

Phylogenetic relationships among Octopodidae species in coastal waters of China inferred from two mitochondrial DNA gene sequences

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ABSTRACT. Octopus in the family Octopodidae (Mollusca: Cephalopoda) has been generally recognized as a "catch-all" genus. The monophyly of octopus species in China's coastal waters has not yet been studied. In this paper, we inferred the phylogeny of 11 octopus species (family Octopodidae) in China's coastal waters using nucleotide sequences of two mitochondrial DNA genes: cvtochrome c oxidase subunit I (COI) and 16S rRNA. Sequence analysis of both genes revealed that the 11 species of Octopodidae fell into four distinct groups, which were genetically distant from one another and exhibited identical phylogenetic resolution. The phylogenies indicated strongly that the genus Octopus in China's coastal waters is also not monophyletic, and it is therefore clear that the Octopodidae systematics in this area requires major revision. It is demonstrated that partial sequence information of both the mitochondrial genes 16S rRNA and COI could be used as diagnostic molecular markers in the identification and resolution of the taxonomic ambiguity of Octopodidae species.

Key words: Molecular phylogeny; Mitochondrial DNA gene sequences; Octopodidae species; COI; 16S rRNA

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INTRODUCTION

The family Octopodidae (Mollusca: Cephalopoda) includes many commercially important species, distributed around the world. Members of the Octopodidae share a basic structural plan (e.g., eight arms, biserial sucker rows, and an ink sac), but our understanding of the phylogeny and taxonomy of this family is currently quite limited. The taxonomy of Octopodidae gets even more confusing when the genus Octopus is considered. Octopus is the largest genus of the Octopodidae family (Sweeney and Roper, 1998). To date, over 90% of the more than 200 species included in the Octopodidae family have been placed in the catch-all genus Octopus, primarily on the basis of historical convention (Norman et al., 2004; Guzik et al., 2005). A number of taxonomic studies based on morphology have recognized distinct species complexes within the genus Octopus as it currently stands (Norman and Hochberg, 1994; Norman and Sweeney, 1997; Norman, 1992, 2000). For example, Robson (1929) identified nine species groups within the genus Octopus, including the Octopus macropus, O. aegina, O. vulgaris, O. pallidus, and O. australis groups, based on distinguishing features such as overall size, arm length, web depth, skin sculpture, and mantle shape. Norman (1992) recognized four primary groups, which includes the O. horridus, O. aegina, O. macropus, and Hapalochlaena species group, in an investigation of the systematic relationships among 16 Great Barrier Reef octopus species using 66 morphological characters. Some of these groups are now being reexamined and are recognized as potentially independent genera (Norman, 1992; Stranks and Norman, 1992; Norman and Sweeney, 1997; Norman and Finn, 2001). Several new genera, such as Abdopus, Aphrodoctopus, Muusoctopus, and Amphioctopus, have been erected from this catch-all genus (Roper and Mangold, 1991; Norman and Finn, 2001; Gleadall, 2004; Huffard and Hochberg, 2005). The polyphyletics of the Octopus genus have been demonstrated by a number of molecular studies (Barriga Sosa et al., 1995; Carlini et al., 2001; Guzik et al., 2005). The reconsideration of generic names and the major revision of these taxa have been proposed by some authors (Norman and Finn, 2001; Gleadall, 2004; Guzik et al., 2005).

The same situation occurs for the systematics of octopus species in China's coastal waters. The octopus species have been quite important target species for China's fishery industry for centuries, but their taxonomy is still far from being certain. Dong (1988) set the basis for our understanding of Octopodidae phylogeny in China's coastal waters late in the last century. He has defined 17 Octopodidae species along the coast of China, which were placed in three genera, Octopus, Cistopus, and Callistoctopus. Fifteen of them were placed in the genus Octopus. Since then, only a few revisions have been made for the systematics of octopus species in China's coastal waters. For example, Lu (1998) recognized 22 species from Taiwanese waters, of which 13 were new to science. Zhu et al. (2005) added one more new species, O. tankahkeei, into genus Octopus. Recently, Chen et al. (2009) removed O. maculosa from the genus Octopus to Hapalochlaena, but still left other species in the genus Octopus. However, according to Norman's revision of octopus taxonomy (Norman and Hochberg, 2005), many octopus species in China's coastal waters, traditionally placed in genus Octopus, should be renamed or removed to other genera. For instance, O. striolatus should be regarded as the junior synonym of Amphioctopus marginatus, O. guangdongensis should be moved to genus Abdopus, and O. dollfusi should be recognized to be the synonym of O. aegina and moved to genus Amphioctopus. Many more revisions have been recommended by Norman for Dong's systematics of octopus species in his study (Norman and Hochberg, 2005).

