



Tracing tiger nut (*C. esculentus* L.): functional food from the colossal *Cyperus* genus

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Abstract

Tiger nuts (also called earth almonds or *chufa*) are the stolons of the sedge *Cyperus esculentus* L. and have been traditionally used as food or as an additive to drinks such as *horchata de chufa*. Recently, *chufa* experienced a hype as functional food because of its richness in dietary fiber and as a source of unsaturated fatty acids. Its close relative, nutgrass (*Cyperus rotundus* L.) has similarly long history of human use, but rather for medical purposes, due to a high content of secondary compounds. Both species are invasive weeds responsible for high yield losses in crop plants, especially in Southeast Asia, Africa, and subtropical America. They are not easily discerned, occur together, and are both traded under similar or identical vernacular names. For the purpose of consumer protection, it is important to discriminate them in commercial samples, which is not trivial by morphological markers and often impossible since they are mostly used in highly processed form. Therefore, a molecular authentication assay was developed using taxonomically authenticated plants as reference material. The plastidic marker *psbA-trnH* *igs* (*intergenic spacer*) and the nuclear marker *Internally Transcribed Spacer* were used for barcoding. To develop a sequencing-free fingerprinting assay, a single-nucleotide polymorphism in the *ITS2* region could be employed to discriminate *C. esculentus* from its possible adulterant *C. rotundus* by a tailored Amplificatory Refractory Mutation System PCR. This approach was successful both, in reference to plants, as well as in highly processed commercial products such as tiger nut flour. We show that by a single PCR we can detect surrogation by nutgrass down to a threshold of 10% in tiger nuts as contribution to safeguard product authenticity against possible adulterations in a growing global market.

Keywords *Cyperus* · *Chufa* · Authentication · *psbA-trnH* · ITS · ARMS

Introduction

Tiger nut (*Cyperus esculentus* L.) or the closely related nutgrass or red nut sedge (*Cyperus rotundus* L.) is a curse and a blessing at the same time—depending on the perspective. As persistent weeds, they cause massive losses in crop yield every year with an effective control strategy still urgently needed [1]. On the other hand, both species have a long history of human use, although the applications differ, while *C. esculentus* is mainly used for its nutritional value (for reviews see [2, 3]. *C. rotundus* is less palatable, but richer

in secondary compounds and, thus, more used as medicinal plant), for review, see [4].

The subterranean edible stolons of *C. esculentus*, called tiger nuts or earth almonds, were cultivated already 6000 years ago in the African Mediterranean area [5], and are still important as food or medicine to the present day. The “grains of Al-Aziz” (*Hab’ el aziz*—the current Egyptian name [6]) are used in different countries: as part of *Horchata de chufa* (powder of stolons with water and sugar resulting in a refreshing drink) in Spain [7], as addition to local beverages (like *Kunnu*) in Nigeria and Ghana to increase their nutritional value [8], as general remedy to prevent malnutrition [9], or lately, popularized in Europe in form of complete stolons or flour that can be added to several dishes as functional food. For such applications, only the species *C. esculentus* is used. This species is morphologically variable with a blurred border between domesticated and wild or even weedy types. The domesticated forms are referred to as *var. sativus* [10]. More recently, the term *var.*

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chufa has been proposed [2]. In addition, several wild and weedy forms with distinct geographic distributions have been described. A thorough taxonomic study by Schippers et al. [11] arrives at four forms, namely *C. esculentus* var. *esculentus*, *C. esculentus* var. *leptostachyus* (Boeckler), *C. esculentus* var. *hermannii* ((Buckley) Britton), and *C. esculentus* var. *macrostachyus* (Boeckler). Some authors also delineate *C. esculentus* var. *esculentus* as domesticated form from *C. esculentus* var. *sativus* [Flora of China, efloras.org]. While the tastes of the domesticated and the wild or weedy forms were shown to be very similar during a comparative study [2], the domesticated var. *sativus* was more enjoyable because it was found to be less fibrous [12]. A comparison between two accessions of *C. esculentus* from Nigeria that differed in stolon color [13] found a comparable abundance of essential elements, fatty acids, and carbohydrates [9, 13]. While it is not clear whether these forms belonged to the same type of *C. esculentus*, the Nigerian study suggests that intraspecific variation apparently does not have big effect on nutritional composition. This is good news for consumer protection, narrowing the target for authentication to the species level (*C. esculentus*), not calling for the need to differentiate within this species.

However, there is a clear need to draw a line between *C. esculentus* and *C. rotundus*, although the contents of bulk nutrients are comparable: Values for carbohydrates in *C. esculentus* range from around 45% [8] to 59% [13], comparable to the 59% reported for *C. rotundus* [16]. For lipids, values from 24% [13] to 35% [14] have been found in *C. esculentus* versus 24% in *C. rotundus* [16]. For protein content, 4% [15] to 7% [14] in *C. esculentus* seem to be comparable to the 4.7% reported for *C. rotundus* [16]. In contrast, the two species differ significantly when the composition of essential oils is considered. The main component of *C. esculentus* is oleic acid (some 70%), which is comparable to olive oil [8, 15]. In contrast, *C. rotundus* shows drastically lower contents of oleic acid, only around 5% [17]. Further differences concern palmitic acid that accounted for ~15% of the essential oil in *C. esculentus* [8, 15]), but was not even detectable in *C. rotundus* [17]. An inverse situation was seen for linoleic acid, which was significantly lower, around 8%–9%, in *C. esculentus* [8, 15] but very abundant, >20%, in *C. rotundus* [17].

Thus, while the two species are similar in nutritional values, they already differ qualitatively in oil composition. These differences are even more pronounced if the secondary compounds are considered that are linked with the rich ethnomedicinal tradition for *C. rotundus* (for review see [4]). As often in traditional medicinal plants, there are numerous applications, especially in Ayurvedic and other Indian systems of medicine, and the link with the respective active compound is usually unknown (for review, see [18]). However, a few associations have been investigated in more

detail, for instance phytoestrogenic activities attributed to β -sitosterols [19] that would explain the use of *C. rotundus* to cure menstruation-related problems in women [20]. A second group of compounds are phenylpropanoids, such as quercetin or chlorogenic acid that might be responsible for the effect of *C. rotundus* against inflammatory diseases of the skin [21]. These medically active secondary compounds are not seen in *C. esculentus*. As long as it is clear that *C. rotundus* is used in a medical context, where dosage and formulation are controlled by a protocol of use, the use of this species is to be seen as beneficial. The problem arises when this plant becomes isolated from its traditional context of healing and is shifted into a different context, namely, that of *C. esculentus*, which is used for nutrition. Thus, for the sake of consumer safety, it is important that commercial products from the two species are declared correctly.

