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Morphological and genetic differentiation of two loliginid squids, *Uroteuthis (Photololigo) chinensis* and *Uroteuthis (Photololigo) edulis* (Cephalopoda: Loliginidae), in Asia

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ABSTRACT

The squids *Uroteuthis (Photololigo) edulis* and *Uroteuthis (Photololigo) chinensis* (family Loliginidae) are commercially important fishery species in many coastal regions of Asia. The morphologies of these two squids are very similar, and identification based on morphology has been inadequate. The occurrence of cryptic species in the family Loliginidae has been reported. The widely distributed *U. (P.) chinensis* and *U. (P.) edulis* are believed to comprise several cryptic species. In this study, the taxonomic status of the two species in East Asia was elucidated by morphological and genetic analyses. Analysis of *U. (P.) chinensis* from Hong Kong and Xiamen (China) and *U. (P.) edulis* from Yamaguchi (Japan) and Shanghai (China) was performed in order to determine the effectiveness of different morphometric variables in discriminating between the two species. Multivariate analysis of 27 morphometric indices revealed no new morphological characters for the taxonomic identification of the two taxa, which can be distinguished by the teeth shape and number on arm sucker rings, and the percentage of hectocotylized part of left arm IV of males. The morphometric differences between *U. (P.) edulis* individuals from the two localities is most probably due to differences in the maturity stages of the sampled individuals between the two localities. Genetic analysis based on the mitochondrial COI and 16S rRNA genes revealed a high divergence of 15.5% and 7.5% respectively, indicating that *U. (P.) edulis* and *U. (P.) chinensis* are distinct species.

1. Introduction

Uroteuthis (Photololigo) chinensis (Gray, 1849) and *Uroteuthis (Photololigo) edulis* (Hoyle, 1885) support a very important commercial fisheries industry in China on the continental shelf off Guangdong, southern Fujian and around the Pescadores Islands in the Taiwan Strait (Voss and Williamson, 1971). *Uroteuthis (Photololigo) chinensis* accounts for up to 90% of the loliginid catch in several parts of China and 15-40% of the trawl catch in the Gulf of Thailand (Roper et al., 1984).

Uroteuthis (Photololigo) chinensis and *U. (P.) edulis* were formerly classified under the genus *Loligo* Lamarck, 1798, as *L. chinensis* and *L. edulis*. Natsukari (1984) subdivided the genus *Loligo* and established *Photololigo*. Since both *L. chinensis* and *L. edulis* have two photophores on the ventral side of the ink sac, they were designated as *P. chinensis* and *P. edulis* by Natsukari (1984). As the two squid taxa have similar generic characters as *Uroteuthis* Rehder, 1945, *Uroteuthis* is used as the genus name instead of *Photololigo, which* is considered to be a valid subgenus of *Uroteuthis* (Vecchione et al., 2005).

The most useful characters for distinguishing the two species are the teeth of the arm sucker rings and the hectocotylized length of arm (Voss and Williamson, 1971). In *U. (P.) chinensis*, the rings of arm suckers have distally 10-18 sharp and conical teeth (Gray, 1849) (Fig. 1A). The hectocotylized part of the left arm IV in males occupies 33-40% of the arm length. In *U. (P.) edulis*, the arm sucker rings have distally 6-12, more often 6-8, obtuse teeth (Hoyle, 1885) (Fig. 1B). The hectocotylized part accounts for 50-67% of the arm length. There is a longitudinal cutaneous ridge along the middle of the ventral mantle in mature males. However, the longitudinal ridge is regarded as a character that is too variable to be used in generic systematics (Vecchione et al., 1998).

It is not surprising that *U. (P.) chinensis* and *U. (P.) edulis* are sometimes regarded as the same species, as they are morphologically very similar. In the description of *U. (P.) chinensis* and *U. (P.) edulis* in Hong Kong (Voss and Williamson, 1971), the size of both species is similar (maximum size 30 cm in mantle length), the colour is the same (colourless translucent, bright red or any intermediate shade), and both species are important to commercial fisheries. The geographical

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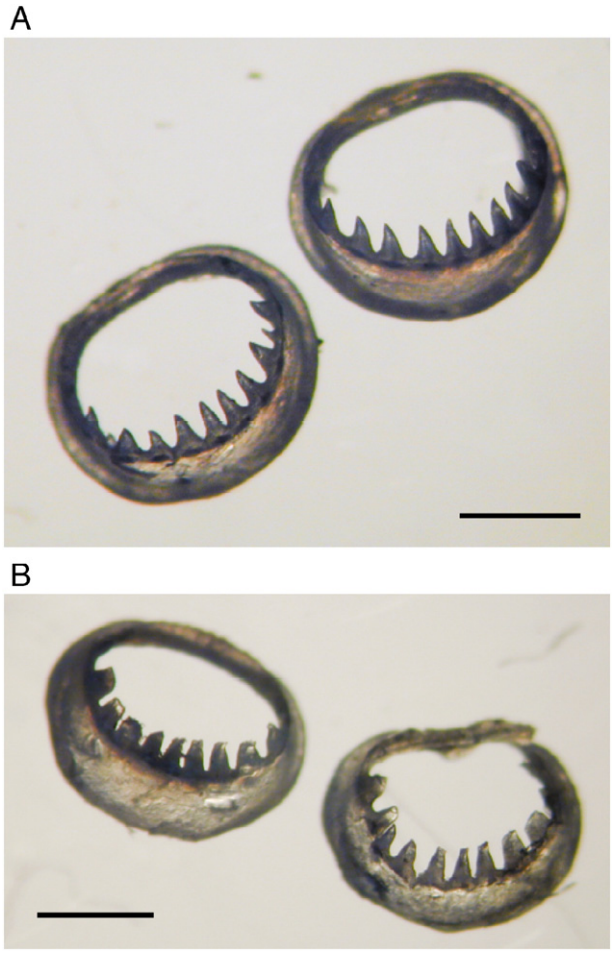

Fig. 1. Arm sucker ring of (A) *U. (P.) chinensis*, with sharp teeth, and (B) *U. (P.) edulis*, with blunt teeth. Scale bar 1 mm.