For testing of Norman's recommendation, in this study, we report the phylogenetic rela-

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tionships of 11 species in the family Octopodidae, collected from China's coastal waters, based on two mitochondrial genes, cytochrome c oxidase subunit I (COI) and 16S rRNA. In particular, we aimed to resolve the phylogenetic relationships among species within the genus *Octopus*.

MATERIAL AND METHODS

Eleven Octopodidae species were collected from the coastal waters of China, and the collection data are shown in Table 1. One individual of each species was used for the nucleotide sequence analyses. The living or frozen octopus specimens were transferred to the laboratory, and crude DNA was extracted from muscle tissue by proteinase K digestion following a standard phenol-chloroform method. The 16S rRNA region of the mtDNA was amplified following the procedure used by Zheng et al. (2004) in decabrachia cephalopods with the primer set P16SF (5'-CGCCTGTTTAHYAAAAACAT-3') and P16SR (5'-CCGGTCTGAACTCAGMTCAYGT-3'). The COI region of the mtDNA was amplified using the primer set PCOIF (5'-TAAACTTGAGGGTGACCAAAAAAT-3') and PCOIR (5'-GGTCAACAAATCATAAAGATATTG-3'). PCR amplification was carried out in 50-µL reaction mixtures containing 50 ng template DNA, 1X reaction buffer, 2.0 mM MgCl., 0.2 mM dNTPs. 0.2 µM of each primer, and 4.0 U Taq DNA polymerase (Promega, Madison, WI, USA) in a PTC-200 (Bio-Rad, USA) PCR machine. The reaction mixtures were preheated at 94°C for 5 min, followed by 40 cycles of amplification (94°C for 1 min, 51°C for 1 min, and 72°C for 1 min), and a final extension at 72°C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide to confirm the amplification. The PCR products were purified with the Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai, China) and sequenced with both forward and reverse primers in Invitrogen Ltd. (Shanghai, China) following the standard cycle sequencing protocol.

1	, <u>,</u>	X	, .
Species	Collection location	COI GenBank accession No.	16S rRNA GenBank accession No.
O. striolatus Dong, 1976	Zhejiang, East China	JX456262	JX456251
O. variabilis Sasaki, 1929	Liaoning, North China	JX456263	JX456252
O. tankahkeei (unknown)	Zhejiang, East China	JX456264	JX456253
O. ovulum Sasaki, 1917	Zhejiang, East China	JX456266	JX456254
O. aegina Gray, 1849	Zhejiang, East China	JX456265	JX456255
O. dollfusi Robson, 1928	Guangdong, South China	JX456267	JX456256
O. oshimai Sasaki, 1929	Zhejiang, East China	JX456268	JX456257
C. indicus Orbigny, 1840	Guangdong, South China	JX456269	JX456258
O. vulgaris Cuvier, 1797	Fujian, East China	JX456270	JX456259
O. ocellatus Gray, 1849	Shandong, North China	JX456271	JX456260
O. fusiformis Brock, 1887	Guangdong, South China	JX456272	JX456261

Table 1. Octopodidae analyzed for the partial cytochrome oxidase subunit 1 (COI) and 16S rRNA genes.

 $O_{\cdot} = Octopus; C_{\cdot} = Cistopus.$

The nucleotide sequences obtained using both the forward and reverse primers were edited and aligned using Clustal W (Thompson et al., 1997). All the nucleotide sequences obtained were deposited in GenBank under the accession numbers shown in Table 1. The number of polymorphic sites, nucleotide composition, and number of transitions and transversions between species were determined using DnaSP version 5.10 (Rozas et al., 2003). Sequence variations of the COI and 16s rRNA genes were analyzed. The distance matrix was computed using the Kimura 2-parameter method with the MEGA 3.1 software program (Molecular Evo-

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lutionary Genetics Analysis) (Kumar et al., 2004). Both COI and 16s rRNA sequences were used to carry out the phylogenetic analysis using MEGA 3.1 to construct the unweighted pair-group method with arithmetic averages (UPGMA), neighbor-joining (NJ), and maximum parsimony (MP) phylogenetic trees. Homologous sequences from *Cistopus taiwanicus* (family Octopodidae) (Dai et al., 2012) were used as the outgroup. To verify the robustness of the internal nodes the of NJ, UPGMA, and MP trees, bootstrapping with 1000 replications was used to assess the reliability of all nodes on each tree.

