

1 **DNA barcoding of tuberous Orchidoideae: A resource for identification of orchids used in Salep**

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3 Abdolbaset Ghorbani<sup>1,2</sup>, Barbara Gravendeel<sup>3,4</sup>, Sugirthini Selliah<sup>5</sup>, Shahin Zarré<sup>6</sup>, Hugo de Boer<sup>1,3,5,\*</sup>

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5 <sup>1</sup> Department of Organismal Biology, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18D, SE-75236,  
6 Sweden

7 <sup>2</sup> Traditional Medicine & Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran,  
8 Iran

9 <sup>3</sup> Naturalis Biodiversity Center, Darwinweg 2, 2333 CR Leiden, The Netherlands

10 <sup>4</sup> University of Applied Sciences Leiden, Leiden, The Netherlands

11 <sup>5</sup> The Natural History Museum, University of Oslo, P.O. Box 1172 Blindern, 0318 Oslo, Norway

12 <sup>6</sup> Department of Plant Sciences, University of Tehran, Iran

13 \* Corresponding author: Hugo de Boer, Natural History Museum, University of Oslo, P.O. Box 1172 Blindern, 0318  
14 Oslo, Norway. Email: [hugo.deboer@nhm.uio.no](mailto:hugo.deboer@nhm.uio.no)

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20

21 **Abstract**

22 Tubers of terrestrial orchids are harvested and traded from the eastern Mediterranean to the Caspian Sea for the  
23 traditional product Salep. Over-exploitation of wild populations and increased middle-class prosperity have escalated  
24 prices for Salep, causing overharvesting, depletion of native populations and providing an incentive to expand  
25 harvesting to untapped areas in Iran. Limited morphological distinctiveness among traded Salep tubers renders species  
26 identification impossible, making it difficult to establish which species are targeted and affected the most. In this study,  
27 a reference database of 490 nrITS, *trnL*-F spacer and *matK* sequences of 133 taxa was used to identify 150 individual  
28 tubers from 31 batches purchased in 12 cities in Iran to assess species diversity in commerce. The sequence reference  
29 database consisted of 211 nrITS, 158 *trnL*-F, and 121 *matK* sequences, including 238 new sequences from collections  
30 made for this study. The markers enabled unambiguous species identification with tree-based methods for nrITS in 67%  
31 of the tested tubers, 58% for *trnL*-F and 59% for *matK*. Species in the genera *Orchis* (34%), *Anacamptis* (27%) and  
32 *Dactylorhiza* (19%) were the most common in Salep. Our study shows that all tuberous orchid species in this area are  
33 threatened by this trade, and further stresses the urgency of controlling illegal harvesting and cross-border trade of Salep  
34 tubers.

35

36 **Introduction**

37

38 Tuberos terrestrial orchids have long been used as medicine and dietary supplements in different parts of the world  
39 (Bulpitt 2005; Bulpitt *et al.* 2007; Hossain 2011; Chinsamy *et al.* 2011). In the eastern Mediterranean, Asia Minor and  
40 the Middle East, tubers of different orchid species are collected indiscriminately from the wild and are traded for  
41 production of Salep tuber powder (Kasperek & Grimm 1999; Ece Tamer *et al.* 2006; Sandal Erzurumlu & Doran 2011;  
42 Ghorbani *et al.* 2014a; Kreziou *et al.* 2015). Harvested tubers are washed in water, boiled in either water or milk, sun-  
43 dried and traded as dried tubers (Kasperek & Grimm 1999). The tubers are ground into a powder and used in preparing  
44 a hot beverage known as Salep or Salepi and also in ice cream production (Sezik 2002a; Ece Tamer *et al.* 2006; Starin  
45 2012). Salep drink was once common in Europe (Landerer 1850), but is now consumed mainly in Turkey and Greece  
46 (Bulpitt 2005; Ece Tamer *et al.* 2006; Starin 2012). It is estimated that as much as 30 tons of orchid tubers are harvested  
47 annually in Turkey, which requires the destruction of 30-120 million orchid plants (Kasperek & Grimm 1999; Sezik  
48 2006). Increasing popularity of Salep has increased the demand for Salep tubers, which in turn has led to further  
49 overharvesting of wild orchid populations (Sezik 2002b; Kreziou *et al.* 2015). Scarcity of wild orchids in Turkey has  
50 forced traders to tap into new sources in adjacent countries (Ghorbani *et al.* 2014b). In Iran, where orchid tubers are  
51 traditionally hardly consumed, an orchid boom is underway in which an estimated 5.5-6.1 million orchids are harvested  
52 annually for export to Turkey (Ghorbani *et al.* 2014a). Conservation concerns have made orchid tuber collection illegal  
53 in Greece, Turkey and Iran, but collection bans are poorly enforced (Ghorbani *et al.* 2014b; Kreziou *et al.* 2015). All  
54 orchid species are included by the Convention on International Trade of Endangered Species of Fauna and Flora  
55 (CITES) on Appendices I or II (CITES 2014), which means that international trade of these species and derived  
56 products is regulated. Most of the Salep tuber trade from Iran to Turkey takes place without CITES permits, and tubers  
57 are often mislabeled as low-value nuts or other products to circumvent taxes and permit requirements (Kasperek &  
58 Grimm 1999; Ghorbani *et al.* 2014b; Kreziou *et al.* 2015). This large-scale, yet poorly visible trade makes it difficult to  
59 ascertain which species are targeted and in what quantities. Morphology-based approaches for identification are  
60 insufficient and cannot even accurately distinguish dried tubers from different genera. Other methods for salep  
61 identification, such as GCMS, HPLC, gravimetric, absorbance and rheological analyses, all indicate that identification  
62 to species level is not possible using only chemical analyses (Dogan *et al.* 2007; Tekinşen & Güner 2010; Babbar &  
63 Singh 2016). Adequate monitoring would enable identification of priority species for conservation measures such as  
64 curbing overexploitation, and targeting high-value species for cultivation.

