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Title: An insight into the Chinese traditional seafood market: species characterization of cephalopod products by DNA barcoding and phylogenetic analysis using COI and 16SrRNA genes.

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Keywords: squid; cuttlefish; octopus; processed seafood; molecular species characterization.

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Abstract: Squids, cuttlefish and octopus are used for the preparation of traditional products sold on the Chinese market without a specific denomination. In this study DNA barcoding and phylogenetic distance analysis of COI and 16S rRNA genes' fragments were used to characterize the most commonly processed species in dried whole, grilled shredded and salted cephalopod preparations. Ninety-five products (23 sold as cuttlefish, 4 as octopus and 68 as squid) purchased in Chinese local markets were analyzed. Overall, the study identified 10 different species: Sepia pharaonis, S. esculenta, S. recurvirostra, S. lycidas in cuttlefish; Amphioctopus marginatus in octopus; Uroteuthis chinensis, U. edulis, Ommastrephes bartramii, Illex argentinus and Dosidicus gigas in squids. This latter species, characterized by a low commercial value, was found in the majority of the samples (50.5%) and in all the shredded products. By comparing the molecular results with the declared macrocategory (cuttlefish, octopus and squid), two cases of misdescription were pointed out, involving shredded cuttlefish and octopus which were identified as D. gigas. Our results are of particular interest in the light of the scarcity of data regarding the identification of cephalopods on international markets and considering that China is one of the leading cephalopod-producing countries.

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Dear Editor,

Please find enclosed the manuscript entitled **"An insight into the Chinese traditional seafood market: species characterization of cephalopod products by DNA barcoding and phylogenetic analysis using COI and 16SrRNA genes**" to be considered for publication in Food Control.

Cephalopods represent an important resource for human nutrition. The global production largely depends on several Asian countries and, among them, China is one of the major producer, importer and exporter. While on the international market cephalopods are generally sold fresh or frozen whole or sliced products (rings and arms, tubes and wings), the offer of these products on the Chinese internal market consists of traditionally processed specialities. Their typology varies among different areas, according to consumers' preferences and salted, dried and grilled cephalopods are largely available on the market.

Although different species of squid, cuttlefish and octopus are used in the processing, products are sold under these three macro-categories' names and without a specific denomination. Thus, at present, notwithstanding the raising interest also of Chinese consumers' in food traceability and labelling, information on the single species involved is not available. The lack of a specific legislation for seafood denominations in China also poses major limits for the international trade, exposing the market to potential frauds.

The study aimed at the molecular characterization of variously processed cephalopod products, purchased on the internal market, by DNA barcoding and phylogenetic distance analysis using *COI* and *16S rRNA* genes. An insight on the species most frequently used for these traditional seafood preparations was given and their geographical distribution, conservation status and commercial value were investigated. The national cephalopod production, import and export was investigated and discussed in relation to the specific information on the cephalopods species retrieved by the study.

Ten different species were identified in the three macro categories: *Sepia pharaonis*, *S. esculenta*, *S. recurvirostra*, *S. lycidas* in cuttlefish; *Amphioctopus marginatus* in octopus; *Uroteuthis chinensis*, *U. edulis*, *Ommastrephes bartramii*, *Illex argentinus* and *Dosidicus gigas* in squids. This latter species was retrieved in more than 50% of the samples and, interestingly, it was the only species found in shredded products. Among them two case of misdescription involving shredded cuttlefish and octopus which were identified as *D. gigas* were found by the comparison of the molecular

results with the declared macrocategory. Our results are of particular interest in the light of the scarcity of data regarding the identification of cephalopods on international markets and considering that China is one of the leading cephalopod-producing countries. The present study sheds some light on the internal market enlarging the information already obtained on cephalopods exported from China to western countries and particularly to the EU market, recently published in your journal (Guardone L, Tinacci L, Costanzo F, Azzarelli D, D'Amico P, Tasselli G, Magni A, Guidi A, Armani A, DNA barcoding as a tool for detecting mislabeling on incoming fishery products from Third countries: an official survey conducted at the Border Inspection Post of Livorno-Pisa (Italy) Food Control (DOI: 10.1016/j.foodcont.2017.03.056).

The manuscript has not been published elsewhere nor is it being considered for publication elsewhere. All authors have approved this manuscript, agree to the order in which their names are listed, declare that no conflict of interests exists and disclose any commercial affiliation.

Best regards

Andrea Armani

An insight into the Chinese traditional seafood market: species characterization of cephalopod products by DNA barcoding and phylogenetic analysis using *COI* and *16SrRNA* genes.

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ABSTRACT

Squids, cuttlefish and octopus are used for the preparation of traditional products sold on the Chinese market without a specific denomination. In this study DNA barcoding and phylogenetic distance analysis of *COI* and *16S rRNA* genes' fragments were used to characterize the most commonly processed species in dried whole, grilled shredded and salted cephalopod preparations. Ninety-five products (23 sold as cuttlefish, 4 as octopus and 68 as squid) purchased in Chinese local markets were analyzed. Overall, the study identified 10 different species: *Sepia pharaonis*, *S. esculenta*, *S. recurvirostra*, *S. lycidas* in cuttlefish; *Amphioctopus marginatus* in octopus; *Uroteuthis chinensis*, *U. edulis*, *Ommastrephes bartramii*, *Illex argentinus* and *Dosidicus gigas* in squids. This latter species, characterized by a low commercial value, was found in the majority of the samples (50.5%) and in all the shredded products. By comparing the molecular results with the declared macrocategory (cuttlefish, octopus and squid), two cases of misdescription were pointed out, involving shredded cuttlefish and octopus which were identified as *D. gigas*. Our results are of particular interest in the light of the scarcity of data regarding the identification of cephalopods on international markets and considering that China is one of the leading cephalopod-producing countries.

Keywords: squid; cuttlefish; octopus; processed seafood; molecular species characterization.

1. Introduction

Cephalopods are short-lived organisms, characterized by a rapid growth significantly influenced by environmental conditions. In particular, the oceans warming and the decrease of fish competitors and predators, due to intensive fishery practices, have positively affected cephalopod populations leading to a substantial increase in their worldwide biomass (Doubleday *et al*., 2016).