RESULTS

Analysis of 16S rRNA sequences

The alignment of 16S rRNA sequences from all samples, including outgroups, produced 458-bp fragments with insertions or deletion gaps counted. Of the 458 sites, 274, 184, 66, and 26 were conserved, variable, parsimony informative, and singleton, respectively. The polymorphic sites are given in Figure 1. The analysis revealed nucleotide frequencies of A = 34.90%, T = 39.20%, G = 17.10%, and C = 8.80%. Average transversional pairs (sv = 59) were more frequent than transitional pairs (si = 38), with an average ratio of 1.55. The pairwise genetic distance values (Kimura 2-parameter), based on 16S rRNA using MEGA 3.1, are given in Table 2. The interspecies distance among Octopodidae ranged from 0 to 0.151, with an average of 0.106. The highest interspecies genetic distance (0.151) was between O. variabilis and O. vulgaris, and the lowest genetic distance (0) was between C. taiwanicus and C. indicus. All the NJ, UPGMA, and MP trees revealed identical phylogenetic relationships among the species (Figure 2). Four major clusters were obtained, with the 1st cluster formed by the five species O. striolatus, O. aegina, O. ovulum, O. dollfusi, and O. ocellatus (Group A); the 2nd cluster formed by O. oshimai and O. vulgaris (Group B); the 3rd cluster formed by the species C. taiwanicus, C. indicus, and O. tankahkeei (Group C); and the 4th cluster formed by species O. variabilis and O. fusiformis (Group D). In all the trees, these clusters were supported by high bootstrap values (NJ = 79 to 100%; UPGMA = 85 to 100%; MP = 70 to 100%).

Analysis of COI sequences

The alignment of COI sequences from all samples, including outgroups, produced 625-bp fragments with no insertions, deletions, or stop codons observed. Of the 625 sites, 428, 197, 170, and 27 were conserved, variable, parsimony informative, and singleton, respectively. The polymorphic sites are given in Figure 3. Most of the variable changes among the species were 3rd codon position transitional substitutions. The analysis revealed nucleotide frequencies of A = 30.30%, T = 36.30%, G = 14.60%, and C = 18.80%. Average transitional pairs (si = 89) were more frequent than transversional pairs (sv = 56), with an average ratio of 1.59. The pairwise genetic distance values based on COI sequences using MEGA 3.1 are given in Table 3. The interspecies distance with COI ranged from 0.000 to 0.203, with an average of 0.162. The highest genetic distance (0.203) was between *C. taiwanicus* and *O. fusiformis*, *O. fusiformis* and *O. oshimai*, and *O. fusiformis* and *O. vulgaris*, whereas the lowest interspecies distance (0.000) was again found between *C. vulgaris* and *C. oshimai*. All the NJ, UPGMA, and MP trees revealed identical phylogenetic relationships among the species (Figure 4).