distributions of *U. (P.) chinensis* and *U. (P.) edulis* overlap (Fig. 2). Both are dominant squid species on the continental shelf between Taiwan and Hainan. *Uroteuthis (Photololigo) edulis* has been recorded in central Japan, the northern part of the South China Sea, the Philippine Islands and northern Australia, at depths ranging from 30 to 170 m (Roper et al., 1984; Voss and Williamson, 1971). The range of *U. (P.) chinensis* extends from the South China Sea and the East China Sea to Japan, the Arafuru Sea and northeastern Australia to New South Wales, at water depths of 15-170 m (Roper et al., 1984; Voss and Williamson, 1971). However, the widely distributed *U. (P.) chinensis* and *U. (P.) edulis* probably comprise several allopatric cryptic species, some of which may be endemic to Australia (Yeatman and Benzie, 1994). The presence of cryptic species is common in cephalopods (Soller et al., 2000). Two previous biochemical genetic studies carried out on squids have shown the presence of cryptic species (Brierley et al., 1993a; Carvalho et al., 1992). This is also common in *Loligo* species (Augustyn and Grant, 1989; Brierley et al., 1993b; Yeatman and Benzie, 1993, 1994). Furthermore, species misidentification due to difficulties in discerning the differences between and within species (Smith et al., 1981) presents another problem. This suggests that there are problems in identification based on current morphological criteria, which cannot adequately discriminate species (Yeatman and Benzie, 1994).

Although it is sometimes difficult to collect morphological data from soft-bodied animals, morphometric studies have been applied to squids for many years (Haefner, 1964). Morphometry has also been employed to identify differences between subspecies or species in some squids (Augustyn and Grant, 1989; Barón and Ré, 2002b; Martinez et al., 2002; Sanchez et al., 1996). The population structure of *Loligo gahi* in Falkland waters was determined using a combination of morphometric and biochemical genetic studies (Carvalho and Pitcher, 1989). Populations of other loliginid species have also been identified by morphometric studies (Cohen, 1976; Kashiwada and Recksiek, 1978). Morphometric measurement is an important tool for the identification of species in the digestive contents of many predators (dosSantos and Haimovici, 1998).

The application of molecular systematics to cephalopods is a relatively young science. Nucleotide sequence data from mitochondrial 16S rRNA gene has been used for phylogenetic analysis of decapod cephalopods (Bonnaud et al., 1994) and octopods (Allcock and Piertney, 2002; Piertney et al., 2003). The cytochrome *c* oxidase subunit I (COI) and 16S rRNA (16S) genes have also been used to examine the phylogeny of squids (Anderson, 2000; Lindgren et al., 2005; Strugnell et al., 2005). The molecular evidence provides an approach to review the taxonomy based on morphological data.

The aim of the present study was to elucidate the morphometric relationships of *U. (P.) chinensis* and *U. (P.) edulis* from East Asia and to determine the relative efficiency of different morphometric variables in species identification. The mitochondrial COI and 16S rRNA genes were used in this study to determine whether the two taxa in East Asia were (1) conspecifics exhibiting clinal variations, (2) two distinct species, or (3) more than two species with the presence of cryptic species.

2. Materials and methods

2.1. Sample collection

Specimens of *U. (P.) chinensis* were collected from Hong Kong and Xiamen (Fujian province, China) (Fig. 2). Specimens of *U. (P.) edulis* were collected from Yamaguchi (Japan) and Shanghai (China). *Uroteuthis (Photololigo) duvauceli* collected from Hong Kong and Shanghai was included in the genetic analysis for comparison. *Uroteuthis*

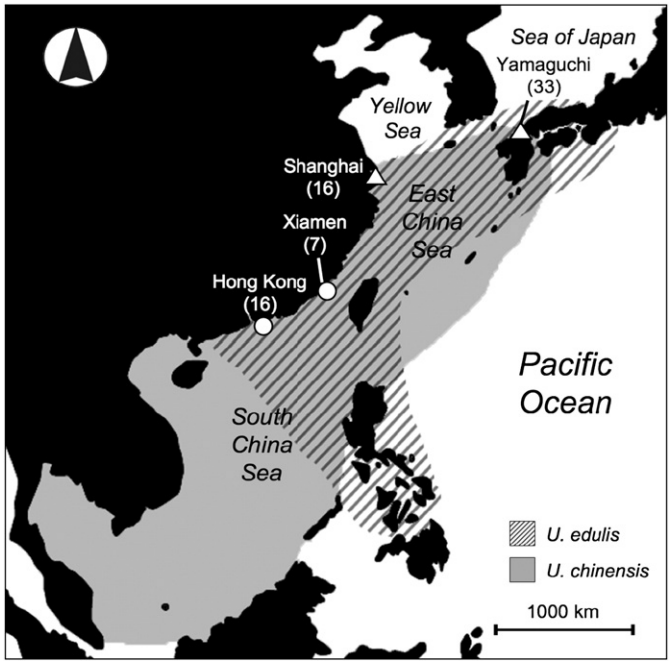

Fig. 2. Distribution of *U. (P.) chinensis* and *U. (P.) edulis* in East Asia and sampling locations of this study. The known geographical distributions of *U. (P.) chinensis* and *U. (P.) edulis* are shown in light grey and parallel diagonal lines, respectively. Their distribution ranges extend to northern Australia (not shown in the figure). The sampling locations of specimens identified as *U. (P.) chinensis* and *U. (P.) edulis* are indicated as circles and triangles respectively. The number of samples for morphometric multivariate analyses is shown in parentheses.

Table 1
Morphological characters recorded on *Uroteuthis (Photololigo) chinensis* and *U. (P.) edulis*