Cephalopods represent an important resource for human nutrition, constituting 4% of the total volume of the fisheries world trade (http://www.fao.org/3/a-i5555e.pdf). Thanks to an excellent palatability, high nutritional value and to an increasing demand for alternative fishery products, cephalopods are encountering consumers' favour (Zlatanos *et al*., 2006; Wen *et al*., 2015a). The species of main economic interest belong to two distinct orders (Decapodiformes and Octopodiformes) and, for commercial and catch statistics purposes, they are conventionally grouped in three macro categories: squids (short-fin; long-fin and bobtail squids), cuttlefish and octopus (Arkhipkin *et al.*, 2015). Squids' category, the most represented of the three macro categories in the global market, reached a total production of 3385003 tons, followed by octopus (400404 tons) and cuttlefish (331824 tons) in 2015 (http://www.fao.org/fishery/topic/16140/en). The global production largely depends on major producers belonging to Asian (China, Vietnam, Thailand, Indonesia, India), North African (Morocco, Mauritania), North American (California) and South American (Argentina, Mexico and Peru) countries (Globefish highlights, 2016). To date, China is ranked both as a leading cephalopod-producing country, with total

70 catches of more than 1.3 million tons, representing about 29% of the total world
71 cephalopods catches, and as one of the major cephalopod importer countries
72 (http://www.fao.org/3/a-i5555e.pdf).

73 On the international market cephalopods are generally commercialized as fresh or
74 frozen whole or sliced products (rings and arms, tubes and wings). The offer of
75 cephalopod products on the Chinese market varies among different areas, according to
76 consumers' preferences
77 (http://www.nmfs.noaa.gov/mb/sk/saltonstallken/investigation.pdf) and to traditional
78 processing methods (Li, 2009). Within this variety salted and dried cephalopods are
79 largely available on the market (Fig. 1).

80 Major food safety incidents that occurred in China in the latest 15 years have
81 increased the general awareness of consumers towards food safety issues and boosted
82 the interest in food traceability and labelling (Liu *et al*., 2013). However, a specific
83 legislation for seafood traceability, such as a seafood labeling system and an official
84 list of reference seafood trade names, is still missing. Therefore, seafood products are
85 sold on the market without a specific denomination, paving the way to inaccurate
86 labelling (Xiong *et al*., 2016).

87 Species identification of whole fresh cephalopod specimens can be achieved by
88 visual inspection according to the morphological keys available in specific FAO
89 catalogues (Jereb & Roper, 2005, 2010; Jereb *et al*., 2016). However, this requires a
90 high level of expertise because morphometric characters may be influenced by
91 environmental factors (Martinez *et al*., 2002). Moreover, due to their soft bodies,

cephalopods can be easily damaged during collection and a morphological identification is completely unfeasible in case of processed seafood where anatomic features have been removed or altered.

Alternatives tools for the authentication of cephalopods' species are represented by DNA based techniques mainly targeted on mitochondrial DNA (mtDNA) genes' fragments analysis. Cytochrome *c* oxidase I (*COI*) and 16s ribosomal RNA gene (*16SrRNA*) have been successfully used for molecular characterization (Anderson, 2000; Dai *et al*., 2012; Gerhardt and Knebelsberger, 2015; Galal-Khallaf *et al*., 2016). In addition, mtDNA genes have been applied for the identification of traditional Chinese seafood, such as sea cucumber (Wen *et al*., 2011), dried shellfish (Chan *et al*., 2012, Wen *et al*., 2017),fish maw (Wen *et al*., 2015b) and salted jellyfish (Armani *et al*., 2013).

The aim of this study was to identify variously processed cephalopod products collected from the Chinese market by DNA barcoding and phylogenetic distance analysis using *COI* and *16S rRNA* genes. An insight on the species most frequently used for these traditional seafood preparations was given. Their geographical distribution, conservation status and commercial value were investigated, in order to provide specific information on the cephalopods species marketed in China.

2. Materials and Methods

2.1. Sample collection, DNA amplification and sequencing

2.1.1 Sample collection. A total of 95 traditional processed cephalopods products were directly purchased in three cities: Guangzhou and Zhanjiang (Guangdong

114 province) and Zhuzhou (Hunan province). The samples consisted of 23 cuttlefish
115 products, 4 octopus products, 68 squid products (Table 1). Each sample was registered
116 by an internal unique code and photographed. Tissue samples were collected and
117 stored at -20°C until further analysis. Details on the type of product (name used by the
118 vendor) and on the production origin (producers' location) are summarized in Table 1.
119 *2.1.2 DNA extraction and PCR amplification.* Total DNA extraction was performed
120 starting from 30 mg of tissue samples using the TIANamp Marine Animals DNA Kit
121 (TIANGEN, China) according to the manufacturer's instructions. Total DNA
122 concentration and quality were assessed using a ND-1000 UV-Vis Spectrophotometer
123 NanoDrop (Thermo Fisher Scientific Inc, USA). The *COI* gene was used as the
124 elective marker. The universal primer pair LCO1490 and HCO2198, proposed by
125 Folmer *et al*., (1994) for the amplification of a fragment of 658bp of the *COI* gene
126 metazoan invertebrates, was selected according to its proved efficiency in the
127 amplification of phylogenetically distant cephalopod species (Anderson, 2000; Dai *et*
128 *al*., 2012; Gerhardt and Knebelsberger, 2015). The *16S rRNA* gene, already applied to
129 cephalopods molecular based identification (Anderson, 2000; Chapela *et al*., 2002;
130 Dai *et al*., 2012; Galal-Khallaf *et al*., 2016; Sanchez *et al*., 2016) was selected as an
131 alternative molecular target and used for the amplification of those DNA samples that
132 failed sequencing and post sequencing analysis using the *COI* barcode. The universal
133 primer pair 16Sar and 16Sbr, by Palumbi (1996), was chosen for the amplification of
134 a ~ 550 bp gene fragment according to previous assessments in cephalopods' DNA
135 amplification (Galal-Khallaf *et al*., 2016; Giusti *et al*., 2016).