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	111111	2367788011	1222233334	5566677780	0111111112	222222333	444445555	55566
	1236156789	0154545945	6356734581	0856701757	9234567890	2345689789	1234592456	78901
0.striolatus	GGGTGATTTA	TAACTGAAAT	ACATTCATGG	TGTGGGAAAA	AATATCTTAA	TATAA		TT
0.variabilis	GAAG	AG	.TGTGA	.AAA.GGT	ATATG.	ATAAT	TATATGATTA	TATG.
0.tankahkeei		GAA	TT.C	.A.AA	ATAA		AATAA	ATAAA
0.ovulum	A.		G.TT	CA	T.ATG			
O.aegina			A.		T			
0.dollfusi	A.		G.CT	A	CT	T		
0.oshimai	A.	TC.GGTA	TA	.A.TATT	GTATAG		ATTAT	GTA.A
C.indicus		TC.GA	T.GTA	.A.AAAG	ATATT	GTATTTAATA	ATATAATTAT	ATA.A
0.vulgaris	TTAA.GA	.G.TC.GGTA	TA	.A.TATT	GTATAG		ATTA-	-TA.A
0.ocellatus	A.A.		TACTA	CA.AAA	TGT	.TT		A
0.fusiformis	A.GAAG	AG	.TGTA	.A.AAAGT	.GGTGT	G-GGG	TGTTTTTAGA	TGG
C.taiwanicus		TC.GA	T.GTA	.A.AAAG	ATATT	GTATTTAATA	ATATAATTAT	ATA.A
	22222222222	2222222222	22333333333	33333333333	33333333333	33333333333	33344444	
	2222222222 6666777788	2222222222 8888899999	22333333333 9900223333	33333333333 3334444444	33333333333 4466666666	33333333333 6777777888	33344444 99900124	
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0.striolatus	2222222222 6666777788 3456678902 GTTTTGA	2222222222 8888899999 3467901234 TTATTAAATA	2233333333 9900223333 7827890123 TGAG-TAT	3333333333 333444444 4590134567 ATTTAT	3333333333 4466666666 8912345678 ATGATAGTAT	3333333333 677777888 9035679023 ATATAAAATT	33344444 99900124 45979079 ATAGAGAA	
O.striolatus O.variabilis	2222222222 6666777788 3456678902 GTTTTGA AAATA.AG	222222222 8888899999 3467901234 TTATTAAATA .AT.G	2233333333 9900223333 7827890123 TGAG-TAT GT.A.A	3333333333 333444444 4590134567 ATTTAT A	3333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A	3333333333 677777888 9035679023 ATATAAAATT GAG.TGA.	33344444 99900124 45979079 ATAGAGAA G.TTGG	
O.striolatus O.variabilis O.tankahkeei	222222222 6666777788 3456678902 GTTTTGA AAATA.AG A.A	222222222 8888899999 3467901234 TTATTAAATA .AT.G A.GTG.	2233333333 9900223333 7827890123 TGAG-TAT GT.A.A GTA	3333333333 333444444 4590134567 ATTTAT A G.GTATA	3333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A .AAT.TTATA	3333333333 677777888 9035679023 ATATAAAATT GAG.TGA. TGTGG.	33344444 99900124 45979079 ATAGAGAA G.TTGG TTTG	
O.striolatus O.variabilis O.tankahkeei O.ovulum	222222222 6666777788 3456678902 GTTTTGA AAATA.AG A.AG	222222222 8888899999 3467901234 TTATTAAATA .AT.G A.GTG. 	223333333 9900223333 7827890123 TGAG-TAT GT.A.A GTA AA.TA	3333333333 333444444 4590134567 ATTTAT A G.GTATA G	333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A .AAT.TTATA .AT.GT.	333333333 677777888 9035679023 ATATAAAATT GAG.TGA. TGTGG. TATT.G	33344444 99900124 45979079 ATAGAGAA G.TTGG TTTG T	
O.striolatus O.variabilis O.tankahkeei O.ovulum O.aegina	222222222 6666777788 3456678902 GTTTTGA AAATA.AG A.AG G	222222222 8888899999 3467901234 TTATTAAATA .AT.G A.GTG. 	2233333333 9900223333 7827890123 TGAG-TAT GT.A.A GTA AA.TA 	3333333333 333444444 4590134567 ATTTAT A G.GTATA GG.	3333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A .AAT.TTATA .AT.GT. T	3333333333 6777777888 9035679023 ATATAAAATT GAG.TGA. TGTGGG. TATT.G A	33344444 99900124 45979079 ATAGAGAA G.TTGG TTTG T A.A	
O.striolatus O.variabilis O.tankahkeei O.ovulum O.aegina O.dollfusi	222222222 6666777788 3456678902 GTTTTGA AAATA.AG A.AG G AG	222222222 8888899999 3467901234 TTATTAATA .AT.G A.GTG.	2233333333 9900223333 TGAG-TAT GT.A.A GTA AA.TA A.TA	333333333 333444444 4590134567 ATTTAT A G.GTATA G.GTATA GG. A.	333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A .AAT.TTATA AT.GT. T T.ATG.	333333333 677777888 9035679023 ATATAAAATT GAG.T.GA. TGTGG. T.AT.T.G A T.AGT.T.G	33344444 99900124 45979079 ATAGAGAA G.TTGG TTTG A.A T	