Variable	Abbreviation	Description
Dorsal Mantle Length	DML	Length of dorsal mantle from anterior to posterior extremes
Mantle Width	MW	Greatest width of mantle
Fin Length	FL	Length of fins from the midpoint of an imaginary line joining anterior margin of fins to posterior extreme of mantle
Fin Width	FW	Greatest width of fins between lateral margins
Head Length	HL	Length of head from anterior margin of nuchal cartilage to the base of the arm I
Head Width	HW	Greatest width of head
Eye Diameter	ED	Diameter of eye
Arm Length I	ALI	Length of arm I from the junction of arms to the distal extreme
Arm Length II	ALII	Length of arm II from the junction of arms to the distal extreme
Arm Length III	ALIII	Length of arm III from the junction of arms to the distal extreme
Arm Length IV	ALIV	Length of arm IV from the junction of arms to the distal extreme
Hectocotylus Length	HcL	Length of hectocotylus
Hectocotylized proportion of Left ALIV	HL%	Percentage of total arm length of left arm IV that is modified by hectocotylization
Tentacle Club Length	TCL	Length of tentacle club
Tentacle Club Width	TCW	Greatest width of tentacle club
Tentacle Central Sucker Diameter	CSD	Ring diameter of largest sucker on the carpus of tentacle
Tentacle Marginal Sucker Diameter	MSD	Ring diameter of sucker lying at lateral margin of the largest central sucker on tentacle's carpus
Tentacle length	TL	Length of tentacle from junction of arms III and IV to distal extreme
Nuchal Cartilage Length	NcL	Length of nuchal cartilage from anterior to posterior ends
Funnel Locking Cartilage Length	FcL	Greatest length of right funnel locking cartilage from anterior to posterior ends
Gladius Length	GL	Length of gladius from anterior to posterior ends
Gladius Width	GW	Greatest width of gladius between its lateral margins
Rachis Length	RL	Length of rachis from anterior extreme of vanes to anterior end
Rachis Width	RW	Width of rachis at the level of anterior end of the vanes
Arm sucker ring teeth number		Number of teeth on the sucker ring of the largest sucker on arm III

(Photololigo) chinensis and *U. (P.) edulis* were discriminated according to the shape of arm sucker ring teeth. Sex was established by examining the reproductive organs. A five-stage system based mainly on the development of reproductive organs and position of spermatophores or eggs (Boyle and Ngoile, 1993) was used to assess individual maturity. The specimens from all localities were stored at −20 °C immediately after collection. Tissues from the arm and mantle of each specimen were preserved in 95% ethanol until DNA extraction. The number of individuals employed in the morphometric analysis is indicated in Fig. 2.

2.2. Morphometric characters

Twenty-three morphometric characters and one meristic character (teeth number of the largest arm sucker ring on arm III) were recorded (Table 1). Measurements were made on the frozen samples with a ruler or a pair of calipers to the nearest 1 mm. The shape of arm sucker ring teeth was observed under a light microscope. To avoid the effect of size difference, most characters were analysed in proportion to the dorsal mantle length (DML). A total of 27 indices were calculated and used for analysis (see Table 1 for abbreviations): MW/DML, FL/DML, FW/DML, FW/FL, HL/DML, HW/DML, HW/HL, ED/DML, ALI/DML, ALII/DML, ALIII/DML, ALIV/DML, TCL/DML, TCW/DML, TCW/TCL, CSD/DML, MSD/DML, MSD/CSD, TL/DML, NcL/DML, FcL/DML, GL/DML, GW/DML, RL/DML, RW/DML, GW/GL, and RW/RL. Most of these indices have been used routinely in previous morphometric studies of squids (Barón and Ré, 2002b; Haefner, 1964).

2.3. Multivariate analysis of data

Variations in the morphometric characters between *U. (P.) chinensis* and *U. (P.) edulis* were analysed using multivariate analysis (PRIMER 6, Plymouth Routine in Multivariate Analysis) (Clarke, 1993). The program PRIMER is principally designed for studies of changes in biotic communities using species data matrices, but it can also be applied to numerical taxonomy, in this case using matrices of individual squids based on morphometric indices. Data were standardized (Clarke, 1993) before a resemblance matrix was created based on the morphometric indices between the *Uroteuthis* samples using the Euclidean distance measure. Non-metric Multidimensional Scaling (MDS) ordination has proved to be robust in representing the high dimensional data (Chan et al., 2007) indicated by the stress values. MDS was conducted to generate two-dimensional plots of the morphometric indices between individuals of the two species. Analysis of Similarity (ANOSIM, Clarke, 1993) was conducted to test the degree and significance of differences between groups in terms of morphometric indices in the MDS plot. ANOSIM was used to calculate a test statistic (R) that is equal to 1 if all individuals within a population are more similar to each other than to any individual in another population, and 0 if there is no difference between populations. The similarity percentages (SIMPER) procedure was used to calculate the percentage contribution of each morphological character to the overall difference between groups.

2.4. DNA extraction, PCR amplification and sequencing

Mantle tissue of the squid samples was used for total genomic DNA extraction with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). After extraction, the DNA was eluted in 200 μl of double distilled H_2O (ddH_2O). The DNA extracts were evaluated by 1% agarose gel electrophoresis and ethidium bromide staining. The extracted DNA was kept at −20 °C for further analyses.

Polymerase chain reaction (PCR) was performed to amplify partial segments of the mitochondrial genes coding for COI and 16S. The 16S gene was amplified using the universal primers 16Sar and 16Sbr (Simon et al., 1994). The primer pair LCO1490 and HCO2198 (Folmer et al., 1994) was used for COI gene amplification. An annealing temperature of 52 °C was used in standard 3-step PCR, with 32 cycles, for both genes.

The size and quality of PCR products were assessed in 1% agarose gel electrophoresis. Prior to sequencing, amplification products were purified using a gel purification kit (Qiagen, Hilden, Germany) in

Table 2
Specimen sampling localities, GenBank accession numbers, and the number of individuals studied for COI and 16S rRNA sequences

Species	Sample locality	Sequence no.	
		COI	16S rRNA
Uroteuthis (Photololigo) chinensis	Hong Kong, China	10 (EU349429–EU349438)	10 (EU349467–EU349476)
	Xiamen, China	8 (EU349439–EU349446)	4 (EU349477–EU349480)
	Gulf of Thailand	1* (AF075394[a])	1* (AF110091[a])
'*Loligo chinensis*'	South China Sea	1* (AY185505[b] #)	1* (AF369955[b] #)
	unknown	–	1* (AJ000105[c])
Uroteuthis (Photololigo) edulis	Yamaguchi, Japan	10 (EU349447–EU349456)	5 (EU349481–EU349485)
	Shanghai, China	6 (EU349457–EU349462)	4 (EU349486–EU349489)
'*Loligo edulis*'	unknown	–	2* (AF369956[b] #, AJ000103[c] #)
Uroteuthis (Photololigo) duvauceli	Hong Kong, China	2 (EU349463–EU349464)	2 (EU349490–EU349491)
	Shanghai, China	1 (EU349465)	1 (EU349492)
	Gulf of Thailand	–	1* (AF110093[a])
	Andaman Sea	2* (AF075398[a], AF075400[a])	1* (AF110092[a])
'*Loligo duvauceli*'	unknown	–	1* (AJ000101[c])
Uroteuthis etheridgei	Moreton Bay, Australia	1* (AF075389[a])	1* (AF110094[a])
Uroteuthis noctiluca	Australia	1* (AF075403[a])	1* (AF110095[a])
'*Photololigo noctiluca*'	Sydney, Australia	1* (AY293706[d])	1* (AY293656[d])
'*Loligo formosana*'	Rayong, Thailand	1* (AY557524[f])	–
Photololigo sp.	Museum Victoria, Melbourne, Australia	1* (AY616889[e])	1* (AY616881[e])
Loligo vulgaris	Plymouth, U.K.	1* (AF075397)	1* (AF110082)
Lolliguncula mercatoris	South Africa	1* (AF075390)	1* (AF110085)
Total		48	40

