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Article

Trade in Zambian edible orchids – DNA barcoding reveals use of unexpected orchid taxa for *chikanda*.

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Abstract: In Zambia wild edible terrestrial orchids are used to produce a local delicacy called *chikanda*, which has become increasingly popular throughout the country. Commercialization puts orchid populations in Zambia and neighbouring countries at risk of overharvesting. Hitherto, no study has documented which orchid species are traded on local markets, as orchid tubers are difficult to identify morphologically. In this study, the core land-plant DNA barcoding markers rbcL and matK were used in combination with nrITS to determine which species were sold on Zambian markets. Eighty-two interviews were conducted to determine harvesting areas, as well as possible sustainability concerns. By using nrITS DNA barcoding, a total of 16 orchid species in six different genera could be identified. Both rbcL and matK proved suitable to identify the tubers up to genus- or family level. *Disa robusta*, *Platycoryne crocea* and *Satyrium buchananii* were identified most frequently and three previously undocumented species were encountered on the market. Few orchid species are currently listed on the global IUCN Red List. Local orchid populations and endemic species could be at risk of overharvesting due to the intensive and indiscriminate harvesting of *chikanda* orchids and we therefore encourage increased conservation assessment of terrestrial African orchids.

Keywords: CITES; Chikanda; Conservation; DNA barcoding; Orchids; Species delimitation;

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1. Introduction

Terrestrial orchids have been used for medicinal and culinary purposes for centuries [1], with the most notable example being the use of orchid tubers to make *salep*, a traditional Turkish creamy drink or ice cream, consumed in Asia Minor and several countries on the Balkan peninsula **[2–5]**. In south-eastern Africa terrestrial orchid tubers are mixed with peanut flour, salt, baking soda and chili powder to make a traditional Zambian meat-like cake known as *chikanda* or African polony **[6–8]**. Although initially not highly regarded [9], *chikanda* has more recently become popular throughout the country. It is sold as a snack along the streets, on markets, in supermarkets and on the menu of high-end restaurants [10] and recipes as well as cooking tutorial videos can be found online [11]. Orchids used for *chikanda* are harvested exclusively from the wild, and although it is unlikely that traditional village consumption poses a serious threat to orchid populations, the increased popularity and subsequent commercialization of *chikanda* has led to the exhaustion of Zambian orchid resources [8]. Collecting tubers means the end of a perennial and generally long-lived orchid, since the entire plant is removed in the harvesting process.

Soweto market in the Zambian capital Lusaka is the hub of the *chikanda* trade. Surveys performed on this market have shown that a large part of the *chikanda* tubers sold are sourced from Tanzania and that Zambian *chikanda* orchids are collected from the Luwingu, Mporokoso and Kasama districts in the Northern Province and Serenje in the Central province [7,8]. According to local *chikanda* vendors, another region with a flourishing *chikanda* trade is the Kitwe region in the Copperbelt Province, but so far no surveys have been performed there. Despite international legislation (CITES) banning prohibiting cross-border trade, an estimated 2–4 million orchid tubers are transported annually from Tanzania to Zambia [8,12]. Import from the surrounding countries of Angola, DRC, Malawi and Mozambique is also documented [8,10]. Orchid species originally reported as ingredients for *chikanda* are *Disa robusta* N.E.Br. and *Satyrium buchananii* Schltr. [12,13], whereas recently at least 32 species belonging to the genera *Brachycorythis*, *Disa*, *Eulophia*, *Habenaria*, *Roeperocharis* and *Satyrium* were suggested to be used for *chikanda* production based on collections in the field [12,14–19] and one metabarcoding study of ready-made *chikanda* cakes [19]. To date, however, no study identified the orchids traded at the local markets, since the tubers lack sufficient morphological characters for taxonomic identification to species level [8,19]. Local classification systems categorize the tubers based on texture, harvesting locality, soil colour and phenology, but these are not likely to be congruent with scientific classifications [6,15].

Knowing which orchid species are currently being collected for the expanding *chikanda* trade enables the identification of species susceptible to overharvesting and can inform conservation planning . Molecular methods such as DNA barcoding can be applied to identify samples when morphological diagnostic characters for identification are lacking [20]. DNA barcoding and metabarcoding has proven to be effective in the authentication of commercial wood species (Jiao, 2018), medicinal plants [21] and *salep*-producing orchids on Iranian markets [5]. The analysis of ingredients in Tanzanian *chikanda* cake with DNA metabarcoding revealed the presence of 21 different orchid species [19], but a DNA barcoding approach has not yet been applied to individual orchid tubers used to make this product. The aim of this study was to test to what extent species delimitation using standard molecular markers yields robust identification of *chikanda* orchid tubers traded on Zambian markets. Molecular identification can enable mapping of harvesting and trade of specific Zambian orchid species and facilitate identification and implementation of targeted conservation strategies. Within that framework this study also aimed to identify conservation issues associated with the *chikanda* trade, and addresses the following questions: 1) Which species are used for *chikanda* production in the Lusaka and Kitwe districts of Zambia and what is their geographic origin? 2) Can *chikanda* tubers be identified up to species level using DNA barcoding? 3) How do local classification systems relate to scientific species concepts? 4) What are the main conservation issues associated with *chikanda* trade in the Lusaka and Kitwe districts?