Both the PCR reactions were set in a final volume of 20 µl containing 2 µl of a 10x buffer (5Prime, Gaithersburg, USA), 100 mM of each dNTP (Euroclone, Pavia, Italy), 250 nM of forward primer, 250 nM of reverse primer, 25 ng/mL of BSA (New England BIOLABS® Inc. Ipswich, MA, USA), 1.25 U PerfectTaq DNA Polymerase (5Prime, USA), 30 ng of DNA template. The PCR were run on PeqSTAR 96 Universal Gradient thermocycler (Euroclone, Milan, Italy). After the initial denaturation at 94°C for 3 min, a primers specific cycling step of 40 cycles and a final elongation at 72°C for 10 min were performed. The two cycling programs for the amplification of the *COI* gene and the *16S rRNA* gene fragments were set as follows: denaturation at 94°C for 30 s, annealing at 46°C for 30 s, extension at 72°C for 40 s and denaturation at 94°C for 25 s, annealing at 54°C for 30 s, extension at 72°C for 15 s. The PCR products were checked by 1.8% agarose gel electrophoresis (GellyPhorLE, Euroclone SPA, Milano) prestained with GelRed™ Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA); the presence of the expected band was assessed by a comparison with the standard marker SharpMass™50-DNA ladder (Euroclone SPA, Milano). PCR products were purified with EuroSAP PCR Enzymatic Clean-up kit (EuroClone Spa, Milano) and stored at -80°C prior to the sequencing.

2.1.3 DNA sequencing and sequences analysis. The sequencing of PCR products was carried out by the Experimental Institute of Zooprophylaxis of Piedmont, Liguria and Aosta Valley (Turin, Italy) to obtain forward and reverse direction sequences for each PCR product. The sequencing reaction was performed by the use of a 4-capillary 3130 Genetic Analyzer (Applied Biosystems) and the BigDye®

158 Terminator v3.1 Cycle Sequencing kit (Life Technology, Thermo Fisher Scientific
159 Inc.). All the complementary sequences were checked and manually edited with
160 Bioedit 7.0 software (Hall, 1999). All the *COI* sequences were also checked for
161 nuclear mitochondrial pseudogenes (numts) following the quality control proposed
162 by Song *et al*., 2008).

163 ***2.2 Post sequencing: DNA barcoding and phylogenetic distance analysis***

164 The final sequences were queried against the reference sequences available in
165 BOLD (http://www.boldsystems.org/) and GenBank (http://www.ncbi.nlm.nih.gov)
166 databases by the use of the Identification System (ID's) and the Basic Local Analysis
167 Search Tool (BLAST), respectively. As regard BOLD ID's the sequences were
168 queried to search Species Level Barcode Records. In case of no match, the query was
169 enlarged to All Barcode Records on BOLD. Concerning the *COI* gene identification
170 of a sample at species level was assigned when the identity rate showed less than 2%
171 difference with reference sequences of a given species (Barbuto *et al.*, 2010). In case
172 of *16S rRNA* the identity score of 100% was set as the cut-off parameter for the
173 species assignment (Armani *et al.*, 2015a). The results obtained from the comparison
174 with the databases were then verified by Neighbor Joining clustering analysis (Saitou
175 & Nei, 1987) by the application of the p-distance method according to Katugin *et al*.,
176 (2017). For this purpose, reference sequences of the *COI* and *16SrRNA* genes were
177 collected from BOLD and GenBank for 104 species belonging to Sepiidae,
178 Octopodidae, Loliginidae and Ommastrephidae families (Table 1SM). The sequences
179 obtained from commercial samples, together with those retrieved from the databases

(from 1 to 5 for each species), were used to produce 6 distinct sequences alignment datasets as 2 datasets (1 for the *COI* gene and 1 for the *16SrRNA*) were obtained for each of the three macro categories (squid, octopus and cuttlefish). The commercial samples were included in the dataset according to their preliminary identification by DNA barcoding. Unrooted Neighbour joining (NJ) trees were produced to visualize divergence within families, genera and species and to verify the clustering patterns. Node support was assessed by the bootstrap method using 1000 pseudoreplicates (Felsenstein, 1985). Bootstrap values (BV) equals or higher than 70% were considered suggestive of significant clustarization (Van der Peer, 2009). All the analysis were computed on Mega 6.06 (Tamura et al., 2013) set on the standard invertebrate mitochondrial genetic code.

2.3 Comparison of the molecular results with purchasing information

2.3.1 Comparison of the provinces of origin with the product type and the identified species. The distribution of the identified species in relation to the provinces of origin was investigated.

2.3.2 Comparison of the product description with the identified species. The samples were declared misdescribed when the species molecularly identified did not match with the seafood category (squid, cuttlefish and octopus) declared for that product.

2.4 Characterization of the products identified at species level and trade data analysis

The distribution of the cephalopods species identified by molecular analysis was

202 searched using SeaLifeBase (http://www.sealifebase.fisheries.ubc.ca/), WoRMS
203 (http://www.marinespecies.org/) and EOL (http://eol.org/) in order to determine their
204 geographical origin. Data on the price category, conservation status (IUCN
205 classification) and vulnerability, were also collected from SeaLifeBase. Chinese
206 cephalopod production (2012-2015) was assessed consulting FAO Global Production
207 statistics
208 (http://www.fao.org/figis/servlet/TabLandArea?tb_ds=Production&tb_mode=TABLE
209 &tb_act=SELECT&tb_grp=COUNTRY&lang=en), FAO Global Capture Production
210 (http://www.fao.org/fishery/statistics/global-capture-production/query/en) and FAO
211 Global Aquaculture Production
212 (http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en).
213 Commercial flows regarding cephalopods' import and export patterns to and from
214 China between 2012 and 2015 were searched using Trademap
215 (http://www.trademap.org/Index.aspx) and the UN Comtrade database
216 (https://comtrade.un.org/).