This task is far from trivial since the colossal genus *Cyperus* harbors around 950 species, with newly described species added to this day, such that the taxonomy on *Cyperus* has remained under continuous revision [22]. This is partially due to the limited set of available morphological traits, rendering taxonomic identification often difficult and ambiguous [23]. Further confusion is added by the fact that many species have been described several times under different names. The number of synonyms listed for certain *Cyperus* species in the Kew Garden Plant List (now World Flora Online) is overwhelming: for *C. esculentus*, there are 51 synonyms, for *C. rotundus* even 76 [theplantlist.org]. Of special interest is the occurrence of a monophyletic subclade of *Cyperus* with around 760 species that share C_4 photosynthesis as synapomorphy and comprise both, *C. esculentus* and *C. rotundus* [24], while the species with C_3 photosynthesis seem to be paraphyletic. These phylogenetic relationships are supported by the plastidic marker *psbA-trnH* *igs* [24], but also by the nuclear markers *ETS1f* [24], and *ITS* [25, 26], as well as by complete chloroplast genome data [27].

However, the full diversity of *Cyperus* is not yet covered by genetic barcoding markers in public databases. For instance, in GenBank, so far only 459 sequences for *ITS* and 362 for *psbA-trnH* *igs* have been deposited (status November 2021), among those certain species are represented with many replicates, others only with one. While a couple of genetic barcoding marker regions are used commonly and have been validated for their universality, ease of sequencing, and discriminative power [28, 29], a region that works equally well in all land plants has not been found yet (and mostly likely never will). Hence, which marker is suitable, needs to be tested individually for the taxon of interest. In plants, nuclear and plastidic genome-based barcodes are used preferentially, while mitochondrial markers, common in animal barcoding, are underrepresented. Among the plastidic markers, *psbA-trnH* *igs* has been very powerful, because it is sufficiently universal and often allows

discrimination down to the species level [28, 30]. In the monocotyledons, this region is around 350 nucleotides in average, but can reach fragment sizes up to 900 nucleotides [31]. Since the nuclear *ITS* marker has been widely applied to the genus *Cyperus* in many previous studies, such that a rich body of information is available, we integrated this marker into our study as well.

For practical applications, DNA fingerprinting methods that do not require sequencing, are preferable. However, methods, such as the use of arbitrary primer pairs, so called RAPD [32] often suffer from drawbacks that impinge on reproducibility. The lack of sequence information has the consequence that annealing temperatures of RAPD primers need to be low and, thus, are prone to target ambiguity [32]. Fingerprinting strategies that use genetic barcoding data can circumvent these specificity issues. The use of Restriction Fragment Length Polymorphism (RFLP) allows to generate species-specific banding pattern by treating amplicons of the respective barcoding marker with an appropriate restriction enzyme. This approach successfully allowed to discriminate between two different plant species of the Myrtaceae family that are both commercialized under the term “Lemon Myrtle” [33], and the same strategy was even able to discern different chemotypes of Holy Basil [34]. As alternative to RFLP, single-nucleotide Polymorphisms (SNPs) can be used to design a duplex PCR with the addition of a diagnostic primer that is tailored such that it will bind only in one species, but not in the other. This strategy, termed Amplification Refractory Mutation System (ARMS), provides the

advantage that the full-length amplicon of the barcoding marker serves as a positive control for the success of the PCR as such [35]. In fact, the ARMS method was successfully applied for the identification and authentication of Goji fruits [30], or detecting adulterations of Bamboo Tea [36].

In the current study, we addressed the problem to discriminate *C. esculentus* and *C. rotundus* in commercial samples. We started off by (1) constructing a robust framework of authenticated reference plants for molecular barcoding, then (2) implemented the sequences generated from this set of reference plants into a phylogenetic context, which finally (3) allowed applying this context to authenticate the declared identity of commercial samples. Finally, we developed and validated a robust and convenient ARMS PCR-based assay to confirm the presence of *C. esculentus* and to detect possible adulteration by *C. rotundus* in processed commercial products.

Materials and methods

Plant material

A collection of reference plants for *Cyperus* was assembled in the Botanical Garden of the KIT (Table 1) and is maintained as living voucher specimens. Commercial products of tiger nut (traded as dried or powdered stolons) were sampled from supermarkets and online stores (Table 2).

Table 1 Accessions of *Cyperus* reference plants used in this study, identified by determination keys based on morphological traits, nomenclature updated according to World Flora Online

Voucher KIT	Taxon	GenBank psbA-trnH	GenBank ITS
UNKAR1693	<i>Cyperus diffusus</i> Vahl	OR523166	OR528046
UNKAR3703	<i>Cyperus esculentus</i> L	OR523168	OR528048
UNKAR3704	<i>Cyperus rotundus</i> L	OR523178	OR528057
UNKAR3705	<i>Cyperus iria</i> L	OR523174	OR528054
UNKAR3752	<i>Cyperus involucratus</i> Rottb	OR523172	OR528052
UNKAR5723	<i>Cyperus papyrus</i> L	OR523176	OR528056
UNKAR6739	<i>Oenanthe silaifolia</i> M. Bieb	OR523190	OR528065
UNKAR6740	<i>Cyperus papyrus</i> L	OR523177	–
UNKAR7051	<i>Cyperus involucratus</i> Rottb	OR523173	OR528053
UNKAR7266	<i>Cyperus involucratus</i> Rottb	OR523181	OR528059
UNKAR9377	<i>Cyperus esculentus</i> L	OR523169	OR528049
UNKAR9378	<i>Cyperus esculentus</i> L	OR523170	OR528050
UNKAR9379	<i>Cyperus iria</i> L	OR523175	OR528055
UNKAR9380	<i>Cyperus esculentus</i> L	OR523171	OR528051
UNKAR9385	<i>Cyperus strigosus</i> L	OR523183	OR528061
UNKAR9409	<i>Prunus dulcis</i> (Mill.) D.A. Webb	OR523191	OR528066
UNKAR9565	<i>Cyperus rotundus</i> L	OR523179	OR528058
UNKAR9566	<i>Cyperus rotundus</i> L	OR523180	–
UNKAR9567	<i>Cyperus squarrosus</i> L	OR523182	OR528060
UNKAR9568	<i>Cyperus eragrostis</i> Lam	OR523167	OR528047

Table 2 List of commercial products of tiger nut purchased for this study

ID KIT	Product code	ncbi ID psbA-trnH	ncbi ID ITS
CP1	Commercial Product 1	OR523184	OR528062
CP2	Commercial Product 2	OR523185	–
CP3	Commercial Product 3	OR523186	–
CP4	Commercial Product 4	–	–
CP5	Commercial Product 5	OR523187	OR528063
CP8	Commercial Product 8	OR523188	OR528064
CP9	Commercial Product 9	OR523189	–

The correct identity of the reference plants is crucial for any authentication approach and, therefore, all reference plants were taxonomically determined using published taxonomic keys [37], Flora of China, <http://www.efloras.org>, as well as the virtual herbarium of Valdosta State University (<https://herb.valdosta.edu>). This allowed to verify, in some cases also to revise, the declared identity of these plants [38]. As potential surrogates, almond (*Prunus dulcis*, Rosaceae), which is often traded in mixtures with tiger nut and in Germany shares the vernacular name (*Mandel* versus *Erdmandel*), as well as *Oenanthe silaifolia* (Apiaceae) forming edible, radish-sized stolons of nutty taste, thus, resembling tiger nut, were included into the study as well, even though they belong to other plant families and are not even monocots.