2. Materials and Methods

2.1. Interviews and sample collection

Fieldwork in Zambia was conducted in 2016 in the Kitwe, Kalulushi, Luanshya, Ndola, Mufulira, Chingola and Chililabomwe districts of the Copperbelt Province; the Kapiri Mposhi and Serenje districts in the Central Province and in the capital Lusaka (Figure 1). Semi-structured interviews were conducted with harvesters, middlemen and vendors to obtain insight in *chikanda* commercialization, harvesting times, preferences and availability. The questionnaires consisted of three sections, one on informant and interview characteristics, one with general questions about *chikanda* posed to all informants, and a third section with questions more specifically designed for each interviewee category: harvester, middleman and vendor. All research was conducted in accordance with the International Society of Ethnobiology Code of Ethics [22]. Ethical clearance was obtained from the Humanities and Social Science Research Ethics Committee of the University of Zambia. The interviews were performed in English or Bemba, with a translator affiliated with the University of Zambia and the Copperbelt University. Informants were selected using the snowball technique [23], by asking people whether they could direct us to people harvesting or selling *chikanda*. All informants were provided with information about the study and signed a prior informed consent sheet. Fieldwork took place during June and July, the peak season for *chikanda* [8], to ensure collection of both fresh and dried *chikanda* tubers on the market and in the field. A collection was made each time a specific vernacular *chikanda* type was bought from a specific vendor, and assigned a collection number (SJK1, SJK2, etc.). Each individual tuber within the collection received a subsample number within that collection (SJK1.1, SJK1.2, SJK 1.16, etc.). The fresh tubers were sliced and stored with silica gel in plastic bags. All *chikanda* samples were brought to Sweden under a CITES inter-institutional exchange agreement between the University of Zambia (ZM001) and the Botany Section of the Evolutionary Museum in the Evolutionary Biology Center in Uppsala (SE009). Export permission was obtained from the Zambian CBD and Nagoya Protocol focal point at the Ministry of Natural Resources and Environmental Protection. Upon arrival to Sweden some of the *chikanda* tubers had sprouted. Those were transferred to the Uppsala Botanical Garden for cultivation and subsequent sampling of fresh leaf tissue for DNA barcoding as well as morphological identification.

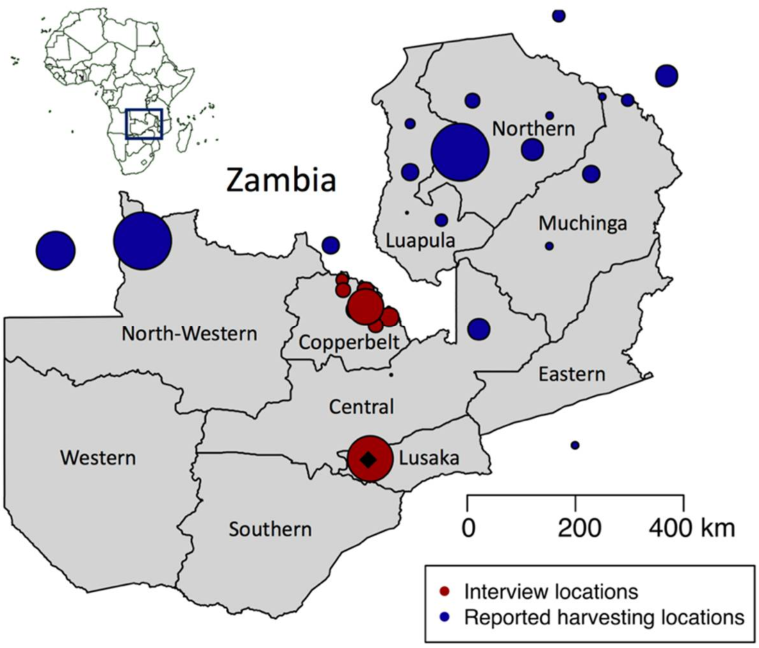

Figure 1. Overview of interview localities and reported provenance of the *chikanda* tubers. Dot size corresponds to the number of informants.

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2.2. Reference taxon sampling

Herbarium specimens were collected with associated silica-dried material for DNA extraction and spirit collections during fieldwork in Zambia in January and February 2017. In addition, putative orchid mycorrhizal fungi were sampled from roots and tubers for isolation, culture and identification at RBG Kew (results not reported here). All material was collected and exchanged in accordance with national and international legislation. The collections are deposited at the Division of Forest Research (Kitwe, Zambia) and RBG Kew (UK) and field identifications verified at the Bews Herbarium (South Africa). A total of 94 novel Orchidaceae reference vouchers were collected for this study, representing 4 *Brachycorythis*, 9 *Disa*, 16 *Habenaria*, 6 *Satyrium* species and 26 species in other orchid genera. Voucher specimens of all taxa sampled are listed in Table S1 (Supplementary Material). In addition, 88 ITS, 71 *matK* and 45 *rbcL Habenaria* sequences generated for a forthcoming phylogenetic study [24], and 510 ITS, 522 *matK* and 213 *rbcL* sequences corresponding to 311, 325 and 100 taxa in the previously mentioned orchid genera downloaded from GenBank were included in the reference database.

2.3. From sample to sequence

Out of the 1284 individual tubers in 48 different sample collections, 304 samples were selected for DNA extraction. A few tubers per sample (2-8) were extracted if the sample was morphologically homogenous, whereas more (6-33) were selected if the sample was diverse. DNA was extracted using a CTAB protocol [25] modified with 3 to 5 extra washing steps with STE buffer (0.25 M sucrose, 0.03 M Tris, 0.05 M EDTA) [26,27], to reduce the gelatinization effect of the large amount of polysaccharides in the starch-rich orchid tubers. Total DNA was stored in 70-100 µl 10mM Tris-HCl buffer, pH 8.0. DNA concentration was measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Oakwood, USA). The core land plant barcoding markers *rbcL* and *matK* were amplified using the primers and protocols described in Kress et al. [28] and Dunning and Savolainen [29] respectively. The reactions were performed in a total reaction volume of 25µl with 14.725µl ddH_2O, 2.5µl DreamTaq Buffer (Thermo Fisher Scientific, Oakwood, USA), 0.5µl 25mM dNTP, 0.65µl 2% Bovine Serum Album (BSA), 0.125µl DreamTaq Polymerase, 2.5µl 5pmol forward and reverse primer and 1.5µl template DNA. Nuclear ribosomal nrITS was amplified using the Sun et al. [30] primers and protocol in a total reaction volume of 25µl containing 15.25µl ddH_2O, 2.5µl DreamTaq Buffer (Thermo Fisher Scientific, Oakwood, USA), 0.5µl 25mM dNTP, 0.125µl 2% BSA, 0.125µl DreamTaq Polymerase (Thermo Fisher Scientific, Oakwood, USA), 2.5µl 5pmol forward and reverse primer and 1.5µl template DNA. For ITS an additional protocol was used with Q5 high-fidelity polymerase: reactions were performed in a total reaction volume of 23.5µl including 10.875µl ddH_2O, 5µl Q5 reaction buffer, 0.5µl 25mM dNTP, 5µl Q5 GC enhancer, 0.125µl Q5 high-fidelity polymerase, 1.5µl 5pmol forward and reverse primer and 0.5µl template DNA. The PCR program for the ITS primers in combination with the Q5 polymerase was an initial heating step of 30s at 98°C; 35 cycles of 10s 98°C, 30s 56°C, 30s 72°C; and a final elongation of 2min at 72°C. PCR products were cleaned using eight times diluted ExoSAP (Thermo Fisher Scientific, Oakwood, USA) and analysed on an ABI3730XL Sanger sequencer by Macrogen Europe (Amsterdam, The Netherlands). The obtained trace files were assembled using Pregap4 and Gap4 [31] as implemented in the Staden package [32]. Sequences shorter than 200 bp were discarded from the analysis and all sequences have been deposited in NCBI GenBank (Table S2, Supplementary Material). The NCBI BLAST algorithm was used to assess the identification of all the obtained query sequences using the Python BLAST tabular parser script (https://github.com/SLAment/Genomics/blob/master/BLAST/BLAST_tabularparser.py). Similarity scores, query coverage, expect value (E-value), and max identity percentage were calculated, and all the information of the top 5 hits were automatically mined and tabulated per marker (Table S3-S5, Supplementary Material), and summarized per individual tuber sample (Table S6, Supplementary Material).