65 DNA barcoding provides an accurate and reliable alternative to morphology-based identification of biological  
66 material (Hebert *et al.* 2003). As a method it can be used to identify and discern species at any developmental or  
67 processing stage from which DNA can be extracted (Hebert *et al.* 2003; Hajibabaei *et al.* 2007), and even from the  
68 minute amounts such as those found in dung (Hibert *et al.* 2013), pollen (Richardson *et al.* 2015), degraded herbarium  
69 vouchers (Särkinen *et al.* 2012), permafrost preserved subfossils (van Geel *et al.* 2008), and ancient sediment cores  
70 (Williams *et al.* 2000; Posadzki *et al.* 2012). Plant DNA barcoding has been applied in many fields, for example  
71 molecular systematics (Liu *et al.* 2011; van Velzen *et al.* 2012), biodiversity inventories (Aubriot *et al.* 2013; Thompson  
72 & Newmaster 2014), wildlife forensics (Deguilloux *et al.* 2002; Ogden *et al.* 2009), bio-piracy control (Parveen *et al.*  
73 2012), and authentication of herbal products (Kool *et al.* 2012; Coghlan *et al.* 2012; Newmaster *et al.* 2013; de Boer *et al.*  
74 2014; Vassou *et al.* 2015).

75 Several genetic regions have been proposed as standard barcodes for land plants, the ideal barcode being both  
76 easily amplifiable and efficiently retrievable from any of the 300,000+ species of plants (Kress *et al.* 2005; Fazekas *et al.*  
77 2008). Most studies now employ a tiered multilocus approach, which is based on the use of a common, easily  
78 amplified and aligned region such as *rbcL*, *rpoC1*, *trnL* or *trnL-F* spacer that can act as a scaffold on which to place  
79 data from a more variable noncoding region such as *matK*, *trnH-psbA*, nrITS, or nrITS2. Most species (approximately  
80 75-85%) can be identified using such an approach, and the subsequent addition of surrogate regions can increase  
81 barcoding success to over 90% in some floras (Ebihara *et al.* 2010; Burgess *et al.* 2011; de Vere *et al.* 2012; Kuzmina *et al.*  
82 2012; Liu *et al.* 2015). In Orchidaceae, several plastid and nuclear molecular markers including *rbcL*, *psaB*, *psbC*-  
83 *trnS*, *rpl16*, *matK*, *ycf1*, *trnH-psbA*, *trnH-trnK*, *trnL-F* and nrITS have been applied for phylogenetic analysis (Cameron  
84 2004; Xiang *et al.* 2011; Parveen *et al.* 2012; Inda *et al.* 2012; Kim *et al.* 2014). These studies suggest that a multi-locus  
85 combination of coding and non-coding regions with different evolutionary rates is necessary for effective identification  
86 of species in Orchidaceae.

87 This study tests the hypothesis that molecular identification using DNA barcoding can be used for  
88 identification of orchid species comprising boiled and dried tuber samples traded in the main export market hubs in  
89 Iran. We address the following research questions: 1) Can DNA be extracted, amplified and sequenced from boiled and  
90 dried Salep tubers? 2) What marker or markers are optimal for the identification of Salep tubers traded in the markets of  
91 Iran? 3) What genera and species are most common among the tubers included in our sampling? 4) Can the most  
92 common traded species be used to predict the main source areas of orchid tubers exported to Turkey? The aim was to  
93 test and establish a DNA barcoding protocol to identify dried orchid tubers from markets and to show the potential of  
94 this technique to curb illegal trade of CITES listed orchid tubers.