217 **3. Results and Discussion**

218 ***3.1. Samples collection, PCR amplification and sequencing***

219 In the current study, sampling was conducted according to the availability of the
220 products on the surveyed markets. Dried squid, a traditionally largely appreciated
221 seafood preparation
222 (http://www.nmfs.noaa.gov/mb/sk/saltonstallken/investigation.pdf; Dong *et al.*, 2013),
223 accounted for the vast majority (71.6%) of the analysed samples, followed by

cuttlefish (24.2%) and octopus (4.2%). This proportion properly reflects the market scenario provided by the analysis of the available commercial data. In fact, by comparing the import-export data and the production data, the national market of octopus in China can be estimated around 1/20 of the market of squid and cuttlefish together (Table 2).

All the samples produced at least one amplicon suitable for sequencing and one readable sequence, with the exception of SS5, for which no PCR products could obtained. The *COI* gene was successfully amplified from 94 samples. PCR products were then purified for further sequencing analysis. Interpretable sequences were obtained for 97.9% (92/94) of the PCR products (Table 2SM). All obtained sequences did not contain insertions, deletions, non-sense, or stop codons; therefore, PCR or sequencing errors, the sequencing of pseudogenes or of *COI* of symbiotic organisms were excluded. The *16S rRNA* gene was used as alternative target for 2 DNA samples for which non readable sequences were obtained with the *COI* gene and for 15 DNA samples for which the post sequencing analysis on the *COI* target did not allow a species-specific identification. Totally, 17 *16S rRNA* gene sequences were obtained.

The *COI* sequences length ranged from 526 to 658 bp, corresponding to 80-100% of the expected amplicons. All the *16S rRNA* sequences reached 100% of the expected amplicon length (from 503 to 513 bp due to the presence of specie-specific insertion and deletions). These results confirm a high quality of the total DNA extracted from seafood products despite their processing (Table 2SM).

3.2 Post sequencing analysis: species identification

In the present study, the simultaneous utilization of two databases (BOLD and Genbank) for the genetic identification of cephalopods species enhanced the accuracy of authentication. Overall, by the combination of BLAST and BOLD ID's analysis, 78 products out of 95 (82.1%) were univocally allocated to a species (Table 2 SM). Seventy-seven of them were effectively identified at species level by the use of the *COI* barcode alone, the remaining 1 by the analysis of the *16S rRNA* alternative target alone (GS19). In 16 cases, even the combination of the molecular data obtained for both molecular targets did not allow species specific attribution. These samples were in fact only identified at a genus level (16.8%). As mentioned above (section 3.2), for 1 sample (1.1%) no PCR products could be obtained and therefore it was not possible to achieve any identification.

The aforesaid results were further verified by the use of the NJ tree method with p-distance model on 1000 boostraps replicates and the visualization of the samples allocation within the clusters. Specifically, 6 trees (3 *COI* and 3 *16S rRNA* dendrograms) were obtained (Fig 1SM-6SM). By the combination of the DNA barcoding and of the phylogenetic distance analysis, 96.8% (92/95) samples were identified to the species level. Only for 2 samples (2.1%), DC3 and DS19, a species level identification failed. The results are discussed below in detail according to the three different macro categories.

3.2.1 Cuttlefish products. About the cuttlefish products, by using the DNA Barcoding 11 samples were allocated to a species while 11 to a genus due to the presence of more than one species with a top identity value between 98-100%. For the

sample DC3 only a top match of 89-90% by the use of *COI* gene and of 94% by the use of *16S rRNA* was obtained against vouchered sequences deposited as *Sepia* sp. This result is likely due to the absence of reference sequences in the databases as observed during the preparation of the datasets for the phylogenetic analysis (Table 1SM). The NJ analysis on whole dried cuttlefish was conducted including sequences of *Sepia* spp. and *Sepiella* spp. (Sepidae family). Both the NJ trees constructed for cuttlefish samples showed specific clusters for all the species, each supported by bootstrap values higher than 70% (Fig. 1SM and Fig. 2SM). Therefore, except for the sample DC3, that produced a separate cluster in both the NJ analysis and could only be confirmed as *Sepia* sp., all the samples were grouped within a species-specific cluster. The sample GSC1, belonging to the only grilled shredded cuttlefish and preliminarily identified as *D. gigas* by the DNA barcoding analysis of the *COI* target, was confirmed belonging to this species by the distance analysis with a BV of 99% (Table 2SM, Fig. 5SM).

Thus, 22 of the 23 products were unambiguously identified as belonging to the following 5 different species: *Sepia pharaonis* (n=6), *Sepia esculenta* (n=7), *Sepia lycidas* (n=4), *Sepia recurvirostra* (n=4) and *Dosidicus gigas* (Table 2SM).

3.2.2 Octopus products. Even by combining the DNA barcoding results for both *COI* and *16S rRNA* targets the 3 DNA samples belonging to whole dried products could not be allocated to a species level due to the presence of two species (*Amphioctopus marginatus* and *Amphioctopus aegina*) showing an overlapping top match of 98-100%. The DNA sample of the only grilled shredded product was

290 unambiguously allocated to species level as *D. gigas*. The NJ analysis of the DNA
291 samples of the 3 whole dried products was performed using the 5 genera (*Octopus*,
292 *Amphioctopus*, *Callistoctopus*, *Cistopus*, *Eledone* sp.) belonging to the Octopodidae
293 family for which a significant alignment was obtained by the barcoding analysis on
294 both BOLD and BLAST analysis systems. The NJ tree produced on the *COI* target
295 showed significant genera and species clustering (BV>70%), with the exception of
296 *Cistopus taiwanicus* and *Cistopus indicus* that produced two overlapping subclades
297 (Fig. 3SM). All the sequences belonging to dried octopus products were grouped
298 within the *Amphioctopus marginatus* clade. On the contrary, the NJ analysis on *16S*
299 *rRNA* target highlighted a less discriminatory pattern within the genera included in the
300 analysis. In particular, 4 major clusters were obtained, not all of them supported by
301 significant BV (Fig. 4SM). The first clade collected on a unique branch *C. taiwanicus*
302 and *C. indicus* in agreement with the results obtained by Lu *et al.,* 2013; the second
303 and the third clades grouped *Amphioctopus* sp. and *Octopus* sp., respectively. A forth
304 clade collected *Eledone* sp., *Callistoctopus* sp. species and *Cistopus chinensis*. Within
305 *Amphioctopus* spp. clade three significant divisions were produced: *Amphioctopus*
306 *fangsiao* subclade, *Amphioctopus ovulum* subclade and a third subclade that grouped
307 *Amphioctopus kagoshimensis*, *A. aegina* and *A. marginatus* on a distinct branch in
308 which all the DO sequences were allocated.
309 The grilled shredded octopus sample, GO1, already identified to species level as *D.*
310 *gigas* by the DNA barcoding analysis was further confirmed to belong to this species
311 by the distance analysis since it clustered within the species-specific clade supported

312 with a BV of 99% (Table 2SM, Fig 5SM).