2.4. Phylogenetic analysis and species delimitation

Alignments for nrITS, *matK* and *rbcL* were made using AliView [33], combing the query

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sequences and local reference databases consisting of sequences from NCBI GenBank and reference collections from fieldwork and unpublished data from collaborators [33]. Species delimitation was performed using the Poisson Tree Processes (PTP) model, as it has been shown to outperform the Generalized Mixed Yule Coalescent (GMYC) approach as well as OTU-picking methods when evolutionary distances between species are short enough [34]. For all alignments a maximum likelihood (ML) search for the best-scoring tree was performed using the RAxML web server [35] to generate input trees for species delimitation analysis using bPTP [34]. GTRCATI was used to implement the CAT approximation, and the final tree was evaluated using the traditional GTR model. The bPTP.py script settings was 100,000 MCMC chain iterations for all trees; sampling interval thinning value of 100; burn-in of 25%; and a random seed of 1234. No outgroup was defined. The generated convergence curve was visually confirmed. The description of each voucher is built up as following: 'sample #_BLAST search result_identification %_reported city/region of the origin_the country,'; for example: ‘SJK04.09_S.buchananii_97.893_Mwinilunga_Zambia’ (Figure S1-3, Supplementary Material). For the reference sequences, accession number and species names are described for *Disa* and *Satyrium*. The reference *Habenaria* species and others are described as species name and voucher (sample) number.

3. Results

3.1. Market surveys and interviews

We visited 25 markets in ten Zambian cities: seven cities in the Copperbelt Province, two in the Central Province, and one in Lusaka (Figure 1). Eighty-two persons involved in *chikanda* trade were interviewed of which 8 were harvesters, 44 middlemen, 29 vendors and one informant was both harvester and middleman. The term ‘harvesters’ refers to people collecting *chikanda* in the field, ‘middlemen’ to people selling either dried or fresh *chikanda* orchid tubers on the market, and ‘vendors’ to informal street vendors selling ready-made *chikanda* cakes contained in a basket carried on the head. Since one of the harvesters also acted as middleman, in total 83 individual interviews were conducted: 72 participants were female and 10 male. The ages of the respondents varied between 18 and 78, with an average of 41 years. The majority of the respondents belonged to the Bemba tribe (62%), while the other the respondents (38%) belonged to smaller ethnic groups. Most interviews were conducted in the Copperbelt Province (55), eight in the Central Province and 19 in Lusaka.

3.2. Local classification system

Fifty-three different vernacular names for the various *chikanda* types were recorded during the interviews. The most common way to distinguish between *chikanda* tubers was by using the terms original (*myala*) and fake (*mbwelenge* or *msekelele*). In some cases it was the shape of the tuber that was used to differentiate between the different tubers: *mshilamshila* means root-like in Bemba and referred to the elongated, root-shaped tubers whereas *mampanda* referred to the heart-shaped tubers. It also appeared common to use the origin of the tuber as a trade name: *mwinilunga*, *chozi*, *luwingu* and *kasama* are for example all Zambian city names, *sumbawanga* and *iringe* are referring to Tanzanian cities (Sumbawanga and Iringa) and *angola* refers to one of the countries bordering Zambia. In some cases the *chikanda* tubers were sold pre-mixed, whereas other vendors marketed the different types of tubers separately. The morphology and size of the tubers varied both within and between collections of a certain *chikanda* type. Tubers could be heart-shaped, rounded, egg-shaped to elongated and almost root-like. The largest tubers were the heart-shaped ones, which could be up to 5 cm long and 6 cm wide. The elongated tubers were up to 9 cm long and maximally 2 cm wide. Harvesters, middlemen and vendors themselves indicated that they distinguish the tubers based on the size of the granules inside the tubers, which can be large or small and in some tubers a concentric ring was said to be present. Figure 2 illustrates the different tuber types and ready-made *chikanda* that were encountered on the local markets, as well as some of the orchid species producing these tubers.