DNA extraction

DNA from fresh leaves of *Cyperus* reference plants (using 50 mg of starting material) was isolated using the Invisorb R Spin Plant Mini Kit (Stratec Biomedical AG), following the instructions of the manufacturer.

The processed commercial products were not amenable to DNA extraction using this kit; hence, those samples were extracted using cetyl tri-methyl ammonium bromide (CTAB) according to [39] with some modifications given in [33]. In brief, the leaf tissue was shock-frozen in liquid nitrogen, and small aliquots (100 mg) were ground using a Tissue-Lyzer (Qiagen, Hilden, Germany). The resulting powder was mixed with 900 μ L of boiled extraction buffer (1.5% w/v CTAB) containing 10 μ L/mL β -mercaptoethanol, and incubated for one hour at 65 °C. Subsequently, the samples were mixed with 630 μ L of chloroform/isoamylalcohol (24:1), shaken horizontally for 15 min, and then spun down for 10 min (17,000g) at room temperature. The upper aqueous phase (which contains the DNA) was transferred into a fresh 2-mL reaction tube, and the DNA was then precipitated with 2/3 v/v of ice-cold isopropanol and subsequently collected by centrifugation (10 min, 17,000g). The sediment was washed with 1 mL 70% EtOH, dried in a vacuum centrifuge

Table 3 List of primers used in this study

Name	5' → 3' sequence	References
psbA	GTTATGCATGAACGTAATGCTC	[44]
trnH	CGCGCATGGTGGATTCAACAATCC	[45]
Sef17 (ITS)	ACGAATTCATGGTCCGGTGAAGTG T	[26]
Ser26 (ITS)	TAGAATCCCCGGTTCGCTCGCCG TTAC	[26]
S2F (ITS2)	ATGCGATACTTGGTGTGAAT	[43]
ITS_CE3_fw	CACTGGCCCTCCGAGCCGCTGG	This study
ITS_CR1_fw	CAGTGGCCCTCCGAGCCGCGTA	This study

for 15 min, and finally dissolved in 50 μ L nuclease-free H₂O (containing 5 μ g RNase A). Concentration and purity of all DNA samples were determined spectrophotometrically (NanoDrop ND-100, Peqlab).

PCR/gel electrophoresis

As barcoding marker regions, the plastidic *psbA-trnH* *igs* and the nuclear *Internally Transcribed Spacer (ITS)* were amplified in a 30 μ L reaction volume containing 20.4 μ L nuclease-free water (Lonza, Biozym), 3 μ L tenfold Thermopol Buffer (500 mM KCl, 100 mM Tris–HCl and 15 mM MgCl₂), 3 μ L bovine serum albumin (10 mg/mL), 0.6 μ L dNTPs (200 μ M, New England Biolabs), 0.6 μ L of forward and reverse primer (200 nM, see primer list in Table 3), 0.3 μ L of Taq polymerase (5 Units, New England Biolabs), and 1.5 μ L of the extracted DNA as template (50 ng/ μ L).

The *psbA-trnH* *igs* region was amplified by initial denaturation at 95 °C for 2 min, followed by 35 cycles with denaturation at 94 °C for 1 min, annealing at 56 °C for 30 s, synthesis at 68 °C for 45 s, and a terminal extension at 68 °C for 5 min prior to storage at 10 °C. The *ITS* region was amplified using a modified protocol [26] starting with a denaturation at 95 °C for 2 min, followed by nine cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min (decreasing by 0.7 °C during each cycle), and synthesis at 68 °C for 1 min. This was continued by 19 cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and synthesis 68 °C for 1 min, followed by a terminal at 68 °C for 7 min, and storage at 4 °C.

The amplicons were evaluated by agarose gel electrophoresis (NEEO ultra-quality agarose, Carl Roth, Karlsruhe, Germany), visualizing the DNA bands by Midori Green (NIPPON Genetics Europe) and blue light excitation (Safe Imager, Invitrogen). The fragment sizes of the amplicons were determined by a comparison with a 100-bp size standard (New England Biolabs). The amplified DNA was purified using the MSB Spin PCRapace kit (Stratec). Sequencing was outsourced to MacroGen Europe (the Netherlands) and

Eurofins (Germany). The quality of the obtained sequences was examined using the software FinchTV Version 1.4.01.

Phylogenetic analysis

For the analysis and trimming of the sequences, the MEGA7 software (Version 7.0.14) was employed [40]. A multiple sequence alignment was constructed using the MUSCLE algorithm of MEGA7, and the evolutionary relationships were inferred by using the Neighbor-joining algorithm with a bootstrap value that was based on 1000 replicates [41, 42]. The species *Prunus dulcis* (almond) and *Oenanthe silaifolia* served as outgroups to root the phylogenetic tree and to contextualize the genus *Cyperus*.

Amplified refractory mutation system (ARMS)

Based on single-nucleotide polymorphisms in the *ITS2* sequences that were unique for either *C. esculentus* or *C. rotundus*, diagnostic primers were designed to trace either of these species. Primer design was performed as described in [30].

For the ARMS diagnostics, the universal *ITS2* primers S2F and Ser26 (Table 3) were supplemented by one of those diagnostic primers, respectively, to enable a duplex PCR that should result in the amplification of two fragments for

the species of interest, while the alternative species should yield only one band. This approach was also used to verify the identity of the commercial tiger nut products. As to trace tiger nut (*C. esculentus*), the species-specific ITS_CE3_fw primer was used, for detection of the surrogate, nut grass (*C. rotundus*), the specific ITS_CR1_fw primer was added instead. The PCR protocol and the reaction mix were as described above, except for the addition of 0.2 µL of the respective diagnostic primers in addition to the primers amplifying the entire *ITS* fragment see above.

Results

Stolon morphology in reference plants and commercial products is variable

The stolons of *Cyperus esculentus* varied in size and form, which can be seen in the reference plants as well as in the commercial products. Within the available reference plants, three types were found: round stolons of pale yellow color as in ID9378; crescent-shaped stolons of pale yellow color as in ID3703; and crescent-shaped stolons of dark color as in ID9380 (Fig. 1). The stolons of commercial products (as judged from those with intact organs that had not been processed to a powder) resembled the round, pale yellow type

