THE RAFFLES BULLETIN OF ZOOLOGY 2005 **53**(1): 157-164

PATTERN OF GENETIC VARIATION OF BOTTLENOSE DOLPHINS IN CHINESE WATERS

Guang Yang, Guoqing Ji, Wenhua Ren, Kaiya Zhou
Jiangsu Key Laboratory for Bioresource Technology, College of Life Sciences, Nanjing Normal University, Nanjing 210097, China
and
Institute of Genetic Resources, College of Life Sciences, Nanjing Normal University, Nanjing 210097, China

Fuwen Wei
Institute of Zoology, Chinese Academy of Science, Beijing 100080, China

ABSTRACT. – Although two species of bottlenose dolphins (*Tursiops* spp.) in Chinese waters have been recognized, their pattern of genetic variation is unclear at present. In the present study, 424-bp of the mitochondrial control region from 30 bottlenose dolphins collected in Chinese waters were sequenced. Forty-seven variable sites were identified from these sequences, and 9 and 11 haplotypes were defined respectively for *truncatus*- and *aduncus*-type, two morphotypes previously recognized for bottlenose dolphins in Chinese waters. These haplotypes were combined with published mitochondrial control region sequences of other bottlenose dolphins from Chinese, Indonesian, Australian, and Atlantic waters etc. Sequence variability comparison and phylogenetic reconstruction using neighbor joining (NJ) and maximum likelihood (ML) algorithms exclusively supported the separation of haplotypes into two monophyletic clades, each of which corresponds to *truncatus*- and *aduncus*-type. This provided strong support to separate the two morphotypes into respective species, *T. truncatus* and *T. aduncus*. No shared haplotype was found between *Tursiops* species, and four fixed diagnostic site differences between them were identified. All samples from the northern part of Chinese waters, i. e. the Yellow Sea and the northern East China Sea, were genetically identified as *T. truncatus* exclusively, whereas most samples from the southern part of Chinese Waters, i.e. the southern part of the East China Sea, the Taiwan Straits, the South Chinese Sea, and the Gulf of Beibuwan, were identified as *T. aduncus*, but with both species overlapping in the Taiwan Strait. This genetic pattern was congruent with the distribution pattern of *Tursiops* as previously revealed by morphological and ecological evidences. Although the distribution of the two species overlapped in the Taiwan Strait (and maybe adjacent waters), there was no evidence of genetic interchange between them, indicating reproductive isolation. The present study strongly argues for treating the two bottlenose dolphin morphotypes / species as separate management units in making up conservation measures, but further study on the intraspecific structure using multiple molecular marker is also urgent for effective conservation.

KEY WORDS. **–** Bottlenose dolphin, *Tursiops*, Chinese waters, mitochondrial control region, variability, conservation.

INTRODUCTION

Bottlenose dolphins (genus *Tursiops*) are cosmopolitan in tropical and temperate waters, including the Pacific, Indian and Atlantic Oceans (Rice 1998). There are many differences in the morphology of *Tursiops* from various waters (Ross, 1977; Walker, 1981; Duffield et al., 1983; Gao et al., 1995), which has caused a lot of confusion in the taxonomic classification of this genus for a long period of time. For example, for South African waters, Ross (1977) concluded that two species, *T. truncatus* and *T. aduncus*, existed, but in a later study, Ross and Cockcroft (1990) denied this result and concluded that the two forms of bottlenose dolphins in South Africa should be treated as a single species (i.e. *T. truncatus*), and that Australian bottlenose dolphins should also be assigned to this species.

Bottlenose dolphins in Chinese waters were identified as two morphotypes: *truncatus*-type and *aduncus*-type initially (Yang, 1976; Zhou & Qian, 1985). *Aduncus*-type, which was mainly distributed in the South China Sea and the eastern border of the East China Sea, has longitudinal elongated dark spots on the ventral surface and its body size is smaller than that of *truncatus*-type mainly found in the Yellow, Bohai and East China Sea (Zhou & Qian, 1985). The two morphotypes were osteologically differentiated and were separated into two species by Zhou (1987). Nevertheless, in a later study using stepwise discriminant analysis with a

group of external and skull measurements, Gao et al. (1995) were unable to find sufficient evidences to support Zhou (1987)'s separation.