0.striolatus 0.variabilis 0.tankahkeei 0.ovulum 0.aegina 0.dollfusi 0.oshimai	222222222 6666777788 3456678902 GTTTTGA AAATA.AG A.A AG AT A.AAG	222222222 8888899999 3467901234 TTATTAAATA .AT.G A.GTG. A.TA.TGTA.	2233333333 9900223333 7827890123 TGAG-TAT GT.A.A GTA GTAA.TA ATC.AA.TA	3333333333 333444444 4590134567 ATTTAT A G.GTATA GG. G. A. AAGG.T.	333333333 446666666 8912345678 ATGATAGTAT .GA.ATTA.A .AT.TTATA .AT.GT. T T.ATG. AT.TTA	333333333 6777777888 9035679023 ATATAAAATT GAG.TGA. TGTGG. TATT.G A TAGT.T.G GTGAG	33344444 99900124 45979079 ATAGAGAA G.TTGG TTTG A.A. T G.TG	
0.striolatus 0.variabilis 0.tankahkeei 0.ovulum 0.aegina 0.dollfusi 0.oshimai C.indicus	222222222 6666777788 3456678902 GTTTTGA AAATA.AG A.AG G A.AT A.AAG A.A.GAG.A.	222222222 8888899999 3467901234 TTATTAAATA .AT.G A.GTG. A.TA.TGTA. .GTAATAT	2233333333 9900223333 7827890123 TGAG-TAT GT.A.A GT.A.A GT.AA.TA AA.TA ATTAT.	3333333333 333444444 4590134567 ATTTAT A G.GTATA G.GTATA GG A. AAGG.T. GAAAATTA	333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A .AT.GT. T.T. T.ATG. .AT.TTA TAAT.TT-TA	333333333 677777888 9035679023 ATATAAAATT GAG.TGA. TGTGGG. TATT.G ATT.G ATT.G GTGAG T.TG.	33344444 99900124 45979079 ATAGAGAA G.TTGG TTTG A.A. T G.TG TTTG	
0.striolatus 0.variabilis 0.tankahkeei 0.ovulum 0.aegina 0.dollfusi 0.oshimai C.indicus 0.vulgaris	222222222 6666777788 3456678902 GTTTTGA AAATA.AG A.AG T A.AG A.A.GAG.A. A.A.GAG.A.	222222222 8888899999 3467901234 TTATTAAATA .AT.G A.GTG. A.TA.TGTA. .GTAATAT A.TA.TGTA.	2233333333 9900223333 7827890123 TGAG-TAT GTA GTA GTA.TA ATCAA.TA ATTAT. ATCAA.TA	3333333333 333444444 4590134567 ATTTAT A G.GTATA G.GTATA GA. AAGG.T. GAAATTA AAGG.T.	333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A .AT.GT. T.ATG. .AT.T-A TAAT.TT-TA .AT.TT-TA .AT.TT-A	333333333 6777777888 9035679023 ATATAAAATT GAG.TGA. TGTGGG. TATT.G ATT.G GTGAG GTGAG GTGAG	33344444 99900124 45979079 ATAGAGAA G.TTGG TTG T G.TG G.TG G.TG G.TG	
O.striolatus O.variabilis O.tankahkeei O.ovulum O.aegina O.dollfusi O.oshimai C.indicus O.vulgaris O.ocellatus	222222222 6666777788 3456678902 GTTTTGA AATA.AG A.AG G A.AG A.A.GAG.A. A.A.GAG.A. A.AAG .CT	222222222 8888899999 3467901234 TTATTAAATA .AT.G A.GTG. A.TA.TGTA. .GTAATAT A.TA.TGTA. 	2233333333 9900223333 7827890123 TGAG-TAT GT.A.A GTA-TA AA.TA AA.TA ATTAT. ATCAA.TA ATTAT. ATCAA.TA ATAT.	3333333333 333444444 4590134567 ATTTAT A G.GTATA GG. A. AAGG.T. GAAAAT.TA AAGG.T. .AA.T.	333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A .AAT.TTATA .AT.GT. T T.ATG. .AT.TTA TAAT.TTA .AT.TTA .TTATA.T.	333333333 677777888 9035679023 ATATAAAATT GAG.TGA. TGTGG. TATT.G AGT.T.G GTGAG GTGAG .A.ATC.T	33344444 99900124 45979079 ATAGAGAA G.TTGG .TTG .T G.TG G.TG G.TG G.TG G.TG G.TG	
O.striolatus O.variabilis O.tankahkeei O.ovulum O.aegina O.dollfusi O.oshimai C.indicus O.vulgaris O.ocellatus O.fusiformis	222222222 6666777788 3456678902 GTTTTGA AAATA.AG A.AG T A.AAG A.A.GAG.A. A.AAG .CT CTTAA-	222222222 8888899999 3467901234 TTATTAAATA .AT.G A.GTG. A.TA.TGTA. .GTAATAT A.TA.TGTA. 	2233333333 9900223333 7827890123 TGAG-TAT GT.A.A GT.A.A GTA.TA AA.TA ATC.AA.TA ATC.AA.TA ATAT. GC.AGA	3333333333 333444444 4590134567 ATTTAT A G.GTATA G.GTATA GG. AAGG.T. .AAGG.T. .AAGG.T. .AA.T. .GG	333333333 4466666666 8912345678 ATGATAGTAT .GA.ATTA.A .AT.G-T. T T.ATG. .AT.TT-A TAAT.TT-A .AT.TT-A .AT.TT-A .AT.TTA.A	333333333 677777888 9035679023 ATATAAAATT GAG.TGA. TGTGG. TAT.T.G A.T.G GTGAG GTGAG A.ATC.T. .A.T.GG.	33344444 99900124 45979079 ATAGAGAA G.TTGG T G.TG G.TG G.TG G.TG G.TG TG ATTGG	