313 *3.2.3 Squid products.* Based on the DNA barcoding analysis alone all the 66 squid
314 products were allocated to the species level with the exception of DS19for which a
315 maximum match of 89% with the species *Uroteuthis edulis* and a top match of 94%
316 with sequences deposited as *Uroteuthis* sp were respectively highlighted by the use of
317 *COI* and *16S rRNA* targets. with the *16S rRNA* gene. The NJ analysis was performed
318 on 8 genera belonging to Loliginidae family and 11 genera belonging to the
319 Ommastrephidae family. The *COI* tree showed significantly separate species clades
320 for all the genera included (BV >70%) while the *16S rRNA* tree showed a lower
321 efficiency in species discrimination. *Loligo vulgaris* and *L. reyinaudi* were clustered
322 together and the three *Illex* sp. species formed a unique clade (Fig. 5SM and 6SM).
323 DC19 was confirmed as a non-identifiable *Uroteuthis* sp. since it produced a separate
324 cluster from the 4 species included in the dataset. Indeed, the lack of reference
325 sequences (Table 1SM) for 7 out of the 13 (54%) valid species belonging to the genus
326 *Uroteuthis* sp. (according to SeaLifeBase) represents a major limit for the
327 identification within this genus.

328 Overall, phylogenetic analysis confirmed the results obtained by DNA barcoding
329 alone and squid samples were identified as belonging to 2 long-fin squid species (*U.*
330 *chinensis* and *U. edulis*) and 3 short-fin squid species (*D. gigas*, *I. argentinus* and *O.*
331 *bartramii*).

332 ***3.3 Comparison of the molecular results with the purchasing information***

333 *3.3.1 Comparison of the provinces of origin with the product type and the*

334 *identified species.* As concerns the province of origin, altogether the products derived
335 from 7 Chinese provinces, all of them located along the coast (Fig. 2). The sample
336 numerosity per province was not homogeneous: the majority of the products
337 originated from Guangdong province (45.2%) that, interestingly, produced 34 of the
338 43 grilled shredded products. The second and the third provinces for numerosity of
339 sampled products were the neighbouring provinces Fujian and Guangxi, with 29.5%
340 and 11.6% of the analyzed products. In addition, Guangdong province accounted for
341 the large majority of products identified as *D. gigas*, all belonging to the
342 shredded/grilled category (see Section 3.3.2), confirming the high vocation of the
343 province for seafood processing plants
344 (http://www.thefishsite.com/articles/1055/china-fishery-products-annual-report/).
345 About cuttlefish products, identified as potentially locally sourced species (see
346 Section 3.4), they all originated from the three provinces of Guangxi, Guangdong and
347 Fujian, characterized by an intense local fishing activity
348 (http://www.thefishsite.com/articles/1055/china-fishery-products-annual-report/). The
349 latter province also accounts for the origin of all the octopus products.
350 *3.3.2 Comparison of the product description with the identified species.* An
351 appropriate labelling is essential for ensuring traceability and the lack of a
352 standardized system for seafood naming generates a situation of great uncertainty
353 (Xiong et al., 2016). However, assessing the mislabelling rate in seafood products in
354 China is not straightforward. Considering the absence of a specific regulation and, in
355 particular, of an official list of commercial denominations, the verification of the

356 information provided at purchasing is not feasible. In this case only the denomination
357 internationally recognized to describe a product macro-category can be used to assess
358 products' conformity.

359 For cephalopods three different term (squids, cuttlefish and octopus) are used to
360 refer to a wide range of different organism of commercial appeal (Arkhipkin *et al*.,
361 2015). These generic terms were used to assess if the products analyzed were put on
362 the market with a correct description. Misdescriptions were highlighted only for 2
363 samples (2.1%), GSC1 (grilled shredded cuttlefish) and GO1 (grilled shredded
364 octopus), that were both identified as *D. gigas* (Humboldt squid), characterized by a
365 low commercial value (Table 3). Noteworthy is the fact that these two products were
366 the only shredded products among cuttlefish and octopus samples. The slicing and the
367 loss of morphological features could have favoured the species' replacing. This is of
368 particular interest in the light of the molecular results obtained for squids. In fact, all
369 the 41 grilled products belonged to the Humboldt squid *D. gigas*. Thus, it appears that,
370 regardless the declared macro category, shredded products are produced with this
371 lower priced species (Fig. 3). Therefore, even in absence of misdescription, the price
372 of the species is connected to the typology of the product (Table 3).

373 Our results are of particular interest if considered in the light of the
374 non-compliances reported by Santaclara et al. (2007) and Espineira et al. (2010) in
375 processed cephalopod products collected on the Spanish market. In both studies, 30%
376 of the analyzed samples were incorrectly labelled. Moreover, a recent survey on
377 fishery products imported from extra-European countries, conducted in collaboration

with the veterinary staff of the Italian Ministry of Health at the Border Inspection Post of Livorno-Pisa (BIP), highlighted mislabelling issues in seafood products imported from China to Italy (Guardone et al., 2017). In particular, cephalopod products were characterized by the highest percentage of mislabeling (43.8%, 95% CI 32.3–55.9) among all the seafood categories analyzed. The latter study, together with the present results, provided some specific information on the cephalopod species marketed by China both at the international and national level. This information is particularly relevant considering that production and trade data are often referred to the whole macro category or even to grouped macro-categories and not to the single species (see section 3.4.3). Finally, it has to be considered that the low misdescription rate highlighted in this study cannot be considered as representative of the real mislabelling rates affecting the Chinese market. In fact, the low misdescription found could be referred to the fact that only the name of the seafood category, and not the commercial denomination, was verifiable.