Asterisk indicates GenBank sequence. [a–f]indicate sequence from different research groups: [a]Anderson (2000); [b]Zheng et al. (2004); [c]Liu, L., Hudelot, C., Boucher-Rodoni, R., Lu, C. and Bonnaud, L; [d]Nishiguchi et al. (2004); [e]Strugnell et al. (2005); [f]Lindgren et al. (2004). # indicates sequences that may be derived from misidentified individuals.

accordance with the manufacturer's instructions. The COI and 16S gene segments were sequenced using the same forward and reverse primers for PCR amplification. Sequences were obtained by dye terminator cycle sequencing using ABI Prism dRodamine terminator (Applied Biosystems, Foster City, CA, USA) and all sequences were produced on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed using ABI SeqEd version 1.0.3.

2.5. Sequence data analysis

A total of 37 individuals of the squid samples were sequenced for COI, and 26 for the 16S gene, respectively (Table 2). Eleven COI sequences and 14 16S sequences were downloaded from GenBank and incorporated in the analysis, including sequences from another *Uroteuthis* species (*U. noctiluca*), and also from *Loligo formosana* and *U. etheridgei*, which are considered to be synonymous to *U. (P.) chinensis* (Sweeney and Vecchione, 1998). Some of the sequences from GenBank were based on samples classified under the generic names of *Loligo* (*L. chinensis*, *L. edulis* and *L. duvauceli*) and *Photololigo* (*P. noctiluca*). The squids *Loligo vulgaris* and *Lolliguncula mercatoris,* from other genera, were used as the outgroups. Alignments of the data sets were conducted using Clustal W (Thompson et al., 1994) with default gap weighting parameters, and then adjusted by eye. Four methods were used to infer phylogenetic relationships: distance (BIO neighbor-joining, BIONJ, Gascuel, 1997), maximum parsimony (MP), and maximum likelihood (ML) performed in PAUP 4.0b10 (Swofford, 2002), and Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Analyses were conducted for each data set separately (COI, 48 sequences; 16S, 40 sequences) and for the combined data set (29 sequences). Before the combined data set was analyzed, incongruence length difference (ILD) test (Farris et al., 1995) referred to as a partition homogeneity test in PAUP 4.0b10 (Swofford, 2002) was used to examine possible incongruence between genes. One thousand replicates of the ILD test were performed. No evidence was presented for phylogenetic conflict between COI and 16S gene partitions ($P=0.436$), justifying a combined data approach.

The best-fit model of nucleotide substitution used for BIONJ, ML and BI analyses was determined with Modeltest version 3.7 (Posada and Crandall, 1998) using the hierarchical likelihood ratio test (hLRT, Huelsenbeck and Crandall, 1997). Heuristic MP and ML searches were made using tree bisection reconnection (TBR) branch swapping with random addition sequence replicates. The starting tree for branch-swapping was obtained by stepwise addition. Gaps were treated as missing characters. Bootstrap analysis, based on a full heuristic search of 1000 and 100 pseudoreplicates using TBR branch-swapping and random addition, was carried out to determine the MP and ML branch support respectively. One thousand bootstrap replicates were performed in BIONJ analysis. In BI analyses, partitioned substitution models were conducted. Data were divided into separate partitions, including 16S and COI in combined analysis and partitions based on gene codon positions (first, second, and third) in COI. There was no difference in topologies from analysis using a single model. A Markov chain Monte Carlo search was initiated with random trees and run for 2,000,000 generations with a sampling frequency of 100 generations. Convergence was checked by plotting likelihood scores against generation, and 6,000 initial trees were discarded as "burn-in". Two separate analyses and four independent chains were executed to check for convergence of topology.

3. Results

3.1. Morphological analysis

The MDS plot based on the 27 morphometric indices (Fig. 3) with a stress value <0.1 can be considered a good representation of the multivariate information, (Clarke and Warwick, 1994). The plot is a two-dimensional 'map' of the multivariate data, and in this case the variables consist of morphometric indices, so that the distance between individuals can be seen as the difference in their morphometric indices. The multivariate analysis of morphological differences between individuals shows high overlapping and no clear separation between the two loliginid squids, *U. (P.) chinensis* and *U. (P.) edulis* (ANOSIM, $R=0.1$, $P=0.034$) (Fig. 3). However, there is significant separation of their morphometric indices among locations ($R=0.692$, $P=0.001$). The pairwise ANOSIM procedure indicates a clear difference ($R>0.75$, $P<0.01$) (Clarke and Gorley, 2001) between Yamaguchi (Japan) and Shanghai (China), Yamaguchi and Xiamen (China), and Hong Kong (China) and Shanghai. The other locality pairings are also significantly different ($0.75>R>0.5$, $P<0.01$), except for the pairing

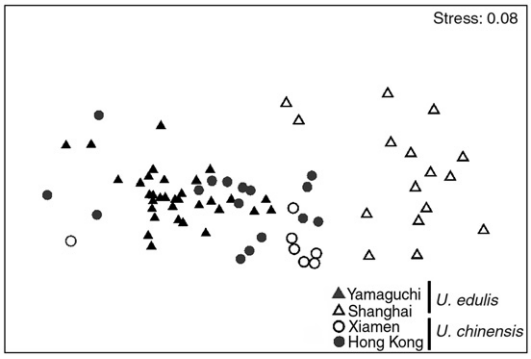

Fig. 3. Multidimensional scaling (MDS) plot on the morphometric indices of *U. (P.) chinensis* and *U. (P.) edulis*, grouped according to localities.

between Hong Kong and Xiamen (R=0.164, P>0.05). The result indicates intraspecific difference between localities in *U. (P.) edulis*, but not in *U. (P.) chinensis*. The results of SIMPER indicate that the character that contributes most to the intraspecific differences in *U. (P.) edulis* between Yamaguchi and Shanghai is the index of tentacle length to mantle length (24.6%).