95

96

97 **Methods**

98

99 *Collection of reference and market material*

100 Flora Iranica vol. 126 (Renz 1978), Flora of Iran vol. 57 (Shahsavari 2008) and Orchids of Europe, North Africa and the  
101 Middle East (Delforge 2006) were used to estimate that a total of 47 orchid species occur in Iran, including 32 species  
102 with tuberous roots that could potentially be targeted for Salep collection. During fieldwork in 2013-2014, a total of 127  
103 herbarium vouchers representing 30 species and subspecies of orchids were collected from natural populations in  
104 different parts of Iran (Suppl. 1). Vouchers were identified (Renz 1978; Delforge 2006; Shahsavari 2008) and deposited  
105 at the herbarium of Tehran University (TUH). Sequences generated from these vouchers (Suppl. 1) as well as selected  
106 vouchered sequences from NCBI GenBank were used to construct a DNA barcode reference library (Suppl. 2).

107 Markets in 12 cities and towns in Iran (Tehran, Kermanshah, Sanandaj, Tabriz, Urmia, Mahabad, Shahindezh,  
108 Kashan, Ardabil, Aq-Emam, Marave-Tappe and Kalaleh) were visited and 31 batch samples of unidentified Salep  
109 tubers containing 15-50 tubers each were purchased. Figure 1 shows the distribution of orchids in Iran at genus level  
110 based on indexed vouchers from TUH and W, plus the location of the 12 main Salep markets. Per sample, tubers were  
111 subsequently categorized based on shape and size, and a total of 150 random tubers were selected as query tubers for  
112 DNA barcoding. Salep tubers in trade are hard to identify, although palmate *Dactylorhiza* tubers differ from those of  
113 other tuberous genera (Figure 2).

114

115 *DNA extraction, amplification and sequencing*

116 For reference samples, total genomic DNA was extracted from silica-gel dried leaf material using a modified CTAB  
117 protocol (Doyle & Doyle 1987). The query tubers were ground into powder using liquid nitrogen, and subsequently  
118 DNA was extracted using a STE-CTAB protocol (Shepherd & McLay 2011). The STE-CTAB protocol was necessary  
119 to reduce gel formation due to the high glucomannan content of tubers. A gelatinous layer, which was formed after  
120 adding CTAB buffer, caused difficulties in extraction procedures and low DNA yields. Extracted DNA was purified  
121 using a GE Illustra GFX<sup>TM</sup> PCR DNA and Gel Band Purification kit following the manufacturer's protocol (GE  
122 Healthcare, Buckinghamshire, UK).

123 Three barcode regions, nrITS (ITS1-5.8S-ITS2), *trnL*-F spacer and *matK* were amplified by a standard  
124 polymerase chain reaction (PCR). The nrITS (ITS1-5.8S-ITS2) region was amplified using the following primers:

125 17SE\_F (5'-ATGGTCCGGTGAAGTGTC-3'), 26SE\_R (5'-CCCGGTTTCGCTCGCCGTTAC-3'), 5.8I-1\_R (5'-  
126 GTTGCCGAGAGTCGT-3') and 5.8I-2\_F (5'-GCCTGGGCGTCACGC-3') (Sun *et al.* 1994). The *trnL*-F spacer was  
127 amplified using the following primers: C\_F (5'-CGAAATCGGTAGACGCTACG-3'), C2\_F (5'-  
128 GGATAGGTGCAGAGACTCAAT-3') and F\_R (5'-ATTTGAACTGGTGACACGAG-3') (Taberlet *et al.* 1991;  
129 Bellstedt *et al.* 2001). *MatK* was amplified using the following four primers: 19\_F (5'-  
130 CGTTCTGACCATATTGCACTATG-3') and 881R (5-TMTTCATCAGAATAAGAGT-3) (Gravendeel *et al.* 2001);  
131 F2\_F (5'-CTAATACCCCATCCCATCCAT-3') (Steele & Vilgalys 1994) and R1\_R (5'-  
132 CATTTCATTGCACACGRC-3') (Kocyan *et al.* 2004). PCR amplification was performed in a 50 µl reaction  
133 volume containing 5 µl reaction buffer IV (10x), 5 µl MgCl<sub>2</sub> (25mM), 1 µl dNTP (10 µM), 0.25 µl Taq-polymerase (5  
134 U/µl), 0.5 µl BSA, 1 µl of each primer (10 mM) and 1 to 4 µl of template DNA. The PCR protocols of 95°C 3 min.,  
135 (95°C 20 s., 55°C 1 min., 72°C 2 min.) x 35, 72°C 10 min., 8°C ∞ for nrITS, 95°C 3 min., (95°C 15 s., 55°C 50 s.,  
136 72°C 4 min.) x 35, 72°C 8 min., 8°C ∞ for *trnL*-F spacer and 95°C 3 min., (95°C 34 s., 59°C 45 s., 72°C 1 min.) x 35,  
137 72°C 7 min., 8°C ∞ for *matK* were applied. Sanger sequencing was performed by MacroGen Europe Inc. (Amsterdam,  
138 the Netherlands) on an ABI3730XL automated sequencer (Applied Biosystems). Primers used for PCR amplification  
139 were also used for sequencing reactions.