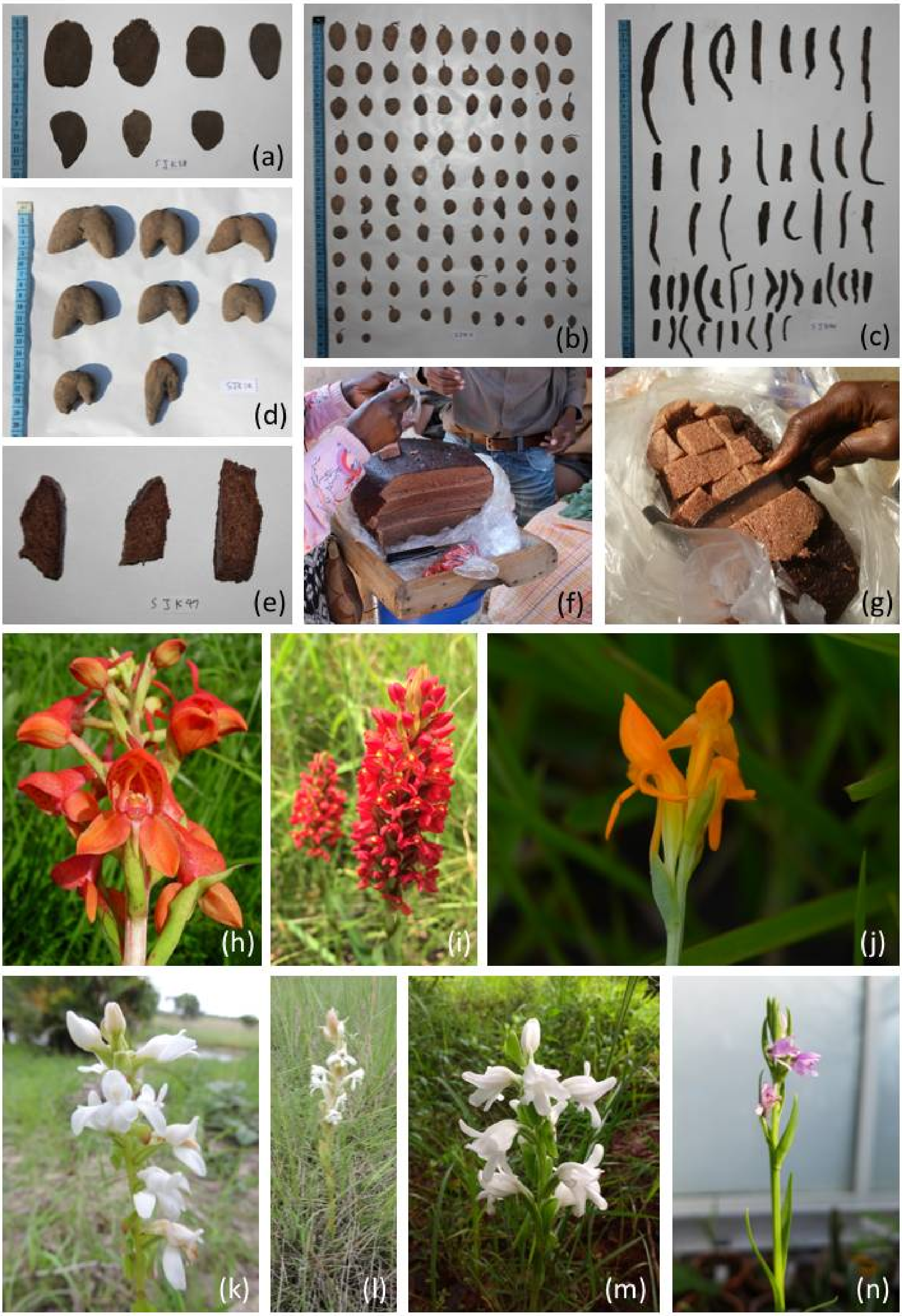

Figure 2. *Chikanda* tubers, cake and orchids. (**a**) Myala – real *chikanda*; (**b**) Mbwelenge – fake *chikanda*; (**c**) *Mshilamshila* – supposedly *Brachycorytis* sp.; **(d)** *Mampanda*. **(e-g)**; *Chikanda* cake; (**h**) *Disa robusta*; **(i)** *Disa welwitschii*; **(j)** *Platycoryne crocea*; **(k)** *Satyrium carsonii*; **(l)** *Satyrium buchananii*; **(m)** *Satyrium kitimboense*; **(n)** *Brachycorytis* cf. *friesii*; Photographs (a-g) by Seol-Jong Kim, (h) by Robert v. Blittersdorff, (i,k and l) by Nicholas Wightman, (j) by Warren McCleland, (m) by Ruth E. Bone and (n) by Sarina Veldman.

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3.3. Chikanda trade and availability

Many participants had a relatively long experience (average 13.5 year) in the *chikanda* trade. They indicated that they were asked by family, friends or neighbors to get involved in the *chikanda* business, or simply because it seemed a profitable industry. Some of the participants, especially harvesters, stated that they started collecting *chikanda* because the plants were easily accessible and were found growing close to their areas of residence. In general, people involved in *chikanda* trade indicated that this work alone did not suffice to fully support themselves and their families, and these traders therefore supplemented their income by trading additional natural products such as fruits, maize, groundnuts, beans, mushrooms, snacks, herbs, and *kapenta* (a dried fish from Lake Tanganyika). In Lusaka, however, income generated with *chikanda* trade seemed to be sufficient for subsistence, and this suggests that there are significant local differences in profits generated with *chikanda* business.

The *chikanda* trade was structured in several different ways. Some of the middlemen loaded the orchid tubers in trucks directly from the harvesting areas and brought them to larger cities such as Lusaka and Kitwe, whereas others relied on agents to gather a certain amount of *chikanda* tubers, which they paid for through the East-African mobile payment system M-Pesa and received as cargo from one of the local buses. In addition to the Tunduma and Nakonde markets that are the trade-hubs between Tanzania and Zambia, other centers of trade were identified on the border with the Democratic Republic of Congo (Chililabombwe and Kasumbalesa) and Angola (Mwinilunga), where both *chikanda* tubers and ready-made *chikanda* cake was sold. An overview of all interview localities and the reported provenance of the *chikanda* tubers is given in Figure 1. Most of the participants indicated that *chikanda* plants are becoming locally rare. Middlemen emphasized the decrease of quantity, whereas harvesters were concerned about the decline in both quantity and quality (size and preferred *chikanda* type), which may also depend on the species. The participants from urban areas stated that access to *chikanda* tubers was managed by the chief of each tribe, who seasonally designated the harvestable *dambo* (wet meadow) within the chiefdom so that the collectors could maintain the quality of the harvests. Nevertheless, interviewed harvesters encountered in the *dambo* areas of Serenje (Central Province) claimed to be free to harvest tubers whenever available.