Figure 1. Alignment of partial DNA sequences of the mitochondrial gene of 16S rRNA from 11 Octopodidae species (both variable sites and gaps are reported), with *Cistopus taiwanicus* used as outgroup.

Table 2 Painvise genetic distances (Kimura 2-parameter) among 11 Octopodidae species based on 16S rRNA

sequences, with Cistopus taiwanicus used as outgroup.												, 11(17)
	O. stri	O. vari	O. tank	O. ovul	O. aegi	O. doll	O. oshi	C. indi	O. vulg	O. ocel	O. fusi	C. taiw
O. stri												
O. vari	0.121											
O. tank	0.101	0.118										
O. ovul	0.058	0.127	0.107									
O. aegi	0.017	0.131	0.114	0.064								
O. doll	0.058	0.127	0.114	0.040	0.064							
O. oshi	0.108	0.127	0.091	0.111	0.117	0.107						
C. indi	0.114	0.111	0.055	0.107	0.120	0.107	0.082					
O. vulg	0.127	0.151	0.111	0.137	0.137	0.134	0.023	0.101				
O. ocel	0.088	0.117	0.111	0.079	0.092	0.076	0.107	0.101	0.127			
O. fusi	0.104	0.064	0.114	0.117	0.114	0.114	0.121	0.104	0.144	0.091		
C. taiw	0.114	0.111	0.055	0.107	0.120	0.107	0.082	0.000	0.101	0.101	0.104	

O. stri = Octopus striolatus; *O.* vari = *O.* variabilis; *O.* tank = *O.* tankahkeei; *O.* ovul = *O.* ovulum; *O.* aegi = *O.* aegina; *O.* doll = *O.* dollfusi; *O.* oshi = *O.* oshimai; *C.* indi = Cistopus indicus; *O.* vulg = *O.* vulgaris; *O.* ocell = *O.* ocellatus; *O.* fusi = *O.* fusiformis; *C.* taiw = *C.* taiwanicus.

Again, four major clusters were obtained with the 1st cluster formed by the five species *O. striolatus*, *O. aegina*, *O. ovulum*, *O. dollfusi*, and *O. ocellatus* (Group A); the 2nd cluster formed by *O. oshimai* and *O. vulgaris* (Group B); the 3rd cluster formed by the species *C. taiwanicus*, *C. indicus*, and *O. tankahkeei* (Group C); and the 4th cluster formed

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by the species *O. variabilis* and *O. fusiformis* (Group D). In all the trees, these four clusters were supported by comparatively high bootstrap values (NJ = 72 to 100%; UPGMA = 82 to 100%; MP = 66 to 100%). The topology of trees based on COI was quite similar to that based on 16S rRNA sequences.



Figure 2. Neighbor-joining (NJ), maximum parsimony (MP) and unweighted pair-group method with arithmetic average (UPGMA) phylogenetic tree of Octopodidae species inferred from DNA sequences of mitochondrial gene 16S rRNA.