However, recent analyses of external morphology, osteology, and mitochondrial (mt) control region variability of sympatric *truncatus*- and *aduncus*-types in Chinese waters unambiguously showed that two distinct species for *Tursiops*, i.e. *T. truncatus* and *T. aduncus*, did exist (Wang et al. 1999, 2000a, b). The two species have significant morphological differences (rostrum length as an absolute measure and as a proportion of total body length or snout-to-eye length, some cranial characters, and the total number of vertebrae had non-overlapping distributions) (Wang et al. 2000a, b). No shared haplotypes were found and seven diagnostic sites were identified for the two morphotypes. The average mitochondrial sequence divergence (4.4%) between the two morphotypes was four times greater than among *Delphinus* species (1.09%, Rosel et al. 1994) (Wang et al. 1999). Wang et al. (1999)'s discoveries influenced Möller and Beheregaray (2001) to unambiguously identify two populations of bottlenose dolphins in the southern Australian waters (i. e. Port Stephens and Jervis Bay) as *T. aduncus*. However, it is worth pointing out that Chinese dolphins examined by Wang et al. (1999, 2000a, b) were mainly collected from the Taiwan Strait and the adjacent Indonesian waters, and thus are not representative of the whole of Chinese waters. The phylogenetic status of bottlenose dolphins in other parts of Chinese waters is still unclear, and the genetic pattern of *Tursiops* species in Chinese waters is still to be investigated to confirm the previous distributional pattern inferred from morphological data.

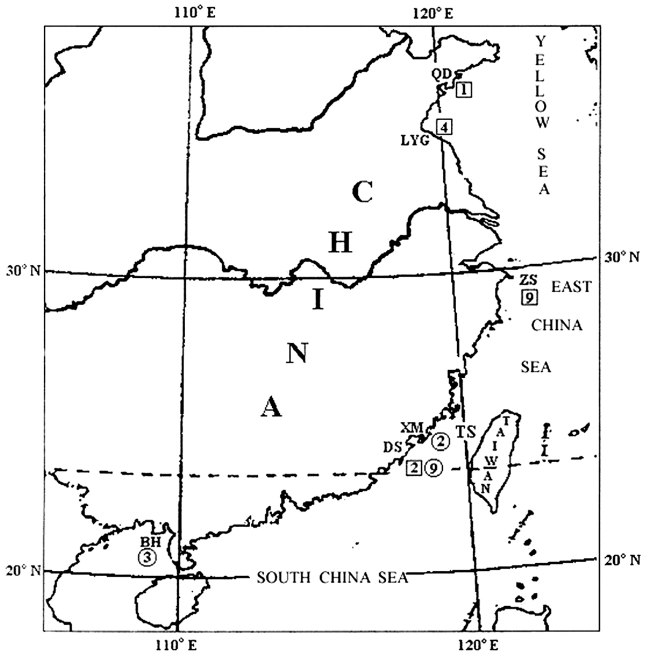

Fig. 1. Locations where bottlenose dolphins were sampled. Numerals within the square and circle symbols represent the sample size for *truncatus*-type and *aduncus*-type, respectively. QD, Qingdao, LYG, Lianyungang, ZS, Zhoushan, XM, Xiamen, DS, Dongshan, TS, Taiwan Strait, BH, Beihai

In the present study, a portion of the mitochondrial control region of 30 bottlenose dolphins collected from the Yellow Sea, East China Sea, Taiwan Strait, and Gulf of Beibuwan was sequenced and combined with previously published homologous sequences of other bottlenose dolphins to address the above mentioned questions.