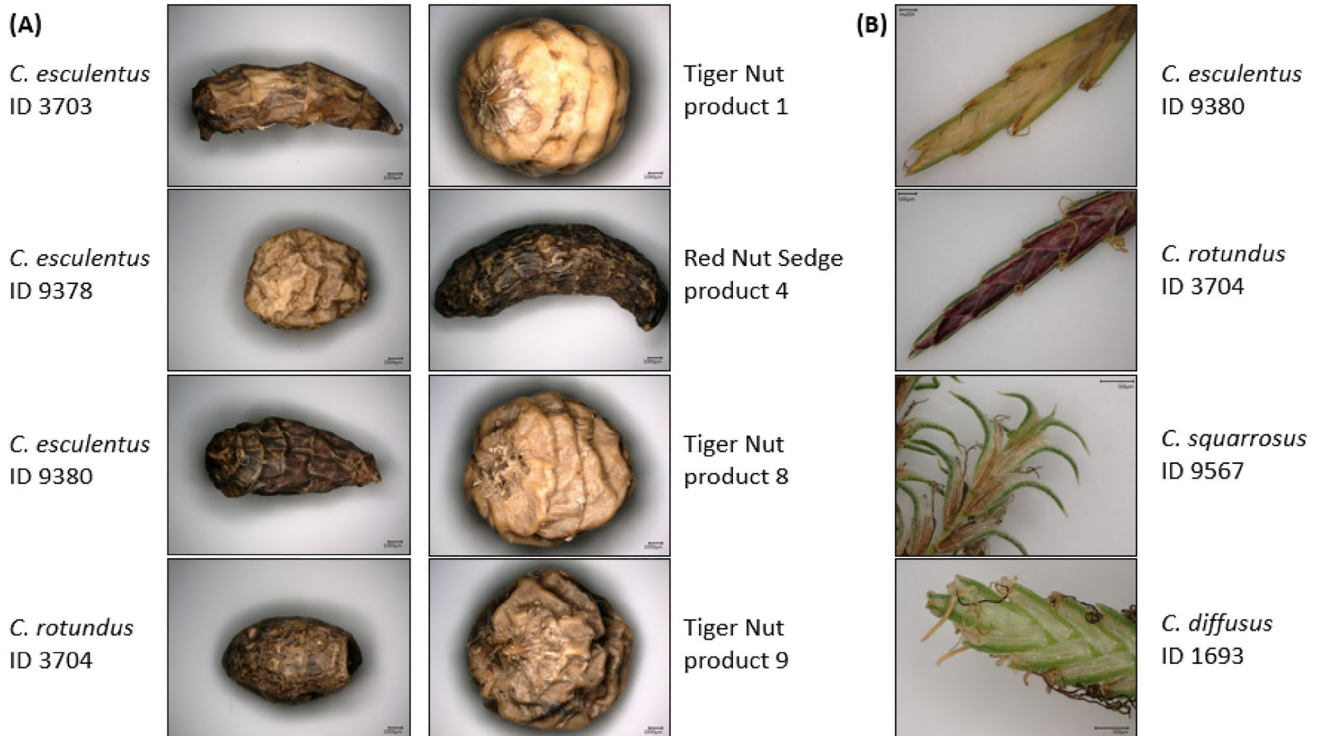


Fig. 1 **A** Morphological variability of stolons from *Cyperus esculentus* and *Cyperus rotundus*. Comparison to commercially available products. Scale bar: 1 mm. **B** Floral traits of *Cyperus* species, among other traits used to assign the species identity. Scale bars: 500 µm

exemplified by ID9378, but were significantly larger. The stolons of ID3704 (*Cyperus rotundus*) were round as well, but of brown color.

The *psbA-trnH* igs marker is long in *Cyperus* and shows a tandem repeat specific for *C. esculentus*

The length of the *psbA-trnH* igs sequences in the genus *Cyperus* ranges from 915 base pairs (after trimming the primer sequences) in the four *Cyperus esculentus* accessions, to slightly shorter fragments in C₃ plants with 875 base pairs in *Cyperus diffusus* to 891 base pairs in *Cyperus eragrostis*. The fragments from the two outgroups were conspicuously shorter, with 325 base pairs in almond (*Prunus dulcis*) and 223 base pairs in *Oenanthe silaifolia*. The multiple sequence alignment of the *Cyperus* accessions including the commercial products had a complete length of 1008 nucleotides with a low G/C content of 23.9% in average. The alignment revealed a tandem repeat which was unique for *C. esculentus* and tiger nut products (Supplementary Figure 1). For all other *Cyperus* species that were part of this study, this motif, of 14 base pairs in length, occurred only once,

while in *C. esculentus* it was doubled to 28 nucleotides in total, which is one reason for the slightly enhanced length of the *C. esculentus* sequences. This sequence pattern can be employed to unequivocally discriminate the earth almond from its close relatives, like *C. rotundus* or *Cyperus iria*.

The *psbA-trnH* igs marker separates C₃ and C₄ *Cyperus* and confirms commercial tiger nut products

The phylogenetic tree inferred from the *psbA-trnH* igs dataset reflected the divergence between C₃ and C₄ *Cyperus* and placed the surrogate species almond (Rosaceae) as well *Oenanthe silaifolia* (Apiaceae) as outgroups (Fig. 2). Within the C₄ clade, the accessions from *Cyperus esculentus* reference plants and the six tested commercial products were clearly separated from a sister clade including, among others, *C. rotundus* and *C. iria*. Even though the commercial products were partially heavily processed, and the fragment length, with around 1000 base pairs, was fairly large, the fragments could be amplified and sequenced readily. Since all six commercial products not only cluster with the four reference plants of *C. esculentus* but show the unique 14-bp

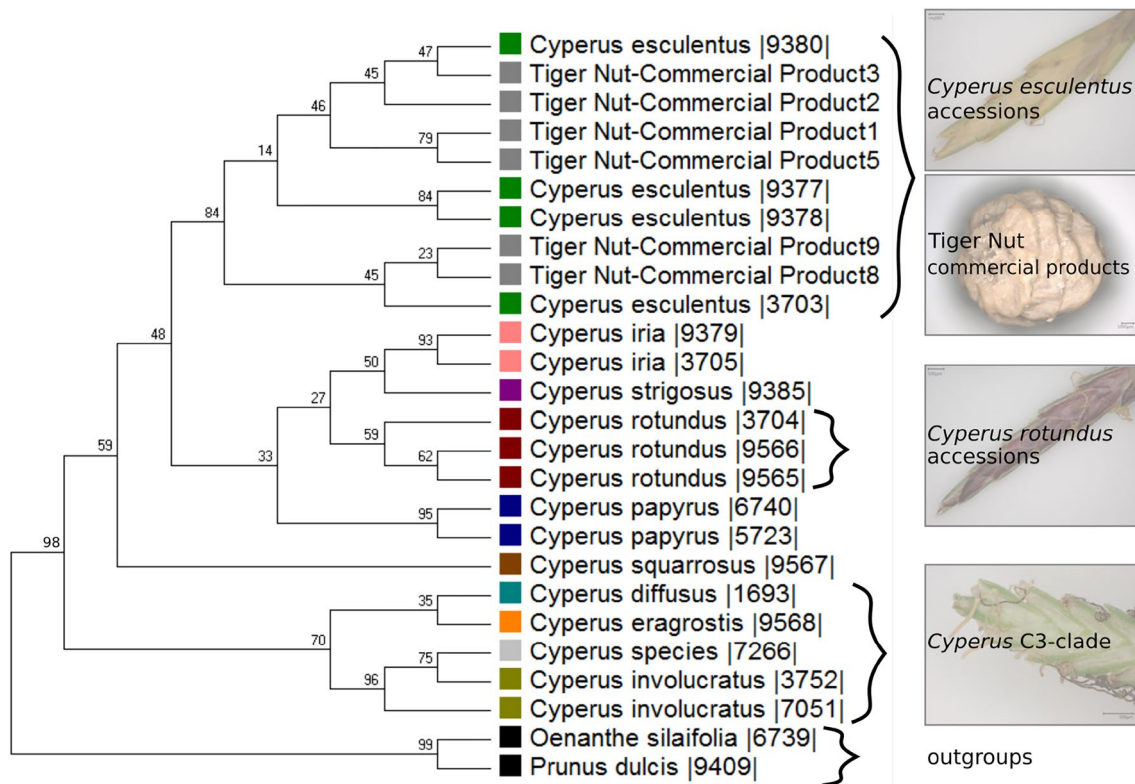


Fig. 2 Phylogenetic tree of the plastid *psbA-trnH* igs comparing selected *Cyperus* reference plants and commercial products of tiger nut. The evolutionary history was inferred using the Neighbor-Joining method [42]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are