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			1	1111111111	1111111112	2222222222	222222
	1112333	3444456667	7889999990	1222334455	5566778890	0001112333	344455
	3492584346	7258943693	8170134699	1069251436	7928170921	4571692147	806958
0.striolatus	TTTCTTTCCC	TTTATTTAAC	AAATCATATT	AAAACTTATC	CTACATAATA	TTATTTGATC	CTTTAC
O.variabilis	AC.TCT.T	CA.T.A.TTT	T.TACC.C	TT.TAT	TA.TTCT	AA.C.	.AT
O.tankahkeei	AC.ATA	CACCT.T	т.с	G.C.CT	.ATATT.T	CAAA.C.	.ATT
0.ovulum	A.T	TC.CT.T	т	т	TATA.CC.	AA	AACCGT
O.aegina					c.	A	
0.dollfusi	TCC.T.T	.ct	TG.AT	т	AT	AACCA	
O.oshimai	ACCA.T	cc	c.cc	T.TC.T	TATCCT	CAAA.CA	.AC.
C.indicus	A.CATA	.ACTTTT	T	TAC.T	.ATT.C	.CT.AAAT	A
0.vulgaris	ACCA.T	cc	c.cc	T.TC.T	TATCCT	CAAA.CA	.AC.
0.ocellatus	A.CTCC	C.AT.CT	тт	TT	TGTTTTCC	CAT.T	
0.fusiformis	AC.TCT.T	CA.T.A.TTT	T.TACCCC	TT.TAT	TA.TTCT	AA.C.	.AT
C.taiwanicus	A.CATA	.ACTTTT	T	TAC.T	.ATT.C	.CT.AAAT	.A
	2222222222	2222222222	33333333333	3333333333	33333333333	3333344444	144444
	6666777778	8888899999	0000011122	3334444555	6666677788	8999900011	122222
	2457036792	3568912347	0123657814	3692568478	0368925814	5036902514	703679
0.striolatus	TATACTATAT	TACCCATCCC	TGCAATCTTA	AACTTCAACT	AATTAAATTC	CATCCCACAA	ATTAA
0.variabilis	CTCTT.CC.C	TAT.AT	ATTATAA.	с.тттс	CT.CA.	A.TT	T
0.tankahkeei	стс	TATTA.	AAT.TAT.AT	T.CCT.	.CGCA	.TCTATCT	.C.CCT
0.ovulum	CA	TATT.	A	.G.AAT.	.ст	TTAT.T	ccc.
O.aegina							
0.dollfusi	A	T.T	TC	.T.C.TT.	.сс.т	TT.CT	
O.oshimai	CCT.TCT.	CTTAT	ATC.A.C	CT.GT.T.	.TTT	.CA.TT	CCT
C.indicus	CTCGC	стт.	AATTAAT	.TTATTC	.CC.G.TA	TT.TTTT	.C.CCT
O.vulgaris	CCT.TCT.	CTTAT	ATC.A.C	CT.GT.T.	.TTT	.CA.TT	CCT
0.ocellatus	TCTCT.	TA.TA	G.AT	TCTATT.	.тссст	ттт.т	T
O.fusiformis	CTCTT.CC.C	TAT.AT	ATTATAA.	C.TTTC	CT.CA.	A.CT	T
C.taiwanicus	CTCGC	стт.	AATTAAT	.TTATTC	.CC.G.TA	TT.TTTT	.C.CCT
	444444444	444444444	5555555555	5555555555	5555555555	5555556666	66666
	3344455555	6788899999	0011122233	3333444445	5566677777	8889990000	11122
	5814703679	8712623589	1736958912	4578013672	5814703679	0581470369	25814
0.striolatus	ACCCCTCCAC	GTTTAATAAT	AATATAAGCC	ATACTTAACA	AAATATTACC	TTCACCTAAC	TTTAA
0.variabilis	TTA.TT	AACA.TCTT.	.TATA	TCTTT	TTTATTA	T.TT.TT.	.CA
0.tankahkeei	.TT.TAT	AACCT.	TCT.ATT	.CTC.TT.	TTTATT	TCTT.C.T	CCA
0.ovulum	CTTTTTGA	AAC.G.C.T.	TCT	c.c	.т	ссст	.CAGG
O.aegina	T				G		
0.dollfusi	C.T.T.TT.T	ACC	TT.TT	.C.TAT.	.TTA	.A.TT.CC.T	т.
O.oshimai	CTTATT.T	ACT.C	C.ATT	.C.TACT.	.CTAT	TT.T.T	т.
C.indicus	.TT.TAAT.T	AACTTC	.T.TATTT	ATT.	CTCAAT	TTT.T	
0.vulgaris	CTTATT.T	ACT.C	C.ATT	.C.TACT.	.CTAT	TT.T.T	т.
0.ocellatus	TTTTT.TT.T	тасст.	CT.TT	ст	.ттст	T.T.C.TT	.CA
O.fusiformis	TTA.TT	AACA.TCTT.	GTATA	TCTTT	TTTATTA	T.TT.TT.	.CA
C.taiwanicus	.TT.TAAT.T	AACTTC	.T.TATTT	ATT.	CTCAAT	сттт.т	

Figure 3. Alignment of partial DNA sequences of the mitochondrial gene, COI of 11 Octopodidae species, with *Cistopus taiwanicus* used as outgroups.

Table 3. Pairwise genetic distances (Kimura 2-parameter)	among 11 Octopodidae species based on COI
sequences, with <i>Cistopus taiwanicus</i> used as outgroup.	

	O. stri	O. vari	O. tank	O. ovul	O. aegi	O. doll	O. oshi	C. indi	O. vulg	O. ocel	O. fusi	C. taiw
O. stri												
O. vari	0.188											
O. tank	0.185	0.176										
O. ovul	0.128	0.193	0.173									
O. aegi	0.006	0.191	0.185	0.124								
O. doll	0.109	0.195	0.180	0.145	0.109							
O. oshi	0.156	0.197	0.171	0.189	0.152	0.149						
C. indi	0.170	0.197	0.123	0.181	0.170	0.168	0.167					
O. vulg	0.156	0.197	0.171	0.189	0.152	0.149	0.000	0.167				
O. ocel	0.149	0.189	0.185	0.143	0.145	0.147	0.179	0.187	0.179			
O. fusi	0.193	0.005	0.180	0.197	0.195	0.197	0.203	0.201	0.203	0.193		
C. taiw	0.172	0.199	0.124	0.183	0.172	0.170	0.169	0.002	0.169	0.189	0.203	

For abbreviations, see legend to Table 2.

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Figure 4. Neighbor-joining (NJ), maximum parsimony (MP) and unweighted pair-group method with arithmetic average (UPGMA) phylogenetic tree of Octopodidae species inferred from DNA sequences of mitochondrial gene COI.