3.4 Characterization of the products identified at species level and trade data analysis

The results allowed to identify 10 different species in the 95 products analyzed (Table 3 and Table 2SM). Observing the range of identified species in the different macro categories, a high variability was observed for cuttlefish (Fig. 4) and squid products (Fig. 3).

3.4.1 Cuttlefish products. The dried whole products were composed of 5 different species of the genus *Sepia*: 4 identified as *Sepia pharaonis, S. esculenta, S. lycidas*

400 and *S. recurvirostra* and 1 not identifiable due to the lack of vouchered sequences in
401 both databases (Table 1SM). All the retrieved cuttlefish species have a similar
402 geographical distribution (Indian Ocean and North West and Western Central Pacific
403 Ocean) (http://www.sealifebase.org; http://eol.org/), a low to low-moderate
404 vulnerability according to Cheung et al. (2005) and a similar high commercial value
405 (Sumaila et al., 2007).

406 The first 3 species are the most commonly caught cuttlefish species of several
407 Asiatic countries (China, Japan, Thailand, Philippines, and Vietnam) and Australia
408 (Jereb & Roper, 2005). Furthermore, in the latest years, in order to sustain the high
409 market demand an intensive research was addressed to the improvement of the
410 aquaculture systems of these species (Barord et al., 2010; Wen et al., 2012) and to the
411 characterization of the nutritional quality between wild and cultured products (Wen et
412 al., 2014, 2015a). The curvespine cuttlefish *S. recurvirostra* has some commercial
413 importance in Hong Kong, where it is caught in multispecies trawls. It is a
414 commercial species in the Gulf of Thailand, South and East China Seas, and Japan
415 (Jereb & Roper, 2005).

416 *3.4.2 Octopus products.* All the dried whole octopus products belonged to
417 *Amphioctopus marginatus*, a species of medium-high commercial value which occurs
418 along the coastal area of the North West Pacific and Indian Ocean (Jereb et al., 2016.)
419 It cannot be excluded that the absence of species variability may be due to the low
420 number of samples analyzed. However, as mentioned, the lower number of this kind
421 of products in comparison with the other macro categories, reflects the internal market

422 demand (Table 2).

423 *3.4.3 Squid products.* For what concerns squid products, a distinction needs to be
424 made between the different type of products. In particular, 5 species (*Uroteuthis*
425 *chinensis, U. edulis, O. bartramii, D. gigas* and *I. argentinus*) were identified in the
426 dried whole category. Two of the identified species (*D. gigas* and *I. argentinus*) were
427 also found in the 6 salted products, while all the 41 grilled/shredded samples were
428 allocated to *D. gigas*. The retrieved species are partially consistent with available
429 studies on the processing of dried cephalopod products attesting the common use of *D.*
430 *gigas* for this kind of preparations (Dong et al., 2013; Zhu et al., 2016). However, the
431 large use of *U. chinensis* and *U. edulis* is unexpected for this kind of products since
432 these high value species are reported to be generally consumed as fresh products or
433 frozen and exported to US and European markets (Guardone et al., 2017, Sunil
434 Mohamed, 2012). Analogously, the scarce presence of *O. bartrami* is surprising
435 considering that this species is reported to be an important resource as a supply of
436 various food products, especially deep-fried squid, soft squid jerky, and semi-dried
437 and seasoned squid (Arkhipkin et al., 2015).

438 *D. gigas*, the largest ommastrephid squid commercially known as Humboldt squid
439 or Jumbo flying squid, was the most frequently represented (46 of the 95 samples,
440 48.4%) and the only species retrieved in shredded and grilled sliced products (Table
441 2). Although this species is not present in the Indo-Pacific area, it has long been
442 exploited by distant water Chinese fleets (Chen *et al*., 2008a). In fact, this pelagic
443 squid is endemic to the eastern Pacific Ocean and is particularly abundant in the

highly productive waters of the Humboldt and California Current systems, and the Costa Rica Dome upwelling (Arkhipkin et al., 2015). After a very intense fishing effort by Asian fleets in the 1980s followed by a fishery collapse (Arkhipkin et al., 2015), Chinese jiggers started fishing this species outside the Peruvian EEZ in 2001 displacing other Asian countries as the main Jumbo squid producer. The effort was then extended to waters outside the Chilean EEZ and later outside the Costa Rican EEZ (Markaida et al., 2016). According to FAO statistics, the Chinese catches of this species increased from 142000 to 323636 tonnes during 2010-2015, representing 21.7% of the total Chinese catches of cephalopods in 2015 (http://www.fao.org/fishery/topic/16140/en). The exploitation of this species is not limited to China's fishing activities. In fact, *D. gigas* has been the most fished cephalopod worldwide since 2004 and it has been among the top FAO 15 single species fisheries for 11 years (2003–2013) (FAO, 2016).

Another species which is not present in the waters of the China Sea is *I. argentinus*, which was found only in 2 dried whole and 2 salted squid products. This species is distributed in the Western South Atlantic (Jereb & Roper, 2010). The development of the Chinese fishery for *I. argentinus* in the Southwestern Atlantic Ocean occurred more recently than for *D. gigas*, since the Chinese jigging fishery began exploiting *I. argentinus* for the first time in 1997, both on the high seas and later in the Argentinean EEZ (Arkhipkin et al., 2015). Based on FAO statistics, the Chinese landing of this species sharply increased from 35000 to 470000 tonnes during 2010-2015. It represented 31.7% of the total Chinese catches of cephalopods in 2015. The yield of

both species mentioned above constitutes more than half (53.1%) of the total Chinese catches of cephalopods in 2015 (http://www.fao.org/fishery/topic/16140/en).