shown next to the branches [41]. *Oenanthe silaifolia* and *Prunus dulcis* form the outgroup. The reference plants of *Cyperus* are labeled by colored squares, e.g., *Cyperus esculentus* (green), *Cyperus rotundus* (red). The evaluated tiger nut commercial products are labeled by gray squares

tandem repeat, they were found to be true tiger nut. Moreover, no sequence ambiguities were observed in the products that could point to possible admixtures. Nevertheless, for the design of a sequencing-free identification assay using ARMS, this repeat is not suited since it almost completely consists of the bases A and T, such that the introduction of destabilizing bases, a central feature of the ARMS strategy, is not possible.

The Internal Transcribed Spacer is informative and allows for identification of *Cyperus*

The barcoding marker region *Internal Transcribed Spacer* could be amplified and sequenced for all reference plants. However, this was not fully successful for the commercial products. In fact, only for one commercial product the complete *ITS* region (comprising *ITS1*, *5.8S* rDNA, and *ITS2*) could be amplified and sequenced successfully. For two other products, only the *ITS2* region could be sequenced.

The *ITS* sequences were longer for the C_3 species such as *C. diffusus*, *C. eragrostis*, and *C. involucratus* with 599, 594, and 593 base pairs after trimming the primer recognition sites, respectively. For the C_4 plants, *C. esculentus* (550) and *C. iria* (563), they were shorter. The *ITS2* fragments recovered from the commercial products 1 and 5, declared as tiger nut, spanned 288 base pairs for both products. Also those partial sequences were included into the dataset that

was used to infer the phylogenetic tree. The length of the two outgroups *Prunus dulcis* (613 base pairs) and *O. silaifolia* (600 base pairs) is similar to the *Cyperus* accessions, however, as dicots due to their large evolutionary distance from the monocot *Cyperus*, the sequences were, not surprisingly, quite deviant. For the four *C. esculentus* accessions, significant intraspecific differences were detected (12 parsimonious informative sites in 550 base pairs). However, there are still diagnostic SNPs common to all these accessions that differ from closely related *Cyperus* species, especially *C. rotundus*. Two SNPs were exclusively found in *C. esculentus* over all tested accessions. Thus, these SNPs qualified as diagnostic trait against all other *Cyperus* species tested in this study. The dataset of the multiple sequence alignment used for inferring the phylogenetic relationship comprises 672 base pairs. The resulting tree places the Rosaceae species *P. dulcis* and the Apiaceae species *O. silaifolia* as outgroups (See Fig. 3), and clearly divides *Cyperus* into a C_3 clade from a C_4 clade. However, the position of some species within the C_4 clade differed from the topology based on the *psbA-trnH* *igs* marker. For instance, *C. iria* was now a sister to *C. esculentus*, while for the *psbA-trnH* *igs*, it had been *C. rotundus* that emerged as sister. Furthermore, the positioning of *C. strigosus* differed significantly. This species had been located with the *C. iria* accessions in the *psbA-trnH* *igs* tree but is now basal to the entire C_4 clade. Both sequences had been generated from the same DNA sample ruling out

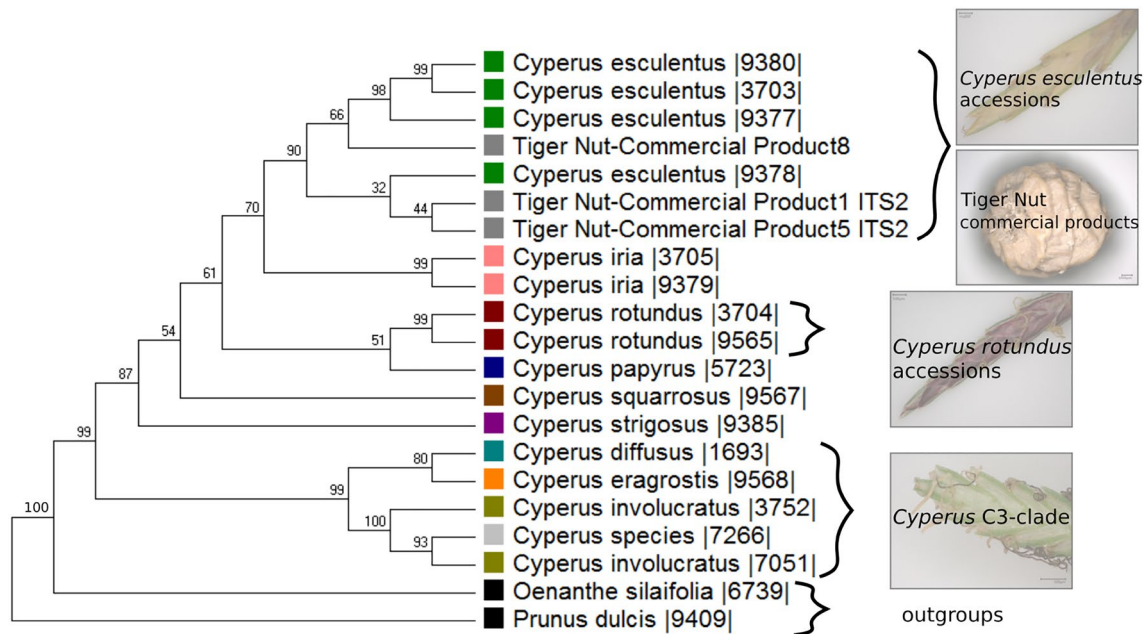


Fig. 3 Phylogenetic tree of the nuclear *ITS* region of selected *Cyperus* reference plants and commercial products of tiger nut. The evolutionary history was inferred using the Neighbor-joining method [42]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown

next to the branches [41]. *Oenanthe silaifolia* and *Prunus dulcis* form the outgroup. The reference plants of *Cyperus* are labeled by colored squares, e.g., *Cyperus esculentus* (green), *Cyperus rotundus* (red). The evaluated tiger nut commercial products are labeled by gray squares

mislabelling of samples. Moreover, both *psbA-trnH* *igs* and *ITS* had been assigned as *C. strigosus* during a BLAST search in GenBank.

The varying topologies using nuclear or plastidic markers in the context of *Cyperus* should be kept in mind when constructing phylogenetic trees and infer the evolutionary relationships of this genus. While all reference plants of *Cyperus esculentus* as well as the three sequenced commercial products cluster together, despite the presence of intraspecific variations, the overall topology inferred for *Cyperus* depends on the type of donor genome (plastidic versus nuclear).