It should be noted that the samples included individuals at different maturity stages (Table 3). For *U. (P.) edulis* of both sexes, >87% of samples from Yamaguchi were at stage V, while ≥60% of the Shanghai samples were at stage I. The variability in composition of *U. (P.) chinensis* individuals was more even in terms of maturity stages, at least for the males. In view of these differences, the result was also analyzed according to maturity stages. The analysis was performed separately for the two species, and only for the males. (The sample size of females was too small, with only four individuals at stage I and two individuals at stage II for *U. (P.) edulis*, and three individuals at stage V and two individuals at stage II for *U. (P.) chinensis*.) The morphometric indices of male *U. (P.) edulis* at different maturity stages were significantly different (Global R value=0.732, P=0.001; Fig. 4A). The pairwise ANOSIM procedure indicated a significant difference (R>0.5, P<0.01) between V and III, V and I, and IV and I. SIMPER indicated that the index contributing most to the difference between these maturity stage pairings was the tentacle length to mantle length (all >23%). On the other hand, the morphometric indices of *U. (P.) chinensis* males at different maturity stages overlapped (Global R value=0.348, P=0.012; Fig. 4B). The pairwise ANOSIM procedure indicated a significant difference between all pairings that included stage V (R>0.5, P<0.01). SIMPER indicated that the tentacle length to mantle length was also the index that contributed most to the difference between these maturity stage pairings (all>33%). This index is higher in individuals at early maturity stages than in those at later stages (Table 4). The sample size in this study was too low for a profitable comparison of species differences at specific maturity stages to be made.

Multivariate analysis between the two species was also conducted with the addition of one sex-specific character, the percentage of total length of left arm IV that is hectocotylized (HL%). This was not incorporated into the morphometric analysis described above, as it is only found in maturing male individuals (stages II to V). Excluding the individuals with broken hectocotylus, 17 *U. (P.) chinensis* and 36 *U. (P.) edulis* were included in this analysis (Fig. 5). Individuals of the same species clustered together (ANOSIM, R=0.928, P=0.001). SIMPER analysis showed that the most discriminative character was HL% which accounted for 92.1% of the dissimilarity between the two species. The mean HL% was 35.7% (range: 25.8-42.0%) in *U. (P.) chinensis* and 59.6% (range: 53.8-67.7%) in *U. (P.) edulis*. There was no significant difference in this character between stages IV and V of *U. (P.) edulis* (Mann-Whitney U test, P>0.05) and between stages II, IV and V of *U. (P.) chinensis* (Kruskal-Wallis test, P>0.05). Hence HL% does not change with maturation. Individuals at stage III for *U. (P.) edulis* (n=2) and *U. (P.) chinensis* (n=2) were not included in the above statistical analyses as their sample sizes were too small.

The only meristic character, the teeth number on arm sucker rings, was significantly different (P<0.001, t-test) between *U. (P.) edulis* (n=66) and *U. (P.) chinensis* (n=25). The mean teeth number on arm sucker rings is 11.4±1.2 (range: 9-14) for *U. (P.) chinensis*, and 9.0±1.0 (range: 7-12) for *U. (P.) edulis*.

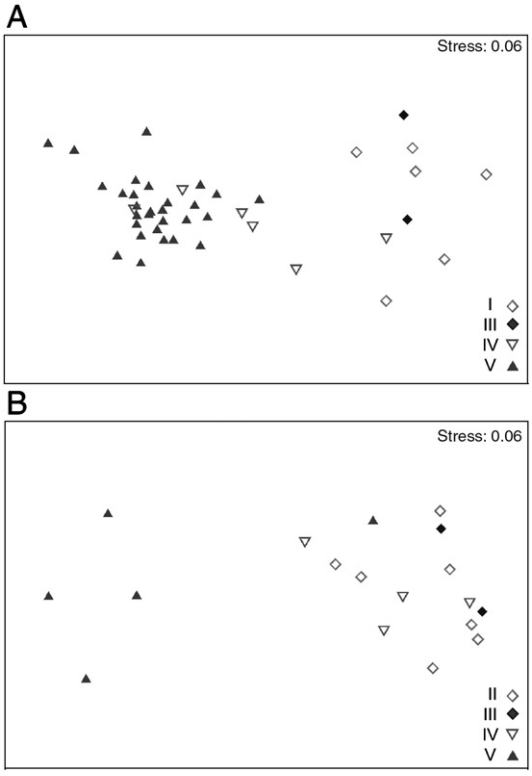

Fig. 4. Multidimensional scaling (MDS) plots on the morphometric indices of (A) male *U. (P.) edulis* and (B) male *U. (P.) chinensis*, grouped according to maturity stages.

Table 3
The percentage of samples for different maturity stages in the four localities

Species	Locality	Sex	n	Maturity stage				
				I	II	III	IV	V
U. (P.) edulis	Yamaguchi (Japan)	M	33	–	–	–	12.1%	87.9%
		F	0	–	–	–	–	–
	Shanghai (China)	M	10	60.0%	–	20.0%	20.0%	–
		F	6	66.6%	33.3%	–	–	–
U. (P.) chinensis	Xiamen (China)	M	5	–	60.0%	20.0%	–	20.0%
		F	2	–	–	–	–	100%
	Hong Kong (China)	M	13	–	30.8%	7.69%	30.8%	30.8%
		F	3	–	66.6%	–	–	33.3%

Table 4
TL/DML ratios at different maturity stages of male *U. (P.) edulis* and *U. (P.) chinensis*

Maturity stage	Mean (range) (No. of samples)	
	U. edulis	*U. chinensis*
I	1.77 (1.56-1.97) (5)	–
II	–	1.50 (1.27-1.73) (7)
III	1.92 (1.84-2.00) (2)	1.53 (1.45-1.60) (2)
IV	1.36 (1.06-1.87) (6)	1.40 (1.16-1.59) (4)
V	1.12 (0.99-1.52) (28)	0.99 (0.85-1.27) (5)

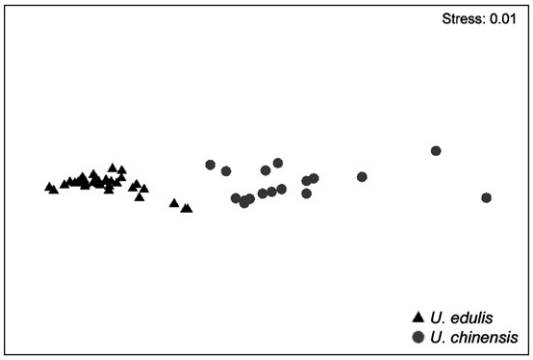

Fig. 5. Multidimensional scaling (MDS) plots on the morphometric indices, including the hectocotylized proportion of left arm IV, of mature male *U. (P.) chinensis* and *U. (P.) edulis*.