The second most represented species in our study was *U. chinensis* (Mitre squid), the largest and the most commonly caught species in the Indo-Pacific region that plays an important role in the marine fishing of China, Vietnam and Thailand (Arkhipkin et al., 2015). As regards China, the fishery accounts for up to 90% of the loliginid catch (Chen et al., 2013).

Swordtip squid *U. edulis*, which was retrieved in 3 dried whole samples, is present in the Yellow and East China Seas, and in the northern waters of Taiwan (Jereb & Roper, 2010). It is particularly relevant for coastal fisheries, as it is caught mainly by the torch-light fishery in Taiwan and by the trawl fishery on the southeast coast of China (Arkhipkin et al., 2015).

Finally, the neon flying squid, *O. bartramii*, identified only in 1 dried whole squid, is an economically important oceanic species widely distributed from subtropical to subarctic waters in the Atlantic, Indian and Pacific Oceans (Jereb & Roper, 2010). This squid has been exploited by Japanese squid-jigging fleets since 1974, and later by South Korea and Taiwan; nowadays it is still fished commercially only in the Pacific Ocean (Arkhipkin et al., 2015). The total annual production of squid caught by Chinese mainland ranged from 36764 to 113200 t from 2003 to 2013 (Wang et al., 2016). The presence of *O. bartramii* only in one sample is surprising since it is traditionally reported as one of the most processed species for traditional Chinese cephalopods preparations (Chen *et al*., 2008b).

Traceability issues mentioned in section 3.3 are further complicated by the intense import-export trade net for squid products: by analysing data from Trademap, it appears that cuttlefish and squids are the most traded category among cephalopods, covering 98% of the total import volumes and 86% of the total export volumes in 2015. Among squids and cuttlefish, the most relevant subcategory is composed by frozen/dried/salted/smoked products, accounting for more than 85% of the import and more than 80% of the export in 2015 (Commodity code 030749), followed by prepared or preserved cuttlefish/squids (160554). Interestingly, according to Trademap and UN Comtrade in 2015 the first category of products was imported from 29 and exported to 95 countries, while the second one was imported from 14 countries and exported to 51 countries.

Conclusion

In the present study, a characterization of the species used in processed cephalopod products widely commercialized within the Chinese internal market was carried out by DNA barcoding and phylogenetic distance analysis. Our results are of particular interest in the light of the scarcity of data regarding the identification of cephalopods on international markets and considering the high mislabelling rate reported in previous studies. The overall results allowed to identify 10 different species in the 95 analyzed products, showing a different frequency depending on the type and on the processing of products. In particular, all the grilled shredded products were composed by the low value Humboldt squid *D. gigas*. The relatively little number of species retrieved per macro category suggests that a more specific labelling system is feasible,

also in the light of the high volume of trade of cephalopods. Conversely, the absence of reference sequences for a high number of sequences still poses limits to an accurate molecular identification and highlights the need to improve the species coverage in the public databases. This work confirms that the molecular inspection of seafood may be a useful support for monitoring international cephalopod trade.

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Fgure captions

Figure 1 Dried whole cuttlefish (a, b), dried whole squid (c, d), dried whole octopus (e), grilled sliced cephalopods (f, g, h), grilled shredded cephalopods (i, j) and salted cephalopods (k, l).

Figure 2 Distribution of the analysed products and of the molecularly identified species in relation to the provinces of origin of the products.

Figure 3 Species molecularly identified in squid products in relation to their processing.

Figure 4 Species molecularly identified in cuttlefish products in relation to their processing.

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***Highlights (for review)**

- Traditional Chinese squid, cuttlefish and octopus products were molecularly characterized
- DNA barcoding and phylogenetic distance analysis on COI and 16S rRNA genes were used
- Ten different species were found, both locally sourced and imported from South America
- *Dosidicus gigas* was the most represented species, constituting all shredded squids

Figure

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Figure

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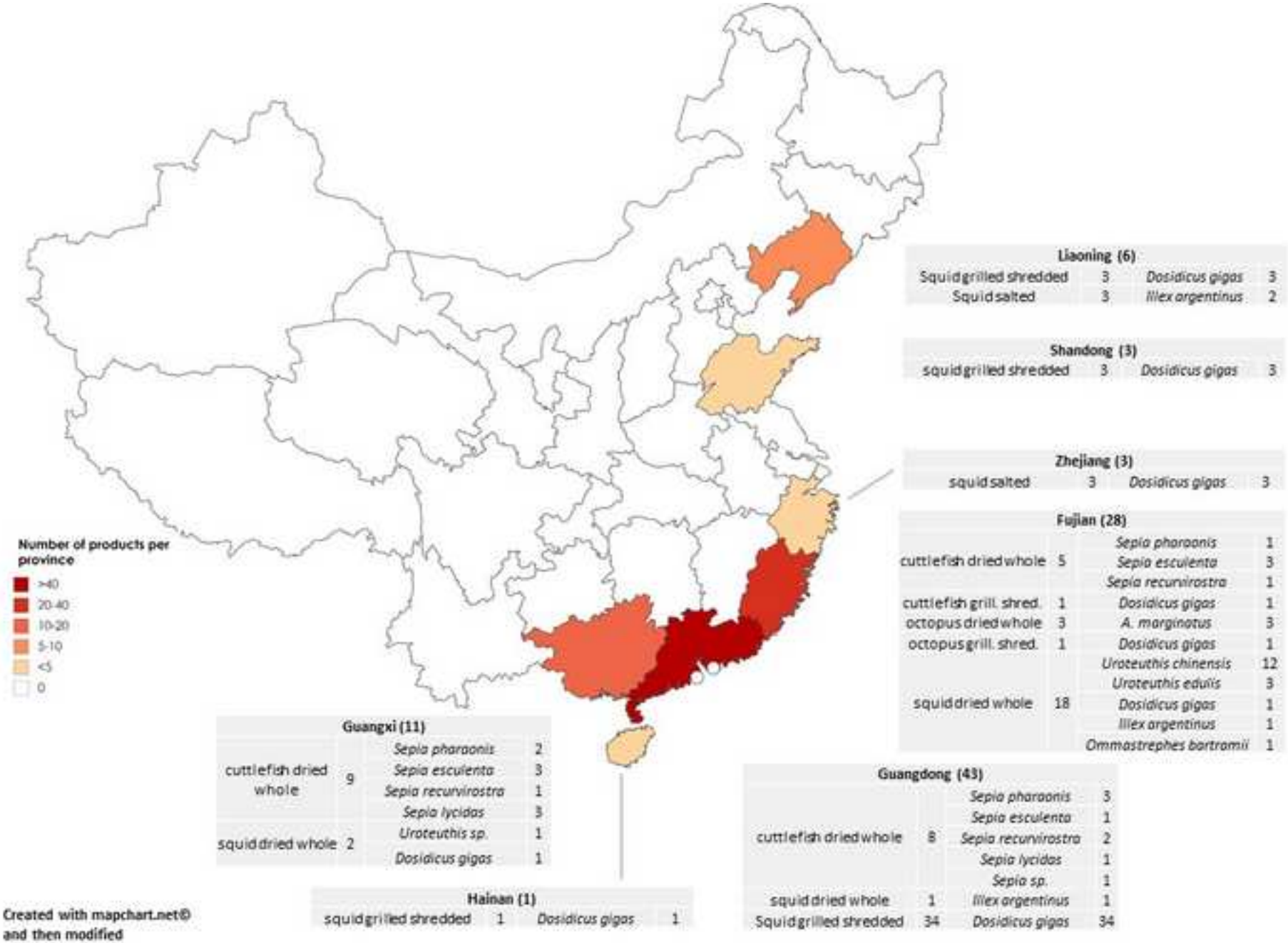