Using species-specific signatures for sequencing-free discrimination of closely related *Cyperus* species

Although there were some difficulties with amplifying and sequencing, the *ITS* region was more suitable for the ARMS approach mainly because the GC content was higher as compared to *psbA-trnH* *igs*, which facilitates tailoring diagnostic primers. One of the diagnostic nucleotide substitutions in the *ITS2* region was used to design species-specific primers. In *Cyperus esculentus*, the *ITS2* fragment (including the complete *ITS2* region and parts of the 5.8S and 28S rDNA) using the primers listed in Table 2 for amplification has a length of 437 base pairs (including fw and rv primers), whereas the fragment in *C. rotundus* is slightly shorter with 425 base pairs. The *ITS2* fragment in *C₃* species of the genus *Cyperus* is slightly larger with 466 base pairs. The decisive SNP (a Guanine) that is diagnostic for *C. esculentus* is positioned at site 164, resulting (together with the length of the primer) in a second (diagnostic) fragment with a length of 294 base pairs, which can be clearly discerned from the full-length amplicon. In fact, a duplex PCR including primers flanking the *ITS2* region along with the diagnostic primer differentially annealing with the species-specific SNP, yielded a second diagnostic band (additionally to the *ITS2* fragment) for all four reference plants of *Cyperus esculentus* (the specific signal is shown for two individuals in Fig. 4b). This diagnostic band was absent in all other species of *Cyperus* that were part of this study, including the closely related species *C. rotundus* and *C. iria*. In the commercial products CP1, CP2, CP3, CP5, CP8 and CP9, the second band could be amplified as well. In contrast, commercial product CP4 lacks this band, even though the full-length *ITS2* amplicon is present demonstrating that the PCR had been successful. While this showed that product CP4 is very unlikely to be *C. esculentus*, it does not tell us, what other species is in the product. We developed, therefore, a complementary assay, using the CR1 primer that is diagnostic for *C. rotundus* and, in fact, generates a second band in *C. rotundus* reference plants (See Fig. 4c). This primer did not amplify such a diagnostic band, neither in the reference plants of *C. esculentus*, nor in the

commercial products that had been verified as *C. esculentus*, further supporting that the closely related *C. rotundus* was absent from those commercial products. However, sample CP4 that had turned out to be not *C. esculentus*, showed the second band with primer CR1, which marks the presence of *C. rotundus*. For both diagnostic primers, an unexpected additional side band was generated at about 1000 bp, that are, however, only amplified in the desired species providing an even clearer signal for *C. esculentus* or *C. rotundus*, respectively.

To determine the detection limit of these ARMS assays, we prepared artificial mixtures of *C. esculentus* and *C. rotundus* DNA, thus simulating surrogations in commercial products that would be otherwise hard to identify, especially in powders. An artificial mixture of 90% *C. esculentus* and 10% *C. rotundus* DNA still showed the second diagnostic band using the CR1 primer, which means that admixtures of *C. rotundus* can be picked up down to 10% (See Fig. 4d).

Discussion

Globalization has not only an economic, but also a botanical component. Plants and plant products are traded globally, entering novel markets. Wealthy, but aging societies develop an increasing awareness for health, while on the other hand, the pressure for economic performance fuel self-optimisation. Both developments drive a boom for functional foods that are consumed for their presumed effect upon human health in the first place. Many of these functional foods originate from traditional medicinal systems, such as Ayurveda, Unani, or Traditional Chinese Medicine that employ food as tools to prevent disease. When these plants are separated from their traditional cultural context and become traded globally, this can result in confusion, or even consumer deception. The attempt to regulate the flow of these new and exotic products by legislation has not been overly successful as illustrated by the fact that meta-studies on adulteration of plant products arrive at estimates of > 25% of cases, where the declaration conflicts with the actual content of a sample (for review, see [46]).

Adulteration is facilitated by situations, where products are entering new markets, whose demand is increasing dynamically, such that production cannot keep pace. These criteria are met by the tiger nut market. The global import volume for tiger nut in 2021 was estimated to range around 2400 million US dollars with a yearly growth rate of close to 20% [47]. Main importer, with around 690 million US dollars, is still the US, but there, growth seems saturated with a five-year increase of only 3.9%. In contrast, China (559 million US dollars, five-year increase of > 150%) and Germany (229 million US dollars, five-year increase of 62%) emerge as new major importers. Under these circumstances,