3.4. Molecular identification of traded orchids

During the DNA extraction many samples formed a thick jelly-like layer in the extraction tubes, despite repeated washing steps with STE buffer. This resulted in a very small water phase and likely negatively influenced the downstream steps of the extraction process. The average DNA concentration of the *chikanda* tuber samples was 4.96 ng/µl, while the average DNA concentration from leaf samples from *chikanda* orchids was 28.5 ng/µl. Out of the 304 samples selected for DNA extraction, 232 samples produced detectable DNA. Amplification was attempted for each barcoding marker for all of the samples. A nrITS sequence was obtained for 159 samples, *rbcL* for 117 samples and *matK* only for 45 samples. Sequences from all three markers were obtained for 40 samples, sequences from two markers for 58 samples, and 55 samples only yielded sequences for a single marker. Analysis of the inter- and intraspecific variation of nrITS was performed for 141 sequences representing 124 *Disa* species, 73 sequences representing 59 *Habenaria* species and 110 sequences representing 67 *Satyrium* species. In the case of *matK* 135 sequences belonging to 122 *Disa* species were included, 507 *Habenaria* sequences belonging to 239 species and 116 sequences belonging to 60 *Satyrium* species. For *rbcL* the available reference material was quite limited: 45 sequences for 37 *Habenaria* species, eight *Satyrium* sequences for two species and four *Disa* sequences for four species. A graphical overview of the inter- and intraspecific variation per genus and per marker can be found in Figure 3. The interspecific variation for nrITS was significantly higher as compared with *matK* and *rbcL* in all three genera: on average 10.2% in *Disa*, 9.51% in *Satyrium* and 8.75% in *Habenaria*. For *matK* the interspecific variation was 2.39% for *Disa*, 2.79% for *Habenaria* and 1.39% for *Satyrium*. The intraspecific distances for nrITS and *matK* were respectively 1.37% and 0.61% for *Disa*, 1.98% and 0.36% for *Habenaria* and 1.45% and 0.48% for *Satyrium*, The limited reference sequences that were available for *rbcL* showed little pairwise interspecific distances (1.44% for *Disa*, 0.82% for *Habenaria* and 1.13% for *Satyrium*) and only allowed for the intraspecifc distance calculation of *Habenaria*

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(0.38%), indicating that *rbcL* is unsuitable as barcode for species level identification of *chikanda* orchids. The calculated thresholds were subsequently used to evaluate the identifications made with blastn.

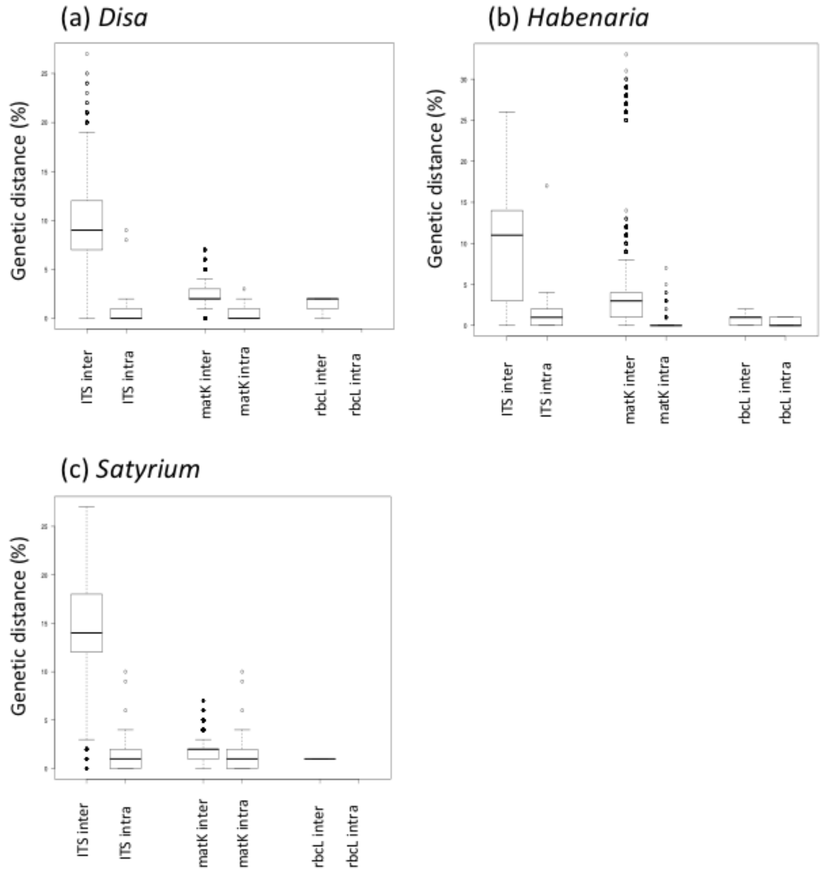

Figure 3. Boxplots showing the inter- and intraspecific variation for *Disa* (**a**), *Habenaria* (**b**) and *Satyrium* (**c**) based on genetic diversity.

In total 15 orchid species were identified using DNA barcoding: *Brachycorytis* sp. SJK7, *Disa caffra* Bolus, *Disa celata* Summerh., *Disa robusta* N.E.Br., *Disa satyriopsis* Kraenzl., *Disa welwitschii* Rchb. f., *Disa* sp. SJK4.1., *Habenaria* sp. SJK31.15, *Habenaria* aff. *helicoplectrum* Summerh., *Habenaria* cf. sp. DO112, *Platycoryne crocea* Rolfe, *Satyrium buchananii* Schltr., *Satyrium carsonii* Rolfe, *Satyrium kitimboense* Kraenzl. as well as one species in an unidentified genus, which seems to be closely related to *Habenaria* based on the similarity-based BLAST identification. Additionally, one orchid that flowered from a sprouting tuber was identified based on morphology as *Brachycorythis* cf. *friesii* (Schltr.) Summerh.

