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

**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;
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 overharaesting 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.
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 10 mM 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 25 μl with 14.725 μl ddH2O, 2.5 μl DreamTaq Buffer (Thermo Fisher Scientific, Oakwood, USA), 0.5 μl 25 mM dNTP, 0.65 μl 2% Bovine Serum Album (BSA), 0.125 μl DreamTaq Polymerase, 2.5 μl 5 pmol forward reverse primer and 1.5 μl template DNA. Nuclear ribosomal nrITS was amplified using the Sun et al. [30] primers and protocol in a total reaction volume of 25 μl containing 15.25 μl ddH2O, 2.5 μl DreamTaq Buffer (Thermo Fisher Scientific, Oakwood, USA), 0.5 μl 25 mM dNTP, 0.125 μl 2% BSA, 0.125 μl DreamTaq Polymerase (Thermo Fisher Scientific, Oakwood, USA), 2.5 μl 5 pmol forward and reverse primer and 1.5 μl template DNA. For ITS an additional protocol was used with Q5 high-fidelity polymerase: reactions were performed in a total reaction volume of 23.5 μl including 10.875 μl ddH2O, 5 μl Q5 reaction buffer, 0.5 μl 25 mM dNTP, 5 μl Q5 GC enhancer, 0.125 μl Q5 high-fidelity polymerase, 1.5 μl 5 pmol forward and reverse primer and 0.5 μl template DNA. The PCR program for the ITS primers in combination with the Q5 polymerase was an initial heating step of 30 s at 98°C; 35 cycles of 10 s 98°C, 30 s 56°C, 30 s 72°C; and a final elongation of 2 min 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
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_5.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, ‘middlesmen’ 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 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 false (mwevelenge or msekete). In some cases it was the shape of the tuber that was used to differentiate between the different tubers: mshilanshila 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: mwiniyanga, 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.
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 intraspecific distance calculation of Habenaria
(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 satyiropsis Kraenzl., Disa welwitschii Rchb. f., Disa sp. SJK4.1., Habenaria sp. SJK3.15, 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
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. verdichii* 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
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. helicoplectum, 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 sale, 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 kasebela 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
Table 1. Overview of the different local *chikanda* classification types, their collections and the identified scientific species.

<table>
<thead>
<tr>
<th>Vernacular name</th>
<th>Collections</th>
<th>Reported origin</th>
<th>barcoding IDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungulwe</td>
<td>SJK16</td>
<td>unknown</td>
<td><em>Disa robusta</em></td>
</tr>
<tr>
<td>Iringe</td>
<td>SJK17</td>
<td>Tanzania</td>
<td><em>Satyrium buchananii</em></td>
</tr>
<tr>
<td>John White</td>
<td>SJK39</td>
<td>Mporokoso, Zambia</td>
<td><em>Satyrium buchananii</em></td>
</tr>
<tr>
<td>Kabula seke</td>
<td>SJK46</td>
<td>Serenje, Zambia</td>
<td><em>Satyrium carsonii</em></td>
</tr>
<tr>
<td>Kapapa</td>
<td>SJK44</td>
<td>Mporokoso, Zambia</td>
<td><em>Habenaria sp. (Clade H. schimperiana, H. kyimbilae, H. microsaccos</em></td>
</tr>
<tr>
<td>Kasebelela, John White and Myala</td>
<td>SJK41</td>
<td>Chinsali and Mporokoso,</td>
<td><em>Platycoryne crocea Rolfe</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zambia and Tanzania</td>
<td></td>
</tr>
<tr>
<td>Kasebulela and Kapapa</td>
<td>SJK11</td>
<td>Luwingu, Zambia</td>
<td><em>Satyrium kitinboense</em></td>
</tr>
<tr>
<td>Mbwelenge</td>
<td>SJK5</td>
<td>Luwingu, Zambia</td>
<td><em>Satyrium buchananii</em></td>
</tr>
<tr>
<td></td>
<td>SJK32</td>
<td>Serenje, Zambia</td>
<td></td>
</tr>
<tr>
<td>Mshilamshila</td>
<td>SJK12</td>
<td>Kawamba, Zambia</td>
<td><em>Brachycorythis sp.</em></td>
</tr>
<tr>
<td></td>
<td>SJK4</td>
<td>Mwinilunga, Zambia;</td>
<td><em>Disa robusta</em></td>
</tr>
<tr>
<td>Myala</td>
<td>SJK18</td>
<td>Sumbawanga, Tanzania</td>
<td><em>Disa welwitschii</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Satyrium buchananii</em></td>
</tr>
</tbody>
</table>


Peer-reviewed version available at Genes 2018, 9, 595; doi:10.3390/genes9120595
<table>
<thead>
<tr>
<th>Location</th>
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<th>Collection Location</th>
<th>Species</th>
<th>Count</th>
</tr>
</thead>
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<tr>
<td>Myala</td>
<td>SJK37</td>
<td>Kawambwa, Zambia</td>
<td>Satyrium buchananii</td>
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<tr>
<td>Myala and nampanda</td>
<td>SJK21</td>
<td>Luapula, Zambia</td>
<td>Disa velvitschii</td>
<td>2</td>
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<tr>
<td>Ntonkonshi</td>
<td>SJK25</td>
<td>Democratic Republic of Congo</td>
<td>Satyrium buchananii</td>
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</tr>
<tr>
<td>Sumbawanga</td>
<td>SJK20</td>
<td>Sumbawanga, Tanzania</td>
<td>Disa satyriopsis</td>
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</tr>
<tr>
<td></td>
<td>SJK31</td>
<td>Serenje, Zambia</td>
<td>Disa caffra</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SJK20</td>
<td>Sumbawanga, Tanzania</td>
<td>Disa robusta</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SJK8</td>
<td>Mwinilunga, Zambia; Mwinilunga, Zambia; Luwingu, Zambia</td>
<td>Habenaria cf sp. DO122 (Clade H. schimperiana, H. kyimbilae, H. microsaccos)</td>
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</tr>
<tr>
<td>mixed</td>
<td>SJK31</td>
<td>Serenje, Zambia</td>
<td>Satyrium carsonii</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SJK19</td>
<td>Luwingu, Zambia</td>
<td>Habenaria aff. helicoplectrum (BB3151)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SJK8</td>
<td>Mwinilunga, Zambia; Mwinilunga, Zambia; Luwingu, Zambia</td>
<td>Disa miniata</td>
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<tr>
<td></td>
<td>SJK9</td>
<td>Luwingu, Zambia</td>
<td>Disa robusta</td>
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</tr>
<tr>
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<td>SJK13</td>
<td>Kawamba, Zambia</td>
<td>Disa velvitschii</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SJK13</td>
<td>Kawamba, Zambia</td>
<td>Satyrium carsonii</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SJK13</td>
<td>Kawamba, Zambia</td>
<td>Disa celata</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SJK13</td>
<td>Kawamba, Zambia</td>
<td>Satyrium buchananii</td>
<td>3</td>
</tr>
</tbody>
</table>
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

Throughout 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 [12,53]. 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
total number of orchid species used for *chikanda* to at least 49. The species most frequently found on
the markets were *Disa robusta*, *Satzrium 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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knowledge and Simon Hultby from the Uppsala Botanical Garden for growing the sprouted *chikanda*
orchid tubers.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design
of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or
in the decision to publish the results.

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S.; Kumar, P.; Masters, S.; Metusala, D.; Roberts, D. L. A review of the trade in orchids


54. IUCN IUCN Red List of Threatened Species. *Version 2017-3*.; 2018;