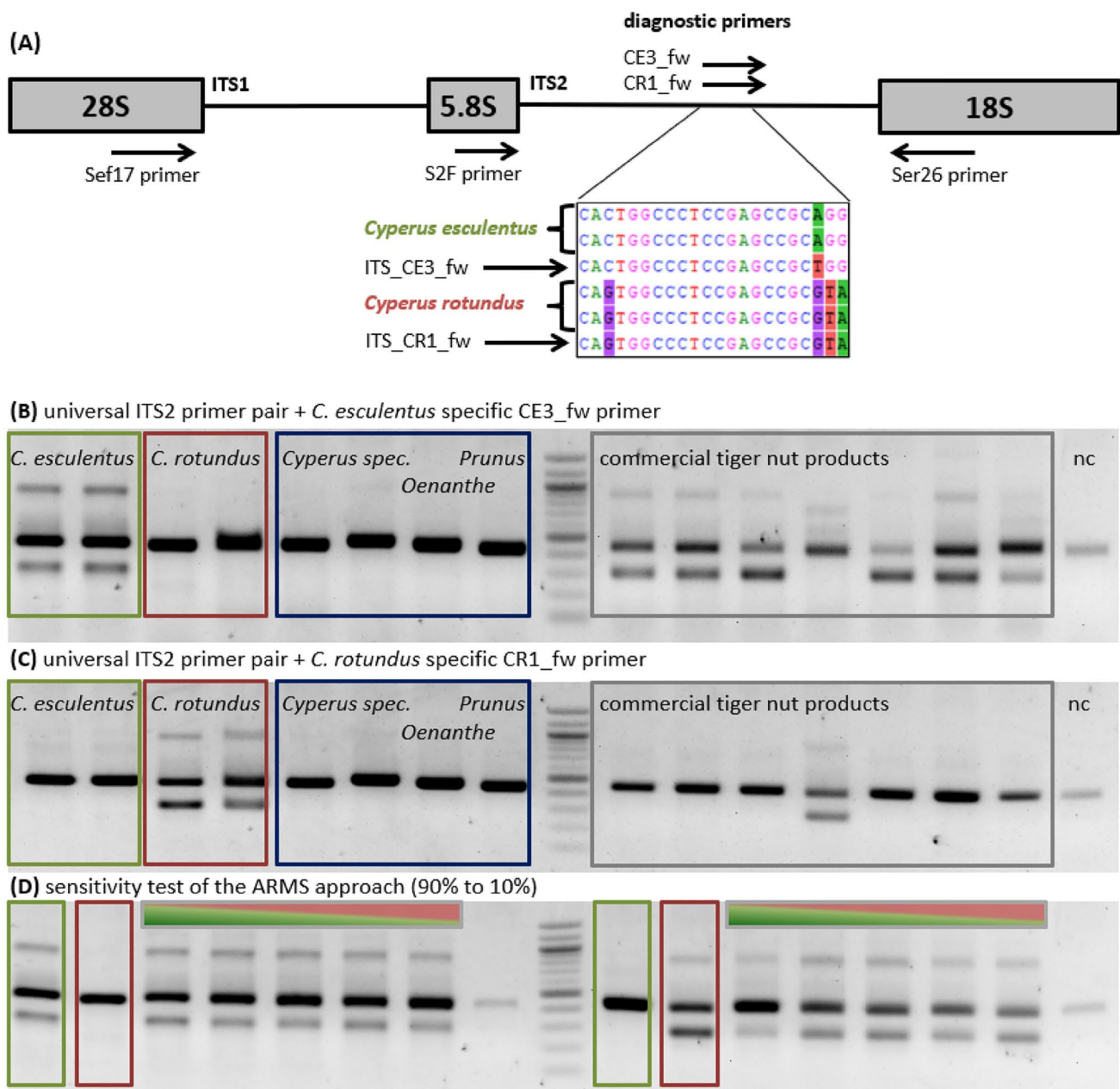


Fig. 4 **A** Schematic illustration of the internal transcribed spacer (ITS) region and orientation of primers used in this study. The decisive nucleotide substitutions were detected in the ITS2 region of *Cyperus* and sequence cutouts of *Cyperus esculentus* and *Cyperus rotundus* as well as the primer sequences are highlighted. **B** ITS2-based agarose electrophoresis of ARMS amplicons from *C. esculentus* (green), *C. rotundus* (red) references and four outgroups (blue, including *C. iria*, *C. strigosus* and *Oenanthe* and *Prunus*). PCR-result using this diagnostic test including all *Cyperus* species that were part of this study are shown in Supplementary Figure 2. Commercial products are labeled in gray. The CE3_fw primer that targets *C. esculentus* was used, see (A). A unique signal in *C. esculentus* individuals as well as in six of seven commercial products could be detected.

C ITS2-based agarose electrophoresis of ARMS amplicons from *C. esculentus* (green), *C. rotundus* (red) references and four outgroups (blue, including *C. iria*, *C. strigosus* and *Oenanthe* and *Prunus*). Commercial products are labeled in gray. The CR1_fw primer that targets *C. rotundus* was used, see (A). A unique signal in *C. rotundus* individuals as well as in one of the commercial products could be detected. **D** AGE of CE3_fw and CR1_fw using reference plants and artificial DNA mixtures of *C. esculentus*/*C. rotundus* (using 90/10, 70/30, 50/50, 30/70, and 10/90 mixtures) in order to test the sensitivity of the approach with respect to the detection of possible adulterations. Independent of concentration, adulteration by the other species could be demonstrated

a controlled regional production would be the best option to control authenticity and avoid adulteration of tiger nut products. However, *C. esculentus* has been included into a list of 150 species that are banned from being introduced to the European Union based on the assessment of the European Food Safety Authority with the intention to contain the phytopathogenic bacterium *Xylella fastidiosa* [48]. Thus, the import of tiger nut products to Europe is expected to grow at similar rates over the next years, such that adulteration will become a progressively pertinent issue. In the following, we will discuss, therefore, what factors make tiger nut prone to fall prey to adulteration (both, intentionally and non-intentionally), how the genetic barcoding strategy developed in the current work can contribute to prevent this, where the limitations of our approach are, and how these could be overcome in future.

Why *Cyperus* causes chaos—in crop fields, in taxonomy and in food products

Stolons derive from underground axillaries and develop by swelling in moist and dark atmosphere [49]. This tuberisation process is not only dependent on genetic factors, but also various environmental conditions, such as soil depth. For instance, for potatoes, cool temperatures [50] and low levels of nitrogen [51] promote tuberisation, while tubers develop more slowly, when it is warm, or when nitrogen is not limiting. These environmental conditions act through modulation of phytohormone synthesis and signaling. For tuberisation in potatoes, especially gibberellic acid (the molecular mechanics are reviews in [52, 53]) and jasmonic acid [54, 55] have been found to be relevant.

Thus, the morphological variability of stolons that was observed for *C. esculentus* in the current study, but also reports from other authors, while certainly also depending on genetic factors [11] is most likely also depending on environmental conditions including abiotic stress responses. For *C. rotundus*, comparative studies have revealed an influence of flooding [56] and differences in rainfall [57] as modifiers of tuberisation. This morphological diversity of stolons seems not to be accompanied by major differences of composition, at least, if bulk compounds are considered [9, 13]. On the other hand, given this variability of stolon morphology, this trait does not qualify as reliable taxonomic marker to delineate the two species *C. esculentus* and *C. rotundus* (see Fig. 1), which might be the reason, why determination keys do not use it as discriminative marker. Anyway, most commercial products are not traded as intact stolons, but often come in processed form, for instance, as flour, such that the anyway shaky identification by stolon morphology is rendered impossible.

Since both species also spread as invasive weeds, the prospect to use a pertinent weed as highly priced functional

foods is certainly too tempting for farmers in developing countries to be missed, and the rapid growth of *chufa* markets promote this tendency even further. A further factor might be that vernacular taxonomy is often ambiguous, as exemplified by the use of the species in Ayurveda. *C. rotundus* plays an important role in Ayurvedic medicine and is designated, among other names, as *musta* and delineated in the Vedic scripts from *jala musta*, representing *C. esculentus* [58], www.easyayurveda.com [59]. However, in current use, this fine line is often ignored and *jala musta* just described as a “variant” of *musta* [60]. It does not need a wild imagination to conceive that, for instance, a German importer might easily fall prey to these pitfalls of traditional nomenclature with the risk of non-intentional adulteration.

psbA-trnH igs and ITS barcoding regions separate *C. esculentus* and *C. rotundus*

The choice for the “best” genetic barcoding marker depends on the species or genus of interest. Many different marker regions have been proposed in theory to be *the* most suitable. However, in practice, the trial of several barcodes is unavoidable. For instance, the “most promising plastid DNA barcode of land plants [61]”, the *ycf1b* region, could not be amplified at all in the *Cyperus* accessions of this study.

The plastidic *psbA-trnH* igs displayed a complete length of around 1000 base pairs in *chufa*, which is remarkably large for this barcoding region, considering the average length for monocotyledons being 357 base pairs, with an estimated maximum of 905 base pairs [31]. The probability of SNPs that can be utilized for molecular authentication through RFLP or ARMS enhances with length; however, low G/C-content in *chufa psbA-trnH* igs limits especially the ARMS approach since the design of viable primers depends among other factors on a sufficient G/C content. While longer barcoding markers have a higher chance to find informative substitutions, the enhanced length of is also a drawback since during the processing of commercial products the DNA can be degraded to such a degree, that a fragment with a size of 1000 base pairs cannot be amplified.