DISCUSSION

As it stands, *Octopus* is the largest genus in the family Octopodidae of the cephalopod species in China's coastal waters (Dong, 1988). However, the monophyly of this genus was also not supported by our results. In all phylogenetic trees based on COI and 16SrRNA gene sequence analyses, four major clusters were obtained and supported by high bootstrap values. This may suggest that at least four "species groups" [as Robson (1929) put it] could be recognized in octopus species from the coastal waters of China. For the "*O. aegina* species group"

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(Group A), Gleadall (2002) suggested a new genus name and argued that the "O. aegina species group" represented a distinct genus, with Amphioctopus being the senior name. In 2005, Huffard and Hochberg formally resurrected the genus name Amphioctopus for the Octopus aegina species complex. Norman and Hochberg (2005) supported this notion and actually removed several species, such as O. aegina, O. burryi, O. carolinensis, O. exannulatus, O. fangsiao, O. granulatus, O. kagoshimensis, O. marginatus, O. membranaceus, O. mototi, O. neglectus, O. ovulum, O. pulcher, O. rex, O. robsoni, O. siamensis, and O. varunae, that were traditionally involved in the genus Octopus to Amphioctopus. According to Norman and Hochberg (2005), O. ocellatus, O. striolatus, and O. dollfusi should be regarded as synonyms of O. fangsiao, O. marginatus and O. aegina, respectively, and should be removed to genus Amphioctopus, together with O. ovulum. Therefore, it is reasonable that the five species formed one cluster in our analysis. However, our results did not support the notion that O. dollfusi should be defined as a synonym of O. aegina, because a considerable sequence divergence was detected between these two species, as shown in Figures 1 and 3. High bootstrap values (90 to 100%) also supported that O. dollfusi be separated from O. aegina in our plotted trees. Considerable morphological characteristics were also noted by Dong (1988) between these two species, O. dollfusi and O. aegina. Therefore, the validity of the species name O. dollfusi should be called into question. For the "O. vulgaris species group" (Group B), we had only two species involved in our study. O. vulgaris is regarded as the type species of genus Octopus (Norman and Hochberg, 2005); therefore, the "O. vulgaris species group" should be placed under genus Octopus. O. oshimai is conventionally included in genus Octopus, but was designated as "unplaced" by Norman and Hochberg (2005). The results of our study supported that O. oshimai be kept in genus Octopus, since it is genetically closely related to the type species of genus Octopus, O. vulgaris. The pairwise genetic distance values (Kimura 2-parameter) based on 16S rRNA and COI between O. vulgaris and O. oshimai were only 0.023 and 0, respectively, as shown in Tables 2 and 3.

With regard to the species *O. variabilis* and *O. fusiformis*, both are long-arm type species (Dong, 1988), which differed from all other species in the study, and our results could only give a less solid conclusion. In all our NJ, UPGMA, and MP trees, the two species formed one cluster, which differed from the other clusters with high bootstrap values (95 to 100%). Norman and Hochberg (2005) designated both of them as genus "unplaced". Our results also support this designation, until more a solid conclusion can be drawn. However, the fact that *O. tankahkeei* clustered with *Cistopus* species in all the plotted trees was quite interesting. *O. tankahkeei* is a new species of cephalopods found in the Southern China Sea (Zhu et al., 2005). It was designated to the genus *Octopus*, family Octopodidae, according to its morphology and some other traits (Zhu et al., 2005; Li et al., 2010). No data of genetics are available for this species, to date. Lin XZ (personal communication), at the Third Institute of Oceanography, State Oceanic Administration, questioned the validity of this species and suspected that *O. tankahkeei* was only the juvenile form of *C. indicus*. The results of our study seem to support this statement, since it stably formed one cluster with *C. taiwanicus* and *C. indicus* in all the trees plotted, but more evidence should be provided before the conclusion can be made.

In conclusion, the data presented here suggest that *Octopus* is not monophyletic and that the systematics of the family Octopodidae in China's coastal waters requires major revision. The present phylogeny revealed strong relationships among closely related species and some information on divergences at the species group level; however, the deeper relationships among the Octopodidae remain unresolved. In particular, the taxonomic status of *O. variabilis*

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and *O. fusiformis* in Octopodidae were not stably placed. However, our results generally are congruent with the taxonomic revision of Octopodidae, based on morphological characters as reported by Norman and Hochberg (2005). Partial sequence information of both the mitochondrial genes 16S rRNA and COI again proved to be useful in the identification and resolution of ambiguous taxa, and may serve as an important tool for future taxonomic revision of Octopodidae species in the coastal waters of China.

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