3.2. Genetic analysis

Of the 587 bp aligned for COI, 209 were variable and 164 were parsimony-informative. With an aligned length of 526 bp (total length ranged from 474 to 510 bp) in the 16S, 161 were variable and 96 were parsimony-informative. There were very few segments with alignment ambiguity, and excluding them from phylogenetic analysis did not change tree topologies or statistical support. The average sequence divergences between *U. (P.) chinensis* and *U. (P.) edulis* were 15.5% (14.7%-16.2%, excluding AY185505) for COI and 7.5% (7.2%-8.7%, excluding AF369956, AJ000103 and AF369955) for 16S. The intraspecific sequence divergences in COI were 0-1.1% within *U. (P.) chinensis* (excluding AY185505) and 0-0.5% within *U. (P.) edulis*, while the values for 16S were 0-1.6% within *U. (P.) chinensis* (excluding AF369955) and 0% within *U. (P.) edulis* (excluding AJ000103 and AF369956). We excluded these GenBank sequences from analysis because they appeared to be based on questionable taxonomic identifications.

In the combined COI and 16S analysis, the best-fit DNA substitution model is the general time reversible model (GTR+G; α=0.163) (Rodriquez et al., 1990). For COI, Modeltest selected the general time reversible model (GTR+G; α=0.199). The transversion model (TVM+I+G; α=0.457; I=0.346) is most appropriate for the 16S data set. MP analysis of the combined data produced four most parsimonious trees, with 684 steps, a consistency index (CI) of 0.681 and a retention index (RI) of 0.872. MP analysis of the COI and 16S data produced 27 and 2 most parsimonious trees, with 478 and 290 steps, CI of 0.603 and 0.766, and RI of 0.903 and 0.915, respectively.

Sequence data of the 16S gene from 40 individuals revealed that each of the four *Uroteuthis* species (*U. (P.) chinensis*, *U. (P.) edulis*, *U. (P.) duvauceli* and *U. noctiluca*) forms a distinct clade (Fig. 6A). The grouping of all *U. (P.) edulis* (except AJ000103 and AF369956) is strongly supported (bootstrap (BP) values 100% in BIONJ, MP and ML analyses; Bayesian posterior probabilities (BPP)=0.99). The clustering of all *U. (P.) chinensis* (except AF369955) is also highly supported (BP=100%; BPP=1.00). *U. etheridgei* (AF110094), synonymous with *U. (P.) chinensis*, forms a well-supported clade with *Photololigo* sp. (AY616881) (BP≥99%; BPP=1.00). This clade clusters with the *U. (P.) chinensis* clade (BP≥86%; BPP=1.0), but with a divergence of 5.5-9.1%. The clade *U. (P.) duvauceli* (including *L. edulis* (AJ000103), which probably is a *U. duvauceli*) is supported (BP=100%; BPP=1.0). *U. noctiluca* (BP=100%; BPP=1.00) also forms a well-supported clade. *U. (P.) chinensis* is closely related to *U. (P.) edulis* (BP≥76%; BPP=0.99).

Sequence data of the COI gene from 48 individuals also revealed that each of the four ingroup species forms a distinct clade (Fig. 6B). The grouping of all *U. (P.) edulis* is also well supported (BP=100%; BPP=1.00). The clustering of all *U. (P.) chinensis* (except AY185505, but including *L. formosana* (AY557524)) is also highly supported (BP≥99%; BPP=1.00). *U. etheridgei* (AF075389) forms a well-supported clade with *Photololigo* sp. (AY616889) (BP=100%; BPP=1.00). The clade clusters with the *U. (P.) chinensis* clade (BP≥82%; BPP=0.98), with a divergence of 11.9-15.6%. *U. (P.) duvauceli* (BP≥96%; BPP=1.00) and *U. noctiluca* (BP=100%; BPP=1.00) each forms a well-supported clade. *U. (P.) chinensis* is closely related to *U. (P.) edulis*, with a weak support (BP≥49% in BIONJ analysis; BPP=0.94).

The combined gene tree, with similar topology to the 16S and COI gene tree, is not shown. The four ingroup species each form a distinct clade. The grouping of *U. (P.) chinensis* and *U. (P.) edulis* is strongly supported (BP=100%; BPP=1.0). The grouping of *U. etheridgei* and *Photololigo* sp. is also well supported (BP=100%; BPP=1.0), and it clusters with the *U. (P.) chinensis* clade (BP≥96%; BPP=1.0). *U. (P.) duvauceli* (BP=100%; BPP=1.0) and *U. noctiluca* (BP=100%; BPP=1.0) also form well-supported clades. The relationships among the four species are identical to those revealed by the 16S and COI tree.