140

#### 141 *Reference database preparation*

142 The reference database was compiled from a total of 490 source sequences of 133 taxa, including both voucher  
143 specimens collected from the field including 85 nrITS sequences (19 species), 90 *trnL*-F (26 species), 63 *matK* (20  
144 species) and publicly available DNA sequences from NCBI GenBank including 126 nrITS sequences (102 species), 68  
145 *trnL*-F (56 species) and 58 *matK* (55 species) (Table 1). All sequences were downloaded from the listed tuberous  
146 genera in the tribe Orchideae (Orchidaceae), including synonymous genera and/or species: *Anacamptis* Rich.,  
147 *Cephalanthera* Rich., *Chamorchis* Rich., *Dactylorhiza* Neck. ex Nevski (including *Coeloglossum* Hartm.), *Gennaria*  
148 Parl., *Gymnadenia* R.Br., *Himantoglossum* W.D.J.Koch (incl. *Barlia* Parl. and *Comperia* K.Koch), *Neotinea* Rchb.f.,  
149 *Neottia* Guett. (incl. *Listera* R.Br.), *Neottianthe* Schltr., *Ophrys* L., *Orchis* L. (incl. *Aceras* R.Br.), *Serapias* L.,  
150 *Limodorum* Boehm., *Platanthera* Rich., and *Steniseiella* Schltr. Representative accessions were included for non-  
151 tuberous genera and tuberous species occurring close to the study area: *Corallorhiza trifida* Châtel, *Epipactis*  
152 *helleborine* (L.) Crantz, *Goodyera repens* (L.) R.Br., *Habenaria macroceratitis* Willd., *Herminium monorchis* (L.)  
153 R.Br., *Pecteilis gigantea* (Sm.) Raf., *Peristylus densus* (Lindl.) Santapau & Kapadia, *Pseudorchis albida* (L.) Á.Löve &  
154 D.Löve, *Satyrium bicornis* (L.) Thunb., *Spiranthes aestivalis* (Poir.) Rich., *Spiranthes spiralis* (L.) Chevall. and *Zeuxine*

155 *strateumatica* (L.) Schltr (Suppl. 2). Where there were more than two accessions per marker per species, only two  
156 accessions were selected, giving priority to those accessions with associated vouchers plus optimal read length and  
157 quality. Representative accessions of *Brownleea parviflora* Harv. ex Lindl., *Disa uniflora* P.J.Bergius and *Disperis*  
158 *lindleyana* Rchb.f. were selected as outgroups based on Inda et al. (2012).

159

#### 160 *Data analysis*

161 Contigs were assembled and edited in SeqTrace (Stucky 2012). All sequences including reference sequences and query  
162 tuber sequences were aligned using MUSCLE (Edgar 2004) as implemented in Aliview v. 1.15 aligner (Larsson 2014).  
163 Final manual inspections were performed and adjustments were done if necessary. Sequences generated for this study  
164 were submitted to NCBI GenBank (Suppl. 1, Suppl. 3).

165 Bayesian inference (BI) and maximum likelihood (ML) analysis were performed for each marker separately  
166 and on concatenated datasets, using RAxML-HPC v.8 (Stamatakis 2014) and MrBayes v.3.2.2 (Ronquist *et al.* 2012) on  
167 CIPRES Science Gateway v.3.3 (Miller *et al.* 2010) and the high performance computing facility available at University  
168 of Oslo, Lifeportal (<https://lifeportal.uio.no/root>). Gaps were treated as missing data.

169 For Bayesian analyses, the model GTR + G was selected for all datasets. Two independent runs with sixteen  
170 MCMC chains were simultaneously performed for 20 million rearrangements initiated with a random starting tree, and  
171 sampling one tree every 1000 generations, except for *matK*. For *matK*, we performed eight MCMC chains and a total of  
172 10 million generations using the default heating temperature. Convergence of runs with default parameters was assessed  
173 on preliminary analyses. Where convergence did not occur, the heating parameter was adjusted to reach a convergence.  
174 Convergence of runs was assessed using Tracer v. 1.6 (Rambaut *et al.* 2014). Twenty-five percent of trees were  
175 discarded as burn-in, and the remaining trees were used to generate a consensus tree with Bayesian posterior  
176 probabilities (PP) values. Only PP values over 0.95 were considered and included for each marker and concatenated  
177 topologies. The number of trees retained for each analysis is presented in Table 2.

