanced web interface to Primer3. *Nucleic acids research*, 35(Web Server issue):71–4.

Veller, C., Nowak, M. A., and Davis, C. C. (2015). Extended flowering intervals of bamboos evolved by discrete multiplication. *Ecology Letters*, 18(7):653–659.

Vieira, R. C., Gomes, D. M. S., Sarahyba, L. S., and Arruda, R. C. O. (2002). Leaf anatomy of three herbaceous bamboo species. *Brazilian Journal of Biology*, 62(4b):907–922.

Wang, C. Z., Li, P., Ding, J. Y., Peng, X., and Yuan, C. S. (2007). Simultaneous identification of Bulbus Fritillariae cirrhosae using PCR-RFLP analysis. *Phytomedicine*, 14:628–632.

Wang, H., Sun, H., Kwon, W. S., Jin, H., and Yang, D. C. (2010). A PCR-based SNP marker for specific authentication of Korean ginseng (panax ginseng) cultivar "Chunpoong". *Molecular Biology Reports*, 37:1053–1057.

Wang, J., Ha, W.-Y., Ngan, F.-N., But, P. P.-H., and Shaw, P.-C. (2001). Application of Sequence Characterized Amplified Region (SCAR) Analysis to Authenticate Panax Species and Their Adulterants. *Planta medica*, 67:781–783.

Ward, J., Gilmore, S. R., Robertson, J., and Peakall, R. (2009). A grass molecular identification system for forensic botany: A critical evaluation of the strengths and limitations. *Journal of Forensic Sciences*, 54(6):1254–1260.

Watanabe, M., Ito, M., and Kurita, S. (1994). Chloroplast DNA phylogeny of Asian Bamboos (Bambusoideae, Poaceae) and its systematic implication. *Journal of Plant Research*, 107(3):253–261.

Weitschek, E., Van Velzen, R., Felici, G., and Bertolazzi, P. (2013). BLOG 2.0: A software system for character-based species classification with DNA Barcode sequences. What it does, how to use it. *Molecular Ecology Resources*, 13:1043–1046.

White, T. J., Bruns, S., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*, pages 315–322. Academic Press, Inc.

Wong, K.-L., But, P. P.-H., and Shaw, P.-C. (2013). Evaluation of seven DNA barcodes for differentiating closely related medicinal Gentiana species and their adulterants. *Chinese medicine*, 8(1):16.

Wu, C. (1962). The classification of Bambuseae based on leaf anatomy. *Bot. Bull. Acad. Sinica.(NS)*, 3:83–108.

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691 Wu, J. N. (2005). *An Illustrated Chinese Materia Medica*. Oxford University Press.

692 Wu, Z., Raven, P., and Hong, D. (2001). Flora of China. Vol. 6: Caryophyllaceae through Lardizabalaceae.
693 *Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis*.

694 Wu, Z., Raven, P., and Hong, D. (2006). Flora of China. Vol. 22: Poaceae. *Science Press, Beijing, and*
695 *Missouri Botanical Garden Press, St. Louis*.

696 Wu, Z., Raven, P., and Hong, D. (2007). Flora of China. Vol. 12: Hippocastanaceae through Theaceae.
697 *Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis*.

698 Yang, D.-Y., Fushimi, H., Cai, S.-Q., and Komatsu, K. (2004). Polymerase chain reaction-restriction
699 fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system (ARMS)
700 analyses of medicinally used Rheum species and their application for identification of Rhei Rhizoma.
701 *Biological & pharmaceutical bulletin*, 27(5):661–669.

702 Yang, H.-Q., Yang, J.-B., Peng, Z.-H., Gao, J., Yang, Y.-M., Peng, S., and Li, D.-Z. (2008). A molecular
703 phylogenetic and fruit evolutionary analysis of the major groups of the paleotropical woody bamboos
704 (Gramineae: Bambusoideae) based on nuclear ITS, GBSSI gene and plastid trnL-F DNA sequences.
705 *Molecular phylogenetics and evolution*, 48(3):809–24.

706 Yang, J. B., Yang, H. Q., Li, D. Z., Wong, K. M., and Yang, Y. M. (2010). Phylogeny of Bambusa and its
707 allies (Poaceae: Bambusoideae) inferred from nuclear GBSSI gene and plastid psbA-trnH, rpl32-trnL
708 and rps16 intron DNA sequences. *Taxon*, 59(4):1102–1110.

709 Zhang, W. and Clark, L. (2000). Phylogeny and classification of the Bambusoideae (Poaceae). *Grasses:*
710 *systematics and evolution*.

711 Zhang, W., Wendel, J. F., and Clark, L. G. (1997). Bamboozled again! Inadvertent isolation of fungal
712 rDNA sequences from bamboos (Poaceae: Bambusoideae). *Molecular phylogenetics and evolution*,
713 8(2):205–17.

714 Zhang, Y.-J., Ma, P.-F., and Li, D.-Z. (2011). High-throughput sequencing of six bamboo chloroplast
715 genomes: phylogenetic implications for temperate woody bamboos (Poaceae: Bambusoideae). *PloS*
716 *one*, 6(5):e20596.

717 Zhang, Y.-X., Zeng, C.-X., and Li, D.-Z. (2012). Complex evolution in Arundinarieae (Poaceae: Bambu-
718 soideae): incongruence between plastid and nuclear GBSSI gene phylogenies. *Molecular phylogenetics*
719 *and evolution*, 63(3):777–97.

720 Zhen, Z. (1995). *Advanced Textbook on Traditional Chinese Medicine and Pharmacology: History, Basic*
721 *Theory, Diagnostics*. New World Press.

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