For the COI sequences from GenBank, AF075394 (*U. chinensis*), AF075398 (*U. duvauceli*) and AF075400 (*U. duvauceli*) cluster with the sequences of the corresponding species determined in the present study in the phylogenetic tree (Fig. 6B). However, AY185505 (*L. chinensis*) is not grouped with the sequences of *U. (P.) chinensis* and is very divergent from them. It is not within the *Uroteuthis* clade and Basic Local Alignment Search Tool (BLAST) result shows that it is more similar to the sequence of *Loligo opalescens* (99%). The sequences of *U. duvauceli* (AF075398 and AF075400) from the Andaman Sea are divergent from the grouping of Hong Kong and Shanghai samples (5.9-6.4%). For the 16S sequences from GenBank, AF110091 (*U. chinensis*), AJ000105 (*L. chinensis*), AF110093 (*U. duvauceli*), AF110092 (*U. duvauceli*), and AJ000101 (*L. duvauceli*) cluster with sequences of the corresponding species in the phylogenetic tree (Fig. 6A), while AJ000103 (*L. edulis*), AF369956 (*L. edulis*) and AF369955 (*L. chinensis*) are not grouped with the sequences of the same species. While AJ000103 (*L. edulis*) is grouped within the *U. duvauceli* clade, AF369956 (*L. edulis*) and AF369955 (*L. chinensis*) are not within the *Uroteuthis* clade. The BLAST results show that the two latter sequences are most similar to those of *Sepia robsoni* (100%) and *Loligo opalescens* (99%) respectively. The sequences of *U. duvauceli* (AF110093 and AF110092) from the Gulf of Thailand and Andaman Sea are divergent from the Hong Kong-Shanghai grouping (2.9-3.1% and 5.7-6.0% respectively) and from each other (4.4%). The three 16S sequences (AJ000103, AF369956 and AF369955) and one COI sequence (AY185505) that are not grouped with the sequences of the same species were reported by two different research groups.

4. Discussion

The soft bodies of squids are easily damaged during trawling, which often makes their morphometric study difficult (Pierce et al., 1994). In this study some specimens had lost their tentacles or other body parts, leading to reduction in the sample size used in multivariate analysis. In the multivariate study, the two loliginid squids, *U. (P.) chinensis* and *U. (P.) edulis*, were highly similar in their morphometric indices as deduced by visual interpretation of the MDS plot (Fig. 3) and ANOSIM. The 27 morphometric indices could not be used to distinguish the two species. Thus morphometric analysis was not able to differentiate the two species effectively.

The analysis demonstrated that the morphometric indices are affected by maturity stages, indicating that the squids grow allometrically. It is known that the size composition of octopus samples can affect morphological indices (Voight, 1991, 1994), and some indices in loliginid squids are highly correlated with size (Pineda et al., 2002). The index that contributes most to the difference among stages is the tentacle length to dorsal mantle length, which decreases with maturity. Tentacles are the pair of specialized arms for prey capture. Long tentacles may facilitate prey capture since the attack distance is longer. Longer tentacles compared to the body length in the early maturity stage are believed to be an advantage in capturing more food for growth. At the late maturity stage, the energy resources

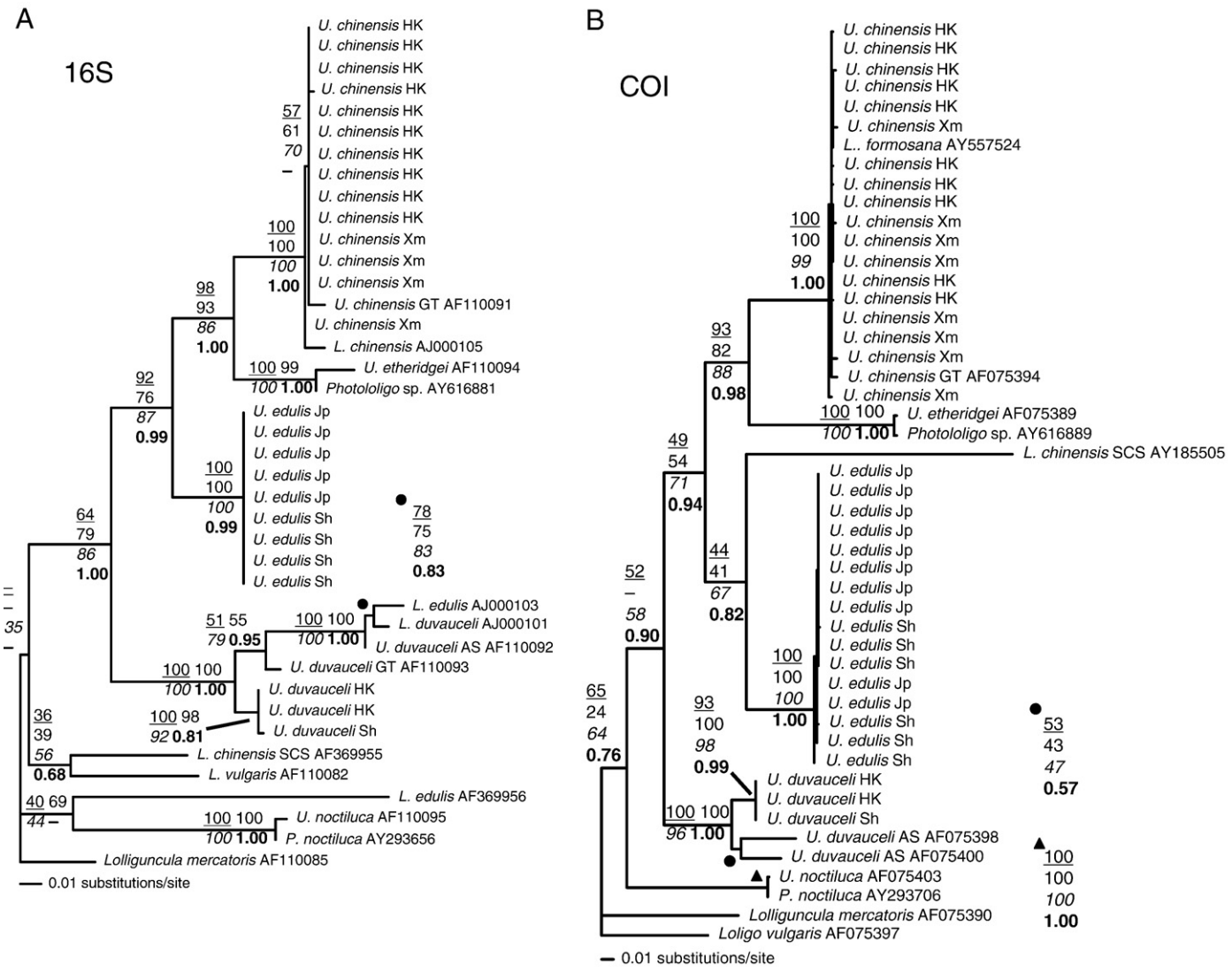

Fig. 6. Phylogenetic tree resolved by maximum likelihood analysis based on (A) 16S sequences and (B) COI sequences. Percentage levels of support are indicated on each branch for BIO neighbor-joining (in normal), parsimony (in italics), maximum likelihood (in bold), and Bayesian posterior probabilities (underlined). HK: Hong Kong (China), Xm: Xiamen (China), Jp: Yamaguchi (Japan), Sh: Shanghai (China), GT: Gulf of Thailand, AS: Andaman Sea, SCS: South China Sea. Accession numbers are shown for sequences from GenBank.

would switch to the expense of reproduction. As the squid matures, the mantle length increases relative to the tentacles. It has been documented in other loliginid squids that the reproductive organs develop quickly at the onset of maturity (Barón and Ré, 2002a).