178 For maximum likelihood analyses with RAxML, the model GTR + G was selected for all datasets, and a rapid  
179 bootstrap analysis with 1000 trees was conducted. Single marker trees were compared for incongruence prior to  
180 concatenation. Datasets were concatenated using Geneious v. 6.1.8 (Kearse *et al.* 2012). Multiple GenBank reference  
181 sequences for a single species were merged in order to obtain one consensus species sequence (cf. Suppl. 2). The unlink  
182 option was used to estimate the parameters for each partition.

183 The BI and ML phylogenetic trees were used to identify the query tubers (Suppl. 4-11). The tubers were  
184 considered successfully identified to species level when they were monophyletically clustered with related individuals

185 of the same species. When tubers were clustered with individuals of different species of the same genus, only a genus  
186 level identification was assigned (Suppl. 12).

187 Sequence similarity search using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) is often  
188 used in DNA barcoding (Little & Stevenson 2007; Sass *et al.* 2007; Kool *et al.* 2012; de Boer *et al.* 2014). BLAST+  
189 (Camacho *et al.* 2009) features implemented in NCBI BLAST were used to query unknown tuber sequences against the  
190 compiled reference database. All top hits less than 15 points lower than the max score were considered for  
191 identification: if the retained top hits (max score -15 points) included only a single species then a species level  
192 identification was estimated; if the retained top hits (max score -15 points) included multiple species in the same genus  
193 then a genus level identification was estimated; if the retained top hits (max score -15 points) included multiple species  
194 in different genera then a family level identification was estimated (Suppl. 12).

195 Final consensus identifications were made based on the results from all markers and methods, BLAST, ML,  
196 and BI (Suppl. 12). Species level identification was assigned if all markers with species level identifications yielded the  
197 same species identification. Genus level identification was assigned if identifications resulted in multiple species of the  
198 same genus.

199

200

## 201 **Results and discussion**

202

### 203 *Amplification and sequencing success*

204 Sequencing success rates were different for reference samples and market tuber samples. For the reference leaf samples  
205 (L), sequencing success was 67% (85 samples) for nrITS, 71% (90) for the *trnL*-F spacer and 47% (63) for *matK* (Table  
206 2). Out of the 127 samples, all three markers could be sequenced for 34 samples, solely nrITS for 29, solely *trnL*-F  
207 spacer for 14 and solely *matK* for 7. For tuber samples (T), sequencing success was 69% for nrITS (104 samples), 63%  
208 for the *trnL*-F spacer (94) and 19% for *matK* (28) (Table 2). Out of 150 tuber samples, all three markers could be  
209 sequenced for 8 samples, solely nrITS for 53, solely *trnL*-F spacer for 29, and none for *matK* only. In general, low  
210 sequencing success might be due to degraded DNA as a result of boiling and drying the tubers during processing.  
211 Sequencing success for nrITS might be affected by fungal contamination during the drying process and orchid  
212 mycorrhizal associations producing a mix of plant and fungal nrITS sequences. *MatK* had the lowest amplification  
213 success, and it has been shown that this locus cannot be amplified with 'universal' orchid primers due to the presence of



214 alternative translation initiation codons in orchids (Barthet *et al.* 2015), and therefore requires 'case by case'  
215 optimization for each genus.

216

217

### 218 *Species identifications*

219 The similarity-based approach using BLAST using nrITS marker data identified 59 out of 104 tuber samples (57%) to  
220 genus level and 45 (43%) to species level. Using *trnL-F* spacer, 61 out of 94 tuber samples (65%) were identified to  
221 genus level and 33 (35%) to species level. Using *matK*, 11 out of 28 tuber samples (39%) were identified to genus and  
222 17 (61%) to species level. The consensus of the BLAST identification of the three markers resulted in genus level  
223 identification in 93 samples (62%) and species level in 57 samples (38%) (Table 3; Suppl. 12).

224 The tree-based approach using RAxML maximum likelihood using nrITS marker data identified 34 out of 104  
225 tubers (33%) to genus level and 70 (67%) to species level (Suppl. 4, Suppl. 12). Using *trnL-F* spacer, 39 out of 94  
226 samples (42%) were identified to genus level and 55 (58%) to species level (Suppl. 5, 12). Using *matK*, 12 out of 28  
227 tuber samples (43%) were identified to genus and 16 (57%) to species level (Suppl. 6, 12). Concatenated data identified  
228 87 samples (58%) to genus level and 63 (42%) to species level (Suppl. 7, 12). The ML consensus identification of the  
229 three markers identified 60 samples (40%) to genus level and 90 samples (60%) to species level (Table 3; Suppl. 12).