MATERIALS AND METHODS

Sample collection and DNA extraction. -In total, 30 samples, 26 muscles and 4 skeletons, of bottlenose dolphins were collected from the Yellow Sea, East China Sea, Taiwan Strait, and Gulf of Beibuwan (Figure 1 and Table 1). All these dolphins were incidentally killed in China's coastal fisheries. Assignment of specimens to different morphotypes of *Tursiops* was on the basis of external and/or osteological diagnostic features described by Zhou (1987) and Wang et al. (2000 b), e.g. *aduncus*-type has ventral spotting with age, the rostrum length of aduncus-type is proportionately longer than the *truncatus*-type; and the *aduncus*-type has 59-62 vertebrae, while *truncatus*-type has 62-67 vertebrae. According to these diagnostic features, 16 individuals of *truncatus*-type and 14 individuals of *aduncus*-type were identified before the molecular analysis.

Total genomic DNA from muscle tissues was extracted following the standard proteinase K digestion and phenol-chloroform extraction procedures as described in Sambrook et al. (1989). For skeletons, 1~2 g samples was carefully washed with 2% H_2O_2, ground into powder by a coppery mortar, and then soaked in 0.5mol/L EDTA (pH 8.0), before proceeding to standard DNA extraction procedures as for muscle samples. Negative controls were conducted to prevent possible contamination.

DNA amplification, sequencing and data analysis. An approximately 550-bp segment of the mtDNA including the highly variable portion in the 5' end of the control region was amplified by the polymerase chain reaction (PCR) using the primer Strobeck (5'-TAATATACTGGTCTTGTAA ACC-3') which was modified from the primer L15926 of Kocher et al. (1989, see Murray et al. 1995) and the primer H00034 (5'-TACCAAATGTATGAAACCTCAG-3') of Rosel et al. (1994). All amplification reactions were performed on a PE2400 (Applied Biosystem Inc.) or PTC200 (MJ Research) thermocycler in a total volume of 100 μl containing 10-100 ng of extracted genomic DNA template, 10 mM of Tris-HCl (pH8.3), 50 mM KCl, 2.5 mM MgCl2, 150 μM of each dNTP, 0.01% gelatin, 3 units of Taq DNA polymerase (Promega), and 0.3 μM of each primer. The temperature profile for the amplifications was 35 cycles at 95°C for 30s, 55°C for 30s and 72°C for 50s followed by an 8-min extension at 72°C. For skeletal samples, the amplified products were purified using WizardTM PCR Preps DNA Purification (Promega), and then used as the template DNA for a second PCR amplification with the primer of Strobeck paired with KZ2 (5'-CCGAAGTAAGA ACCAGATG-3') following the same reaction conditions and

Table 1. Sequences of mtDNA control region of *truncatus*- and *aduncus*-type from different localities used for comparison with bottlenose dolphins from Chinese waters.