The phylogenetic tree based on the plastidic marker *psbA-trnH* igs gives an insight on the maternal evolution of a small fraction from the huge *Cyperus* genus (See Fig. 2) and helps putting the species of interest *C. esculentus* and *C. rotundus* into a phylogenetic context. The clear separation of the *Cyperus* C₃ and C₄ clades has been described earlier using the plastidic *psbA-trnH* igs [24] and is consistent with previous studies that had shown that this genus is *not* monophyletic (see [25, 26]). Within the C₄ clade, *C. esculentus*, *C. rotundus*, and *Cyperus iria* are close, but clearly separated. The topology for the nuclear *ITS* region was fairly close although we encountered some difficulties in addressing the commercial products because either amplification or the

sequencing failed. This does not come as a surprise, since the nuclear genome is only present in two copies, while the plastidic genome is more abundant in cells. The more abundant template can, therefore, explain the higher success rate for the *psbA-trnH igs* marker. Most importantly, irrespective of the marker, the commercial products clustered together with *C. esculentus*.

Although the overall patterns were close, the plastidic and the nuclear marker produced one significant difference in topology. While *C. iria* was clearly placed with *C. rotundus* for the plastidic *psbA-trnH igs* region (see Fig. 2), it is as clearly close to *C. esculentus*, when the *ITS* marker is considered (see Fig. 3). Since plastids are, in most cases, maternally inherited, while nuclear genomes are symmetrically passed on, a straightforward explanation might be that *C. iria* derived from a hybridisation event, where a species close to *C. esculentus*, acted as pollen recipient. Alternatively, more exotic mechanisms, such as chloroplast capture might play a role [62], which have been demonstrated in *Quercus* [63] or *Vigna* [64]. Though chloroplast capture has not been reported in *Cyperus*, the readiness for asexual reproduction through stolons might favor the occurrence of such an event. Generally, one needs to keep in mind that radiation of *Cyperus* was a recent event. For instance, the geographically different subspecies of *C. esculentus* have diverged approximately only 5.1 million years ago [65]. Thus, alleles shared between different species might result in incomplete lineage sorting as it has been shown in other monocot groups such as the subtribe Hippeastrinae from the Amarayllidaceae family [66] or genus *Allium* from the same family [67]. Therefore, more loci, both plastidic and nuclear, would be needed to address a potential discrepancy of plastidic and nuclear phylogenies. However, the scope of the current study was not to address the evolution of the complex genus *Cyperus*, but, quite pragmatically, to use the detected sequence polymorphisms to develop a robust and sequencing-free assay that allows to distinguish *C. esculentus* and *C. rotundus*.

Beyond phylogeny: sequencing-free authentication of *C. esculentus*

The scope of the current study was to develop a robust assay that allows to discern *C. esculentus* and *C. rotundus* by a sequencing-free ARMS strategy. Compared to RFLP as alternative method that can amplify discriminative SNPs into a diagnostic banding pattern [35], the ARMS approach does not require a second step (restriction) after the PCR, and also includes a positive control in form of the full-length amplicon as readout of the successful PCR [68, 69]. Which barcoding marker can be used depends on the species—while the *psbA-trnH igs* marker was successful to authenticate *Lycium barbarum*, traded as ‘Goji’ [30] or the

Peruvian Amaranth (*Amaranthus caudatus*) known under the vernacular name of *kiwicha* [70], this marker was not amenable for ARMS in the current study. Instead, the *ITS2* region that was successfully employed in the current study, did not work in case of Amaranth, also due to un-favorable AT content. Whether a marker can be used for the ARMS strategy is, thus, not only depending on its resolving powers, but also on its base composition. It is virtually impossible to propose a priori a barcode that would allow for ARMS in all species, this can only be decided after aligning the sequences from the target species and its surrogates.

In the current study, we could identify a diagnostic region in the *ITS2* marker and develop two complementary ARMS assays—one produced the diagnostic side band, when the specimen contained *C. esculentus*, the other, when *C. rotundus* was present. The assay worked also for processed material, such as *chufa* flour, underlining the sensitivity of this assay and its importance for consumer safety, and detected admixtures down to 10%. The complementarity is needed to allow for different applications. To verify that a given sample contains tiger nut, the assay is needed, where the side band reports *C. esculentus*. However, in order to verify that this sample is not surrogated by *C. rotundus*, the second assay is useful, because, here, a second band inevitably reports that the sample has been adulterated.

Limitation and potential of the assay

Any identification method, no matter how broad in scale, is only as good as the reference material is reliable. This is also valid for DNA-based approaches. However, in addition to authenticity of the references also, the quality of the DNA is crucial [71]. In commercial samples, processing including high temperature or low pH (reviewed in [72]) will affect the quality of the DNA and thereby all downstream molecular approaches. Degraded DNA may not deliver a functional template for amplification of long (> 500 bp) barcoding marker fragments, such as *rbcLa*, *ycf1b* or *ITS*. This limitation can be circumvented by choosing shorter fragments spanning the informative region. For instance, the nuclear *ITS* region contains the central, highly conserved 5.8S region that can be utilized for amplification of the shorter *ITS1* and *ITS2* fragments. For degraded DNA were templates of > 200 bp are typically rare, it is even possible to shorten the amplicon length to fragments of even only 50–150 bp around the diagnostically relevant polymorphism. The resulting amplicons are still readily separated on an agarose gel. The drawback of such tailored approaches is that not only the ARMS primers, but also the flanking primers need to be designed for the specific assay, and cannot readily be transferred to assays for other species [73, 74].

Recently, hyperspectral fingerprints in the NIR range have been proposed for the differentiation of plants. For

instance, weeds in cereal fields can be detected by this approach (knapweed and weedy barley in wheat: Hermann et al. 2013; Weedy oat in wheat [74]). Even on the subspecies level, varieties of maize could be discriminated by this approach [75]. A similar strategy allowed to discriminate *C. esculentus* (here, in a weed context) from other Cyperaceae, such as *Carex hirta* L. [76]. To what extent, hyperspectral fingerprints can be used to verify tubers or processed products from *C. esculentus* against *C. rotundus* remains unclear. The spectra are highly depending on the reflective properties of the specimen, such that the approach requires extensive calibration and standardization [78], which appears to be a serious limitation compared to a DNA-based approach that is independent of environmental conditions.

Our assay is targeted, it discriminates a specified species against surrogation by a different species. This targeted approach becomes limited, when the commercial product is a mixture of different plant species, because the resulting patterns become complex and difficult to interpret, as demonstrated for mixed samples with Holy Basil [77]. Here, we are currently testing a combination of DNA barcoding and Next-Generation Sequencing that would allow to get statistical information about the abundance of different amplicons.

Conclusions

The rapidly growing markets for botanicals with functional properties bear the possibility for adulterations (accidentally or on purpose). We have developed a convenient, sequencing-free tool to authenticate *C. esculentus* against the closely related *C. rotundus* that is used in a different context, as medicinal plant. Such robust and reliable assays are needed to protect consumer safety against the challenges of botanical globalization.

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Data availability The sequencing data have been deposited in ncbi database.

Declarations

Conflict of interest The authors declare no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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