bPTP analysis for *matK* and *rbcL* showed lumping of several supposedly different species within one clade on several occasions (Figure S1 and S2, Supplementary Material). In case of nrITS the bPTP outcome tree often reflected the expected species boundaries, although the posterior probabilities on the nodes were often too low to determine with confidence if species delimitation had been performed correctly (Figure S3, Supplementary Material). Something that could be observed from both the nrITS BLAST identification, as well as the bPTP analysis, was that some of the identifications showed ambiguity: in collection SJK41, SJK44 and SJK46 some samples were identified unambiguously as *Platycoryne crocea*, whereas others showed a mix of *Habenaria* and *Platycoryne* top hits. The *matK* BLAST identification showed an unambiguous *Platycoryne crocea* identification for these samples, despite the presence of several *Habenaria* species in the reference database and despite the fact that *matK* shows a lower level of interspecific variation. A similar observation could be made for several *Disa* samples, but here a geographic pattern could be observed. The samples that could

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unambiguously be identified as *Disa robusta* were all collected from Tanzania, where the reference sequence originated from. Some of the Zambian samples also showed *Disa robusta* as the closest relative, but not with a high enough percentage identity match to confirm this identification. The bPTP result in this case shows a lumped and mixed clade with several *Disa* species and several other samples included, which confirms that sequence divergence is too limited to resolve the relationship. These samples were identified as *Disa* sp. 1, to reflect the fact that they all grouped together in the same clade. In other cases, such as for certain *Habenaria* species, we named the closely related sequences in the identification, whereas in this case the clade was too large to allow for this, since it contained the following sequences: *Disa engleriana* Kraenzl., *D. erubescens* Rendle., *D. miniate* Summerh., *D. ochrostachya* Rchb.f., *D. satyriopsis*, *D. ukingensis* Schltr., *D. verdickii* De Wild., *D. welwitschii*, *D. zombiaca* N.E.Br., an unidentified *Disa* species and several samples that showed a highest percentage identity match with *D. robusta*, *D. satyriopsis* and *D. welwitschii*. The posterior probability for this clade is only 0.25, and combined with the posterior probabilities present on the within-species nodes, indicates a lot of uncertainty for this identification.

Overall, the most frequently encountered species were *Satyrium buchananii*, with 41 samples in eleven different collections, *Platycoryne crocea* with 19 samples in three different collections and *Disa robusta* with 4-16 samples in two to seven different collections. *Myala* or original *chikanda* seems to contain *Disa robusta*, *D. welwitschii* and *Satyrium buchananii*. *Mbwelenge* or fake *chikanda* seems to correspond only to *Satyrium buchananii* and *mshilamshila* to one or several *Brachycorythis* species. *Kasebelele* and *kapapa* referred to *Habenaria* spp., *Platycoryne crocea*, *Satyrium carsonii* and *Satyrium kitimboense*. The mixed collections contained all of the above-mentioned species as well as an unidentified *Habenaria* species. An overview of the local *chikanda* classification types, their collection numbers, identifications and number of samples can be found in Table 1.

4. Discussion

4.1. Species used for chikanda

Using DNA barcoding as an identification tool for *chikanda* tubers sold on local Zambian markets has allowed us to determine for the first time what orchid species are sold on local markets. Previous studies identifying orchids used for *chikanda* relied on voucher collections made with collectors in the field and their morphological identification, which requires a qualified orchid taxonomist [7,12,14,17,36]. Additionally, relying on local harvesters for details on *chikanda* collection might not always lead to collection in the areas where actual intensive harvest is taking place, since some harvesters do not like to divulge where the best places to harvest are and in some initial harvesting areas such as the Kitulo Plateau in Tanzania's Southern Highlands, collection is now prohibited [37]. The current study identified 16 orchid species present on the markets, including at least three previously undocumented ones: *Brachycorythis* cf. *friesii*, *Platycoryne crocea* and an unidentified species in a genus, which appears to be closely related to *Habenaria*, but is not present in our reference database.

Orchids used for *chikanda* seem to be harvested from several provinces in Zambia, as well as at least two regions in Tanzania. Moreover, three international *chikanda* trade-hubs in towns on or close to the border with the DRC (Chililabombwe and Kasumbalesa) and Angola (Mwinilunga) were identified, in addition to the already known trade-hub Tunduma-Nakonde on the Tanzanian border [8,12]. However, unlike reported in other studies, no harvest from Malawi was mentioned by the people interviewed in this study, which could mean that trade from this country is currently not taking place. Another explanation is that this information is lost on the way and that only Tanzania, being geographically closer and thus more easily accessible for the Bemba people, is mentioned as a region of origin for *chikanda* traded in Zambia.

4.2 DNA barcoding performance

Since the term DNA barcoding was coined in 2003 [20], a plethora of studies applying DNA (meta)barcoding has been performed ranging from retrieving orchids from paleoenvironments [38], preserved in mammoth dung [39] to the identification of Iranian orchid tubers used for *salep* [5]. In