Haplotype codes	Genbank accession number	Locality	Reference
A1-	AF056233	Taiwan, Indo-Pacific	Wang et al. (1999)
A2	AF056234	China, Indo-Pacific	Wang et al. (1999)
A3	AF056235	Taiwan, Indo-Pacific	Wang et al. (1999)
A4	AF056236	Taiwan, Indo-Pacific	Wang et al. (1999)
A5	AF056237	Indonesia, Indo-Pacific	Wang et al. (1999)
A6	AF056238	Indonesia, Indo-Pacific	Wang et al. (1999)
A7-	AF056239	Taiwan, Indo-Pacific	Wang et al. (1999)
A8-	AF056240	Taiwan, Indo-Pacific	Wang et al. (1999)
A9	AF056241	Taiwan, Indo-Pacific	Wang et al. (1999)
A10	AF056242	Taiwan, Indo-Pacific	Wang et al. (1999)
A11	AF056243	Taiwan, Indo-Pacific	Wang et al. (1999)
T1	AF056219	Brazil, SW Atlantic	Wang et al. (1999)
T2-	AF056220	Hong Kong and Taiwan, Indo-Pacific	Wang et al. (1999)
T3	AF056221	Hong Kong, Indo-Pacific	Wang et al. (1999)
T4	AF056222	Mauritania, NE Atlantic	Wang et al. (1999)
T5	AF056223	Taiwan, Indo-Pacific	Wang et al. (1999)
T6	AF056224	Taiwan, Indo-Pacific	Wang et al. (1999)
T7	AF056225	Taiwan, Indo-Pacific	Wang et al. (1999)
T8	AF056226	Taiwan, Indo-Pacific	Wang et al. (1999)
T9	AF056227	Taiwan, Indo-Pacific	Wang et al. (1999)
T10-	AF056228	Taiwan, Indo-Pacific	Wang et al. (1999)
T11	AF056229	Taiwan, Indo-Pacific	Wang et al. (1999)
T12	AF056230	Taiwan, Indo-Pacific	Wang et al. (1999)
T13-	AF056231	Taiwan, Indo-Pacific	Wang et al. (1999)
T14	AF056232	Taiwan, Indo-Pacific	Wang et al. (1999)
T1at	AF155160	Atlantic	Parsons et al. (Unpub.)
T2at	AF155161	Atlantic	Parsons et al. (Unpub.)
T3at	AF155162	Atlantic	Parsons et al. (Unpub.)
T4at	AF268357	NE Atlantic	Parsons et al. (Unpub.)
T5at	AF378176	NE Bahamas	Parsons et al. (Unpub.)
T6at	AF378177	NE Bahamas	Parsons et al. (Unpub.)
T7at	AF378178	NE Bahamas	Parsons et al. (Unpub.)
T8at	U20910	USA, NW Atlantic	Siemann (1994)
T9at	U20911	USA, NW Atlantic	Siemann (1994)
T10at	U20912	USA, NW Atlantic	Siemann (1994)
T11at	U20913	USA, NW Atlantic	Siemann (1994)
T12at	U20914	USA, NW Atlantic	Siemann (1994)
T13at	U20915	USA, NW Atlantic	Siemann (1994)
T14at	U20916	USA, NW Atlantic	Siemann (1994)
T15at	U20917	USA, NW Atlantic	Siemann (1994)
T16at	U20919	USA, NW Atlantic	Siemann (1994)
T17at	U20920	USA, NW Atlantic	Siemann (1994)
A1au	AF287951	Australia, SW Pacific	Moller et al. (2001)
A2au	AF287952	Australia, SW Pacific	Moller et al. (2001)
A3au	AF287953	Australia, SW Pacific	Moller et al. (2001)
A4au	AF287954	Australia, SW Pacific	Moller et al. (2001)
A1cn	AF355576	Xiamen, Taiwan Strait	This study
A2cn	AF355577	Dongshan, Taiwan Strait	This study
A3cn	AF355578	Dongshan, Taiwan Strait	This study
A4cn	AF355579	Dongshan, Taiwan Strait	This study
A5cn	AF355580	Dongshan, Taiwan Strait	This study
A6cn	AF355581	Dongshan, Taiwan Strait	This study
A7cn	AF459520	Gulf of Beibuwan	This study
T1cn	AF355582	Zhoushan, East China Sea	This study
T2cn	AF355583	Zhoushan, East China Sea	This study
T3cn	AF355584	Zhoushan, East China Sea	This study
T4cn	AF355585	Zhoushan, East China Sea	This study
T5cn	AF355586	Zhoushan, East China Sea Lianyungang, Yellow Sea	This study
T6cn	AF355587	Qingdao, Yellow Sea	This study

-: haplotypes from references but also identified in the present study. The capitalized A and T in the haplotype codes represents *aduncus*- and *truncatus*-type, respectively. The haplotype codes representing Atlantic and Australia are at and au, respectively. Haplotypes ending with cn in the code were all identified in the present study.

temperature profile as used in the first amplification. Primer KZ2 was modified from primer H16498 of Rosel et al. (1994). Approximately 450-bp amplified fragments from the first amplification for musle samples and the second amplification for skeleton samples were isolated by gel electrophoresis, then excised and eluted in 30 μL of distilled-deionized water for purification. Purified products were directly sequenced using the primer Strobeck. Cycle sequencing was performed with 150-300 ng of PCR product using the PRISMTM BigDye Terminator Ready Reaction Kit (Applied Biosystems Inc.) for 30 cycles at 96°C for 0.5 min, 50°C for 0.5 min, and 60°C for 4 min. Sequencing reactions were performed with a PTC-200 thermocycler (MJ Research Inc.) and the 310 DNA automated Sequencer (Applied Biosystems Inc.). DNA sequences were determined by the ABI PRISMTM Sequencing Analysis software version 3.3 and the ABI 310 Data Collection program version 1.0.4. Five samples of each morphotype were randomly selected to sequence using primer KZ2 for sequence verification.