While it is common for the morphometric characters of cephalopods to vary according to geographical location (Carvalho and Nigmatullin, 1998), it has also been found that this variability could be insignificant among distant populations of some loliginid squids (Cohen, 1976). In this study, the difference between squids from different localities could be explained by the difference in the composition of individuals at various maturity stages. The *U. (P.) chinensis* samples from Hong Kong and Xiamen are of similar maturity stage composition, and no separation was evident between individuals from these two locations. Most individuals of *U. (P.) edulis* from Yamaguchi were at stage V, while most individuals from Shanghai were at stage I. The observed difference between these two localities is therefore most probably due to the difference in maturity stage composition. This inference is further supported by the observation that the index which contributes most to the difference between stages is also the one that contributes most to the difference between localities.

When HL% was added into the analysis, *U. (P.) chinensis* and *U. (P.) edulis* were clearly distinguishable from each other. This result shows that HL% is the only index under investigation capable of providing sufficient information for species identification of the two species. This result also validates previous observations that the length of the hectocotylized part varied from 33% to 40% in *U. (P.) chinensis* and from 50% to 67% in *U. (P.) edulis*. The hectocotylized lengths of *U. (P.) chinensis* (mean: 35.7%) and *U. (P.) edulis* (mean: 59.6%) in this study are within the reported ranges. HL% is not affected by maturity. However, the limitation of using this morphological character to identify the two species is that it cannot be used to identify females or immature males. Another character previously used to distinguish the two species is the number of teeth on the arm sucker ring, which ranges from 6 to 12 in *U. (P.) edulis* (Hoyle, 1885) and from 10 to 18 in *U. (P.) chinensis* (Gray, 1849). The values of 7-12 in *U. (P.) edulis* and 9-14 in *U. (P.) chinensis* found in the present study are within the reported ranges. Yet this character is not a diagnostic character between the two species, since the two ranges of the teeth number overlap. Because meristic characters are highly sensitive to environmental variations during their formation (Barlow, 1961), teeth number is not a good character for species identification.

Analyses based on two mitochondrial gene sequences clearly separate *U. (P.) chinensis* and *U. (P.) edulis* into two distinct clades. The two genes show a high level of divergence (COI: 15.5%, 16S: 7.5%) between *U. (P.) chinensis* and *U. (P.) edulis*. This divergence falls within the interspecific COI divergence (range: 11-22%, average: ~18%) of *Loligo* species (Herke and Foltz, 2002). This provides evidence for the presence of two distinct species, but does not support the presence of clinal variations among individuals of the same species along the localities included in this analysis. *Uroteuthis (Photololigo) edulis* from Yamaguchi and Shanghai and *U. (P.) chinensis* from Xiamen, Hong Kong and the Gulf of Thailand represent two distinct species. The genetic analysis supports the use of the arm sucker ring teeth shape (Natsukari and Okutani, 1975) in distinguishing the two species, given the overlap in the number of teeth, which is not a diagnostic character.

None of the samples used in this study were misidentified, as all of them were grouped into their own clade. Intraspecific COI divergence (0.15-1.4%) observed in a study of the population structure of the *Loligo* species *Loligo pealei* and *Loligo plei* indicates that the two species do not harbour cryptic species (Herke and Foltz, 2002). All the samples of *U. (P.) chinensis* and *U. (P.) edulis* in this study showed very low COI divergence with other conspecific individuals (range: 0-1.1%). Based on the present data, therefore, it is reasonable to conclude that no cryptic species are found in the Asian region. On the basis of our genetic analysis and also its distribution range, the previously named *L. formosana* is likely to be *U. chinensis*. *U. etheridgei*, which is believed to be synonymous to *U. chinensis* (Sweeney and Vecchione, 1998), shows a divergence corresponding to interspecific difference, though it always clusters with the *U. (P.) chinensis* clade. Thus it is possible that this specimen, together with '*Photololigo* sp.', are cryptic species, as both are from Australia which may harbour some cryptic species (Yeatman and Benzie, 1994). However, since no morphological information is available for these specimens, it is not possible at present to determine whether they have been misidentified or represent cryptic species. To address this question of cryptic species, specimens of the two species from localities throughout their distribution range have to be included in further analysis.

While some of the GenBank DNA sequences included in this study (COI: AF075394, AF075398, AF075400; 16S: AF110091, AJ000105, AF110092, AF110093) are based on correctly identified individuals, others (COI: AY185505; 16S: AJ000103, AF369955, AF369956) appear to have been derived from misidentified specimens. This shows that misidentifications of these squid species are common (assuming that there were no sequence contamination), as the sequences from misidentified individuals have originated from two of the three research groups that reported the sequences included in the present analysis. This highlights the importance of using DNA barcode for species identification of organisms (Hebert et al., 2003), including cephalopods (Strugnell and Lindgren, 2007). DNA barcode is also applicable in the identification of paralarva and immature individuals that cannot be identified morphologically, as well as damaged specimens with missing body parts, which are common in squids (Wakabayashi et al., 2006). However, it is important that DNA barcoding must be backed up by good taxonomy and accurate species identification.

In conclusion, we found that the HL% and the teeth shape and number on arm sucker rings provided useful information for distinguishing *U. (P.) chinensis* and *U. (P.) edulis*. Our results are consistent with observations made by other researchers in previous studies. Other morphometric characters included in the present study do not provide enough information for differentiating the two species, so that no new morphological characters useful for this purpose could be identified. Genetic study of the mitochondrial genes of *U. (P.) chinensis* and *U. (P.) edulis* clearly reveals that they are distinct species. The populations of *U. (P.) chinensis* and *U. (P.) edulis* do not represent the clinal variations among individuals of the same species along the coast of East Asia. No cryptic species were found in this study, but more samples from different localities are needed to resolve this issue, since *U. (P.) chinensis* and *U. (P.) edulis* are widely distributed.

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