230 The tree-based approach using MrBayes Bayesian inference using nrITS marker data identified 33 out of 104  
231 tubers (32%) to genus level and 71 (68%) to species level (Suppl. 8, 12). Using *trnL-F* spacer, 39 out of 94 samples  
232 (42%) were identified to genus level and 55 (58%) to species level (Suppl. 9, 12). Using *matK*, 9 out of 28 tuber  
233 samples (32%) were identified to genus and 19 (68%) to species level (Suppl. 10, 12). Concatenated data identified 48  
234 samples (32%) to genus level and 102 (68%) to species level (Suppl. 11, 12). The BI consensus identification of the  
235 three markers identified 53 samples (35%) to genus level 97 samples (65%) to species level (Table 3; Suppl. 12).

236 The final identification that combines consensus identification results of ML, BI and BLAST approaches  
237 produced an identification of 49 tubers (32.7%) to the genus level and 101 (67.3%) to the species level (Suppl. 12).

238

### 239 *Species composition of Salep*

240 Similarity-based identifications using BLAST showed that *Orchis* (51 samples), *Anacamptis* (40 samples), *Dactylorhiza*  
241 (29 samples), *Ophrys* (18 samples) and *Himantoglossum* (11 samples) and *Steveniella* (1 sample) were constituents of  
242 the studied Salep samples from Iran (Suppl. 12). *Orchis simia* Lam. and *O. mascula* (L.) L. were the main *Orchis*  
243 species in Salep. *Anacamptis pyramidalis* (L.) Rich., *A. coriophora* (L.) R.M.Bateman, Pridgeon & M.W.Chase and *A.*

244 *palustris* (Jacq.) R.M.Bateman, Pridgeon & M.W.Chase were the main *Anacamptis* species. *Dactylorhiza umbrosa*  
245 (Kar. & Kir.) Nevski was the only identified *Dactylorhiza* species. However, 24 out of 29 *Dactylorhiza* samples were  
246 identified only to genus level. It is known that *Dactylorhiza* has a dynamic system of hybridization and allopolyploidy  
247 formation (Hedrén *et al.* 2001, 2008). These allopolyploids show no clear genetic differentiations despite phenotypic  
248 differences (Balao *et al.* 2015) and it is therefore difficult to identify these samples to species level using the applied  
249 markers. Similarly, *Ophrys* was found to be one of the constituents of Salep but discerning the species used as Salep  
250 with the BLAST similarity search was not possible. Species delimitation in closely related taxa of the genus *Ophrys* has  
251 been challenging because of continuous introgression and absence of complete lineage sorting (Devey *et al.* 2008).

252 Tree-based identifications using ML and BI showed similar results: *Orchis* (51 samples for ML and 52 for BI),  
253 *Anacamptis* (40 ML; 39 BI), *Dactylorhiza* (29 ML; 29 BI), *Ophrys* (18 ML; 18 BI), *Himantoglossum* (11 ML; 11 BI)  
254 and *Steveniella* (1 ML; 1 BI) were the constituents of Salep (Suppl. 12). *Anacamptis* species in Salep samples are *A.*  
255 *palustris*, *A. morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase, *A. pyramidalis* and *A. coriophora*. *Orchis* species  
256 contributing to Salep are *O. mascula*, *O. militaris* L. and *O. simia*. It was not possible to identify *Ophrys* and  
257 *Dactylorhiza* samples to species level using the applied markers.

258 Figure 3 shows the species composition of studied Salep tubers based on final consensus identifications  
259 including all markers and methods (Suppl. 12). The phylogenetic relationships among genera is based on Inda *et al.*  
260 (2012). Based on final identification results the genera *Orchis* (51 samples), *Anacamptis* (40 samples), *Dactylorhiza* (29  
261 samples), *Ophrys* (18 samples), *Himantoglossum* (11 samples) and *Steveniella* (1 sample) are the main the constituents  
262 of studied Salep samples. All tuberous orchid species are used for Salep with a preference for species in the genera  
263 *Orchis*, *Anacamptis* and *Dactylorhiza*.