The resultant sequences were aligned with other sequences of bottlenose dolphins from the Taiwan Straits and Indonesian (Wang et al., 1999), Northwestern Atlantic (Siemann, 1994), and Australian (Möller & Beheregaray, 2001) waters etc (Table 1), by using computer software Clustal X version 1.8 (Jeanmougin et al., 1998) with default parameters (i.e. Gap opening=10, Gap extension=0.2, Delay divergent sequences=30%, and DNA transition weight=0.5) and corrected by eye. A sequence from GenBank with accession no U20918 (Siemann, 1994) is much more similar to *Delphinus* than to *Tursiops* species when we did online BLAST search or multi-sequence aligning. For this reason, we regarded the species identity as doubtful and conservatively excluded it from the present analysis. Percent sequence divengence with Jukes-Cantor model among all haplotypes of *truncatus*- and *aduncus*-type were calculated using softwares MEGA (Kumar et al., 2001) and DnaSP (Rozas & Rozas, 1999). The net sequence divergence (D_A) between two morphotypes was calculated based on the haplotypes from Chinese waters using software DnaSP (Rozas & Rozas, 1999). The nucleotide diversity, i.e. sequence diversity within each population and sequence divergence between populations, and haplotype diversity (Nei, 1987) for each population were also computed for dolphins in Chinese and Indonesian waters, where systematic population samplings were available. Neutrality test of variable sites was performed through calculating F* and D* values (Fu & Li, 1993) by the computer software DnaSP (Rozas & Rozas, 1999). Phylogenetic relationships among all available haplotypes were inferred from a median-joining (MJ) network, and two phylogenetic trees reconstructed by maximum likelihood (ML) and neighbor-joining (NJ) algorithms, respectively. The median-joining network approach, containing all most parsimonious trees and displaying the full information content of the sequence data, has become an important tool in intraspecific phylogeny using mitochondrial sequences (Bandelt et al. 1999). The ML and NJ analyses included all the available *Tursiops* haplotypes and some relevant sequences of striped dolphin *Stenella coeruleualba* (Yang et al., 2002, accession nos AY046547-9) and shorted-beaked common dolphin *Delphinus delphis* (accession nos AY422202-4). ML analysis with short-finned pilot whale *Globicephala macrorhynchus* (GenBank accession no AJ226120 and AY168599) as outgroup was performed by using the program DAMBE (4.0.41) (Xia, 2000). Three models (HKY85, F84, and TN93) were compared and the HKY85 model was found to be superior to F84 (χ^2=88, P<0.0001) and TN93 (χ^2=107, P<0.0001) in the present sequence data when fixing the most-parsimonious tree as the tree for test. The HKY85 model was then used and transitions were given the default weighting of 1: 2 relative to transversions. NJ analysis was performed by MEGA version 2.0 (Kumar et al., 2001) with a distance matrix generated according to Tamura-Nei Gamma distance with the options: a=0.99; pairwise deletion. The confidence of each branch was generated through bootstrap resampling with 100 and 500 times replications for ML and NJ methods, respectively.

RESULTS

Of the 424 bp of mitochondrial control region sequences examined for 30 *Tursiops* in the present study (GenBank submission nos AF355576~AF355587), 47 sites were variable, including 39 transitions, five transversions, two insertions/deletions (indels), and one site exhibiting both a transition and a transversion (site 264). These variable sites defined 20 haplotypes, 9 for *truncatus*-type and 11 for *aduncus*-type. These haplotypes were aligned with sequences downloaded from GenBank (Fig. 2). In the alignment, the haplotypes were reduced to 386 bp in length because 14 and 24 bp at the 5' and 3' end of our sequences, respectively, were not available for those downloaded sequences. Of the 62 variable sites identified in the aligned region, 51 were transitions, five were transversions, three exhibited both, one was an indel or a transition, and two were indels. A haplotype identified in the present study was dropped from the alignment and the subsequently analyses because it is identical to haplotype A1 in Wang et al. (1999) only with a transitional difference outside the aligned region. No shared haplotypes were found between the two morphotypes, and 4 diagnostic sites were found between them. That is to say, of the seven diagnostic sites found by Wang et al. (1999), three sites (6, 97, and 261) became non-diagnostic, when new sequences were added in the present analysis. Site 6 was also likely diagnostic, but need to be confirmed by adding the 5' end mitochondrial control region sequences for Australian samples.