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this study a combined use of the core plant markers *matK* and *rbcL* and the nuclear ribosomal ITS region was used to attempt species level identification of tubers traded on Zambian markets. Although genetic distance calculations showed limited interspecific distances between closely related species for all three barcoding markers, DNA barcoding allowed for species-level identification for several of the frequently sold *chikanda* species. The data shows that the core land-plant DNA barcoding markers *rbcL* and *matK* were not suitable because of limited variability between species (*matK* and *rbcL*), amplification problems (*matK*), and/or a limited sequence reference database (*rbcL*). nrITS was shown to be more suitable as a barcode marker to distinguish between different *chikanda* species, but is not discriminative enough to enable reliable species level identification in certain orchid clades, such as the clade with *Platycoryne crocea*, *P. buchananiana* and *Habenaria buchananii;* the clade with *H. schimperiana*, *H. kyimbilae* and *H. microsaccos* and the clade containing *Disa* sp. 1. Another drawback of nrITS are the multiple ITS paralogs present in the ribosomal genome. Usually these copies would show high similarities due to concerted evolution [40,41], but this is not always the case [42–45]. In our case potentially different nrITS ribotypes became fixed in different orchid populations and having only one of them in our reference database could lead to the unresolved identifications observed. Our bPTP results show that even nrITS has too little resolution to reliably delimit species with high posterior probability support using this method. It does demonstrate, however, how valuable the use of tree-based methods can be, since it shows the relations between the sequences and can be used to determine if some of the unidentified samples are likely to belong to the same species or different species within the same genus. Even if no species-level identification can be made for these samples, it is possible to use the clustering to determine the diversity of species used. Although several samples can only be reliably identified as *Habenaria* sp., we find that they are likely to belong to at least three different species (*Habenaria* aff. *helicoplectrum*, *Habenaria* cf. sp. DO122 in the clade with *H. schimperiana*, *H. kyimbilae* and *H. microsaccos* and lastly the *Platycoryne* sp./*Habenaria* sp., which group together with *H. buchananii*, *P. buchananiana* and *P. crocea*. Expansion of the reference database, by including at least one individual per species, and preferably multiple individuals per species from different populations and countries, could ultimately solve remaining challenges, and this seems the way forward in identification of the traded *chikanda* tubers, as well as other species unidentifiable based on morphology. Similar studies using DNA barcoding for the identification of unknown samples show comparable results for the employed barcoding markers. In a study on the identification of orchids used for *salep*, nrITS showed an over three-time higher sequencing success than *matK* as well as a two-times higher species-level identification success [5]. Moreover, the similarity-based approach seemed to outperform the tree-based identification method (ML) in this study as well with 57% and 39% species-level identifications, respectively. nrITS also shows the highest identification performance in studies on the identification of medicinal plants [46,47], which supports the idea that it is recommendable to add a more discriminative marker to the two core land-plant barcodes in studies where it is needed to distinguish between closely-related species. Moreover, our results stress the need for a phylogenetically underpinned taxonomic framework, which is currently available for *Disa* [48] and *Satyrium* [49], but not yet for *Habenaria* and related genera.

4.3. Local versus scientific classification of chikanda

The results of our identifications of *chikanda* orchids traded in the Zambia show that the local classification systems for *chikanda* are not in congruence with the botanical classification of orchid species. The orchids sold on the markets were grouped according to area of origin, tuber consistency preference, or shape of the tuber, but often the tubers offered for sale were mixtures. The different local types of *chikanda* sometimes show a variation in orchid species that are identified within these local grouping. When we look at the *chikanda* type known as *kapapa* for example, SJK44 contains *Platycoryne crocea*, whereas SJK11, which is supposed to be a mix of *kasebelela* and *kapapa,* only seems to contain *Satyrium* species. In case of other chikanda types there seems to be more consistency: *myala* or real chikanda referred to *Disa robusta*, *D. welwitschii* and *Satyrium buchananii*, *mshilamshila* samples

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Table 1. Overview of the different local *chikanda* classification types, their collections and the identified scientific species.

Vernacular name	Collections	Reported origin	barcoding IDs	# samples
Fungulwe	SJK16	unknown	*Disa robusta*	1
Iringe	SJK17	Tanzania	*Satyrium buchananii*	1
			Satyrium carsonii	1
John White	SJK39	Mporokoso, Zambia	*Satyrium buchananii*	1
Kabula seke	SJK46	Serenje, Zambia	*Habenaria* sp. (Clade *H. schimperiana*, *H. kyimbilae*, H. *microsaccos*	1
			Platycoryne crocea Rolfe	7
Kapapa	SJK44	Mporokoso, Zambia	*Platycoryne crocea* Rolfe	6
Kasebelela, John White and Myala	SJK41	Chinsali and Mporokoso, Zambia and Tanzania	*Habenaria* cf sp. DO122 (Clade *H. schimperiana*, *H. kyimbilae*, H. *microsaccos*	4
			Platycoryne crocea Rolfe	4
			Platycoryne sp./*Habenaria* sp.	2
Kasebulela and Kapapa	SJK11	Luwingu, Zambia	*Satyrium kitimboense*	6
			Satyrium carsonii	5
Mbwelenge	SJK5	Luwingu, Zambia	*Satyrium buchananii*	11
			Satyrium sp.	1
	SJK32	Serenje, Zambia	*Satyrium buchananii*	6
Mshilamshila	SJK7	Luwingu, Zambia	*Brachycorythis sp.*	1
	SJK12	Kawamba, Zambia	*Brachycorythis sp.*	1
			Brachycorythis cf. *friesii*	1
Myala	SJK4	Mwinilunga, Zambia;	*Disa robusta*	4
			Disa welwitschii	1
			Satyrium buchananii	4
	SJK18	Sumbawanga, Tanzania	*Disa robusta*	4
			Satyrium buchananii	1

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Myala	SJK37	Kawambwa, Zambia	*Satyrium buchananii*	1
Myala and nampanda	SJK21	Luapula, Zambia	*Disa welwitschii*	2
			Satyrium buchananii	1
Ntonkonshi	SJK25	Democratic Republic of Congo	*Disa robusta*	1
Sumbawanga	SJK20	Sumbawanga, Tanzania	*Disa satyriopsis*	1
mixed	SJK31	Serenje, Zambia	*Disa caffra*	1
			Disa robusta	1
			Habenaria cf sp. DO122 (Clade *H. schimperiana*, *H. kyimbilae*, H. *microsaccos*	1
			Satyrium buchananii	6
unknown-mixed	SJK19	Luwingu, Zambia	*Satyrium carsonii*	1
			Habenaria aff. *helicoplectrum* (BB3151)	1
unknown	SJK8	Mwinilunga, Zambia;	*Disa miniata*	1
			Disa robusta	2
			Disa welwitschii	1
			Satyrium buchananii	2
	SJK9	Luwingu, Zambia	*Satyrium carsonii*	1
	SJK13	Kawamba, Zambia	*Disa celata*	1
			Disa welwitschii	1
			Satyrium buchananii	3

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were identified as *Brachycorythis* species and *mbwelenge* or fake *chikanda* was made of *Satyrium buchananii*. However, our previous study on the analysis of Tanzanian *chikanda* cakes showed that the cake made with fake *chikanda* tubers also contained *Disa miniata*, *Satyrium anomalum*, *S. comptum*, *S. elongatum*, *S. riparium*, *S. shirense* and *S. volkensii*, indicating that there might be some differences between fake *chikanda* samples as well [19]. It is well-known from literature that local species concepts are not necessarily congruent with scientific classifications and that species might be subject to over- or underdifferentiation [50,51]. In this case, the grouping of the orchids according to the area of origin, shape or consistency preference or plainly under the general term *chikanda* is clearly a case of underdifferentiation as a much higher diversity was retrieved when employing DNA barcoding. In order to more reliably identify the orchid species used for a particular *chikanda* type more samples per local classification need to be analyzed.