264

#### 265 *Generic composition of tubers per geographic origin*

266 The analyzed tubers can be geographically categorized into three zones of origin: a western zone (Ardabil, Eastern and  
267 Western Azarbaijan, Kurdistan and Kermanshah provinces), a northern zone (Golestan) and a central zone (Tehran and  
268 Esfahan). Sixty-five tubers originate from the western zone, and these include 26 tubers (38%) of *Anacamptis*, 22 tubers  
269 (32%) of *Dactylorhiza* and 11 tubers (16%) of *Himantoglossum*. The generic composition of the 66 tubers from the  
270 northern zone is different, and these include 42 tubers (64%) of *Orchis* and 15 tubers (23%) of *Ophrys*. The 15 tubers  
271 from the central zone are mainly *Anacamptis* (8 samples, 53%) and *Dactylorhiza* (5 samples, 33%). Although  
272 distribution and abundance of orchids in Iran is poorly documented, the results show that *Dactylorhiza* tubers, that trade  
273 at a lower value in the market, are harvested in the western and central zones, whereas high-value *Orchis* tubers are

274 most commonly collected in the northern zone. Kasperek and Grimm (1999) report the presence of Iranian Salep in  
275 eastern Turkey in the 1990s, and Ghorbani *et al.* (2014a; b) writes that orchid tuber collection in western Iran has a  
276 longer history than in the north and east of Iran, where a recent boom is escalating harvesting and trade. The results  
277 could indicate that the resources for superior quality Salep tubers from *Orchis* species have been depleted in the western  
278 zone, and that Salep collection is now targeting the more inferior quality *Dactylorhiza* tubers. In the northern zone  
279 *Orchis* tubers are still readily available, but as natural populations dwindle collectors will target other genera.

280

281

## 282 **Conclusions**

283 This study has produced a resource of 238 reference sequences and 226 tuber sequences that can be used for  
284 identification of Orchidaceae species in the poorly documented Salep trade in Turkey, Greece and Albania. It also  
285 shows that genomic DNA of sufficient quality can be extracted and sequenced from highly processed Salep tubers.  
286 However, extraction of DNA is accompanied with some difficulties as a result of gel formation due to the high  
287 glucomannan content in the tubers. Post-harvest storage time of the tubers and boiling time during processing may also  
288 affect the quality of extracted DNA. Among the applied markers, nrITS and *trnL-F* spacer were easier to amplify and  
289 sequence than *matK* and these markers also show a higher discriminatory power for most of the genera. However,  
290 *Dactylorhiza* and *Ophrys*, that are known for allopolyploidy and hybridization, are challenging for barcoding using the  
291 applied markers, and a high-throughput sequencing gene capture approach would probably yield the right read depth for  
292 phasing of alleles and accurate species identification (Weitemier *et al.* 2014; Schmickl *et al.* 2015). The results also  
293 show that the genera most affected by Salep harvesting are *Orchis*, *Anacamptis*, *Dactylorhiza* and *Ophrys*. Geographic  
294 clustering of Salep tubers show clear differences in generic composition per zone with significant implications for  
295 harvesting pressure and resource depletion. *Dactylorhiza* and *Anacamptis* are more abundant as Salep tubers from the  
296 western zone, whereas *Orchis* and *Ophrys* are more abundant as Salep tubers from the northern zone. *Himantoglossum*  
297 was only present in Salep from the western zone. The results expose the overharvested species in each region that  
298 should be targeted for tailored conservation activities, and confirms the finding by Ghorbani *et al.* (2014a) that  
299 overharvesting of superior value *Orchis* tubers in western parts has led Salep middlemen and traders to tap into new  
300 areas in northern parts of the country. Conservation measures should be implemented in western, central and northern  
301 Iran to protect wild orchid populations from immediate threats due to unsustainable over-exploitation and to prevent  
302 their disappearance before many of them have even been studied properly.

303

304

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312

313

314 **Author contributions**

315 AG, HdB and BG devised the project. AG carried out the vast majority of the fieldwork, assisted by SZ, HdB and BG.  
316 AG, SS and HdB analyzed the data. AG, SS and HdB wrote the first draft of the manuscript. All authors have read and  
317 approve the final manuscript.

318

319

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479

480 **Data Accessibility**

481 The concatenated sequence matrix of all reference and tubers species, as well as resulting BI and ML phylogenetic trees  
482 (Suppl. 4-11) are deposited in Dryad, <http://dx.doi.org/10.5061/dryad.qb36g>.

483

484 **Tables and Figures**

485 **Figure 1.** Distribution of Salep genera in Iran and location of main markets. Shaded areas show the three zones of  
486 origin: western, central and northern zones.

487 **Figure 2.** Tuber samples of different morphology purchased from the markets. A. Samples of *Orchis/Anacamptis* type  
488 tubers. B. Samples of *Dactylorhiza* type tubers.

489 **Figure 3.** Identifications of screened Iranian Salep tubers. A. Shaded genera occur in Iran. Phylogeny adapted from  
490 Inda et al. 2012. B. Proportion of identified genera. C. Filled circles represent the number of tubers identified to a  
491 particular species.