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Table

Table 1 Sampling information: category, type of processing and production origin (city and province)

Seafood category	n	Type of processing	n	Province of origin	City of origin	n
Cuttlefish	23	dried whole	22	Guangdong	Zhanjiang	8
				Guangxi	Beihai	9
				Fujian	Zhangzhou	5
		grilled/shredded	1	Fujian	Zhangzhou	1
Octopus	4	dried whole	3	Fujian	Zhangzhou	3
		grilled/shredded	1	Fujian	Zhangzhou	1
Squid	68	dried whole	21	Fujian	Zhangzhou	17
					Xiamen	1
				Guangdong	Shenzhen	1
				Guangxi	Beihai	2
		grilled/shredded	41	Guangdong	Guangzhou	9
					Zhanjiang	9
					Jieyang	9
					Dongguan	3
					Foshan	2
					Huizhou	2
				Shandong	Qingdao	3
				Liaoning	Dalian	3
				Hainan	Haikou	1
		salted	6	Zhejiang	Zhoushan	3
				Liaoning	Dalian	3

Table

Table 2 Data on China production (from FAO statistics) and import/export activities (from Trademap and UN Comtrade) for cephalopod products between 2012 and 2015. Values are expressed in tons. The internal market was obtained by subtracting the export volume from the sum of the production and import volumes.

	2012	**2013**	**2014**	**2015**
Octopus				
Production	125800	119169	121325	130245
Import	7805	11368	6966	6217
Export	73499	83417	88945	79796
Internal market	60106	47120	39346	56666
Cuttlefish/squid	**2012**	**2013**	**2014**	**2015**
Production	910237	926696	1225435	1363568
Import	372562	392572	427509	347880
Export	326102	410273	446304	453527
Internal market	956697	908995	1206640	1257921
Ratio octopus/cuttlefish+squid internal market	**15.9**	**19.3**	**30.7**	**22.2**

Table

Table 3 Products' information, molecular identification and characterization of the identified species (data from SeaLifeBase, EOL and WoRMS). DD: data deficient; LC: least concern; n.a.: not available.

Products' information and molecular identification				**Species characterization**			
Category and type	**Identified species**	**n**	**Provinces of origin**	**FAO areas**	**price category**	**vulnerability**	**IUCN status**
Cuttlefish products		**23**					
dried whole (22)	*Sepia pharaonis*	6	Guangdong (3) Fujian (1) Guangxi (2)	51, 57, 61, 71	high	low-moderate (33/100)	DD
	Sepia esculenta	7	Guangdong (1) Fujian (3) Guangxi (3)	61, 71	high	low (10/100)	DD
	Sepia recurvirostra	4	Guangdong (2) Fujian (1) Guangxi (1)	57, 61, 71	high	low (10/100)	DD
	Sepia lycidas	4	Guangdong (1) Guangxi (3)	57, 61, 71	high	low-moderate (28/100)	DD
	Sepia sp.	1	Guangdong	-	-	-	-
grilled/shredded (1)	*Dosidicus gigas*	1	Fujian	67, 77, 87	low	very high (90/100)	DD
Octopus products		**4**					
dried whole (3)	*Amphioctopus marginatus*	3	Fujian (3)	61	low	n.a.	n.a.
grilled/shredded (1)	*Dosidicus gigas*	1	Fujian	67, 77, 87	low	very high (90/100)	DD
Squid products		**68**					
dried whole (21)	*Uroteuthis chinensis*	12	Fujian (12)	57, 61, 71	very high	low (20/100)	not assessed
	Uroteuthis edulis	3	Fujian (3)	51, 57, 61, 71	very high	low-moderate (30/100)	
	Uroteuthis sp.	1	Guangxi				
	Ommastrephes bartrami	1	Fujian	21, 27, 31, 34, 37, 41, 47, 51, 57, 61, 67, 71, 77, 81, 87	medium	n.a.	LC
	Dosidicus gigas	2	Fujian, Guangxi	67, 77, 87	low	very high (90/100)	DD
	Illex argentinus	2	Fujian, Guangdong	41	high	low (19/100)	LC
grilled/shredded (41)	*Dosidicus gigas*	41	Guangdong (34) Shandong (3) Liaoning (3) Hainan (1)	67, 77, 87	low	very high (90/100)	DD
salted (6)	*Dosidicus gigas*	3	Zheijang (3)				
	Illex argentinus	2	Liaoning (2)	41	high	low (19/100)	LC
	not identified	1	Liaoning	-	-	-	-

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