4.4. Orchid availability and conservation

Througout recent decades *chikanda* has made a remarkable leap in popularity. The first record of *chikanda* use made by Audrey Richards [9], described the relish as a poor man's food, eaten in times of famine. Recent studies, from 2002 onwards, show that *chikanda* has emerged as a Zambian snack popular throughout the country. Studies on *chikanda* report that with this rise in popularity, the orchid harvest has escalated and is pressuring local Zambian orchid populations as well as those in neighboring countries [52,12,10,53]. Many people involved in *chikanda* trade indicated that *chikanda* plants were becoming scarce, and many were concerned about both the quality as well as the quantity of the orchids available. Our study also confirmed a significant international trade network for *chikanda* sources in several regions in Tanzania, as well as in the Democratic Republic of Congo and Angola. In our current study, we found at least 16 different orchid species sold as *chikanda* on the Zambian markets and an overview of previous studies contains 46 species reported to be used for *chikanda* (Table S7). This brings us from the use of an initial two orchid species reported for chikanda [13] to a total of 49 species in eight different genera. The increased harvesting pressure in combination with the indiscriminate harvesting and use of many more species than earlier assumed pose a threat to nearly half of the terrestrial orchids occurring in these regions. Despite the establishment of Kitulo National Park in Tanzania, with orchid conservation as a prime concern, it seems that harvesting continues even there, since *iringe* tubers found in this study come specifically from this region [12,53]. Currently there are only seven *Disa* species from Zambia and surrounding countries registered on the global IUCN Red List and no species from other genera used for *chikanda* [54]. Most of the orchid species used for *chikanda* seem to have a widespread distribution, but local populations as well as endemic species could be at risk of overharvesting, and we urge to add the most frequently traded *chikanda* species, such as *Disa robusta* and *Satyrium buchananii* to the IUCN Red List. Although there seems no stopping to commerce, people involved in *chikanda* trade seem genuinely concerned about welfare of local orchids and interested in exploring other options. Since especially the *chikanda* harvesters seem to be in a vulnerable position, where they have to rely on surrounding natural resources to secure their livelihoods [16], it is essential that when trying to protect orchids used for *chikanda*, the situation of the people dependent on the trade is taken into account as well. Currently the development of sustainable cultivation or *chikanda* orchids is attempted in collaboration with the Cape Institute of Micropropagation (Barrydale, South Africa) and possible alternative sources of income for the people involved in *chikanda* trade, such as honey production, are being explored [55]. Alternatively, since the purpose of the *chikanda* orchids mainly is to bind and create an elastic structure to the cake, it might be possible to encourage the use of an alternative source of starch to replace the tubers.

5. Conclusions

DNA barcoding using the nuclear ribosomal ITS marker proved to be useful in identifying terrestrial orchid species traded as *chikanda* on local Zambian markets and outperformed identification using the core land-plant barcoding markers *matK* and *rbcL*. Sixteen orchid species, of which three previously undocumented, were identified from marketed *chikanda* tubers, bringing the

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total number of orchid species used for *chikanda* to at least 49. The species most frequently found on the markets were *Disa robusta*, *Satyrium buchananii* and *Platycoryne crocea*. However, the results are only as good at the reference material is and an increased reference database in combination with an underpinned phylogenetic framework for *Habenaria* and related genera would likely ameliorate the reliability of the identifications. Tubers are harvested from various regions in Zambia and Tanzania, and additional international *chikanda* trade-hubs have been identified on the border with the Democratic Republic of Congo and Angola. People involved in *chikanda* trade indicate that both orchid quality as well as quantity are decreasing and are willing to consider alternatives to *chikanda* trade to secure their income.

Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

Supplementary Materials: The following supplementary figures and tables are available online at www.mdpi.com/xxx/s1

Figure S1. bPTP analysis of all *chikanda* matK query and reference samples.
Figure S2. bPTP analysis of all *chikanda* rbcL query and reference samples.
Figure S3. bPTP analysis of all *chikanda* nrITS query and reference samples.
Table S1. Brahms RDE file of novel voucher specimens of orchid taxa samples in this study.
Table S2. Genbank accession numbers of the chikanda tuber sequences.
Table S3. Hit table with the first 5 BLAST top hits per sample for nrITS.
Table S4. Hit table with the first 5 BLAST top hits per sample for *matK.*
Table S5. Hit table with the first 5 BLAST top hits per sample for *rbcL.*
Table S6. Successfully sequenced samples with their vernacular name, reported origin, identification based on sequence-similarity for nrITS and *matK*, identification based on the tree-based bPTP analysis and the consensus ID.
Table S7. Orchid species used for chikanda according to literature.

Author Contributions: S.V., R.B. and H.d.B. conceived and designed the study in collaboration with S.J.K., D.C., R.V. and N.W.; S.J.K. performed the chikanda collection and interviews in collaboration with D.C., G.M., N.W. and R.V; R.B., N.W. and K.Y. collected reference vouchers in the field, which were identified in collaboration with B.B.; B.B, G.N and F.M. provided the reference sequence database for Habenaria species and some species in closely related genera; S.J.K. performed the labwork for the chikanda collections and M.B.F. for the reference collections; S.J.K. performed the data analysis under supervision of S.V. and H.d.B.; S.V. and S.J.K. wrote the manuscript in consultation with the other authors and the manuscript was edited and reviewed in detail by B.G., T.v.A. and H.d.B.

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