492

493 **Table 1.** Species and samples per genus in sequence reference library.

494 **Table 2.** Sequence matrix and Bayesian analysis data.

495 **Table 3.** Molecular identification of Salep tuber to species and genus level.

496

497 **Supplemental Data**

498 **Supplement 1.** Reference sequences derived from vouchers collected for this study.

499 **Supplement 2.** Reference sequences derived from external NCBI GenBank accessions.

500 **Supplement 3.** GenBank accession numbers of the Salep tubers.

501 **Supplement 4.** RAxML maximum likelihood phylogenetic tree for nrITS.

502 **Supplement 5.** RAxML maximum likelihood phylogenetic tree for *trnL-F* spacer.

503 **Supplement 6.** RAxML maximum likelihood phylogenetic tree for *matK*.

504 **Supplement 7.** RAxML maximum likelihood phylogenetic tree for the concatenated matrix.

505 **Supplement 8.** MrBayes bayesian phylogenetic tree for nrITS.

506 **Supplement 9.** MrBayes bayesian phylogenetic tree for *trnL-F* spacer.

507 **Supplement 10.** MrBayes bayesian phylogenetic tree for *matK*.

508 **Supplement 11.** MrBayes bayesian phylogenetic tree for the concatenated matrix.

509 **Supplement 12.** Molecular identifications of tubers based on similarity- and tree-based approaches.

510

511 **Table 1.** Species and samples per genus in sequence reference library.

Genus	Identification Reference Resource			
	GenBank		Field collections	
	# Samples	# Species	# Samples	# Species
<i>Anacamptis</i>	6	5	17	3
<i>Brownleea</i>	1	1	NA	NA
<i>Cephalantera</i>	3	3	10	5
<i>Chamorchis</i>	1	1	NA	NA
<i>Corallorhiza</i>	1	1	NA	NA
<i>Dactylorhiza</i>	20	18	17	3
<i>Disa</i>	1	1	NA	NA
<i>Disperis</i>	1	1	NA	NA
<i>Epipactis</i>	1	1	7	2
<i>Gennaria</i>	1	1	NA	NA
<i>Goodyera</i>	1	1	NA	NA
<i>Gymnadenia</i>	8	7	NA	NA
<i>Habenaria</i>	1	1	NA	NA
<i>Herminium</i>	1	1	NA	NA
<i>Himantoglossum</i>	10	10	7	2
<i>Limodorum</i>	NA	NA	2	1
<i>Neotinea</i>	5	4	NA	NA
<i>Neottia</i>	3	3	3	1
<i>Neottianthe</i>	3	2	NA	NA
<i>Ophrys</i>	41	23	35	4
<i>Orchis</i>	8	7	21	4
<i>Pecteilis</i>	1	1	NA	NA
<i>Peristylus</i>	1	1	NA	NA
<i>Platanthera</i>	2	2	5	2
<i>Pseudorchis</i>	1	1	NA	NA
<i>Satyrium</i>	1	1	NA	NA
<i>Serapias</i>	8	7	NA	NA
<i>Spiranthes</i>	2	2	NA	NA
<i>Steeniella</i>	1	1	3	1
<i>Zeuxine</i>	1	1	NA	NA

513 **Table 2.** Sequence matrix and Bayesian analysis data.

Markers	No. of sequences			
	Reference (R)	Leaf (L)	Tuber (T)	Total
nrITS	126	85	104	315
<i>trnL-F</i>	68	90	94	252
<i>matK</i>	58	63	28	149
Concatenated	138	135	150	423

514

Markers	Alignment matrix		Bayesian analysis
	Seq length incl. gaps (bp)	Min/max length without gaps (bp)	No. trees retained
nrITS	822	209/722	30 002
<i>trnL-F</i>	1663	287/1032	30 002
<i>matK</i>	1173	365/1105	15 002
Concatenated	3658	209/2677	30 002

515

516 **Table 3.** Molecular identification of Salep tuber to species and genus level.  
 517

<b>Samples for which sequences were obtained</b>										
	ITS		<i>trnL-F</i>		<i>matK</i>		Concatenated		Consensus	
Sequenced samples	104		94		28		150		150	
<b>Similarity (BLAST) identification</b>										
Species	45	43%	33	35%	17	61%	-	-	57	38%
Genus	59	57%	61	65%	11	39%	-	-	93	62%
<b>Maximum likelihood (RAxML) identification</b>										
Species	70	67%	55	59%	16	57%	63	42%	90	60%
Genus	34	33%	39	41%	12	43%	87	58%	60	40%
<b>Bayesian inference (MrBayes) identification</b>										
Species	71	68%	55	59%	19	68%	102	68%	97	65%
Genus	33	32%	39	41%	9	32%	48	32%	53	35%

518