The median-joining networking (Fig. 3) and phylogenetic reconstructions based on different methods (i. e. NJ and ML) (Fig. 4, ML tree was not shown here because its topology is quite similar to that of NJ tree) all separated the *Tursiops* haplotypes into two clades, each of which contained only haplotypes of *truncatus*- or *aduncus*-type. All haplotypes of *truncatus*-type identified in the present study unambiguously clustered with haplotypes for other *truncatus*-type bottlenose dolphins in Chinese and Atlantic waters, whereas all

Table 2. Sequence diversity within (along diagonal) and divergence between (above diagonal) morphotypes, and haplotype diversity (right column) for each morphotype of *Tursiops* in Chinese waters.

	truncatus-type	*aduncus*-type	Haplotype diversity
truncatus-type	(1.67±0.22)%	(5.58±0.26)%	0.91±0.03
aduncus-type		(1.47±0.08)%	0.93±0.03

haplotypes of *aduncus*-type identified in the present study clustered with haplotypes for other *aduncus*-type bottlenose dolphins in Chinese, Indonesian, and Australian waters. In other words, *truncatus* haplotypes and *aduncus* haplotypes constituted respective monophyletic clades. Further, the ML and NJ reconstructions both revealed that there was no direct affinity between *truncatus*- and *aduncus*-type. The *adundus*-type and *Delphinus* + *Stenella* constituted a monophyletic group which had a sister relationship with *truncatus*-type.

Sequence divergence between *truncatus*-type and *aduncus*-type ranged from 3.64% to 6.81%, with an average of 5.17%, when all haplotypes available were examined. The between-haplotype sequence divergence for *truncatus*-type ranged from 0.27% to 5.18%, and averaged 2.88%, whereas for *aduncus*-type ranged from 0.27 % to 3.00%, and averaged 1.51%. The nucleotide diversity p and haplotype diversity for bottlenose dolphins in Chinese waters were presented in Table 2. Both morphotypes in Chinese waters had comparable

	Nucleotide positions								Individual numbers					
	1 1111111122	3455677899	9001333558	1112222222	2222222222	2222233333	333333							
haplotypes	1234567890	1234567869	0807118416	7151467580	1120591251	2691456790	1102713126	047890	QD	LYG	ZS	XM	DS	BH
T1at	GTACAGTTAC	CATAACATCC	GTGA-TTTAT	AGATTCATGA	TCTACATCGT	CCATTTTCTC	TCATTTAGAC	CCTCTC						
T2at	....	....	....-.....	....	....	.....T..	........AG.	......						
T3at	....	....	....-.....	....	....	.....C....	..G....A..	......						
T4at	....	..C....C..	....-....C	G..-.T....	.......T..	..T.C.CCT.	.......A..	....CT						
T5at	....	..C....C..	....-....C	G..-.T....	.......T..	AT.C.CCT..	.......A..	....CT						
T6at	....	..C.....	T...-.....	G..-......	.......T..	A..C.C.TC.	.TG....A..	.T..CT						
T7at	....	..C.....	T...-...G.	G..-......	...C...CT.	A..-.C.TC.	..G...GA..	.T..CT						
T8at	....	..C.....	C...-..C..	G..-......	C.C....T..	AT.C.C.T..	.TG....A..	.T..CT						
T9at	....	..C....C..	T...-.....	G..-......	C.C....T..	A..C.C.TC.	..G....A..	....CT						
T10at	....	..C....C..	....-..C..	G..-......	C.C....T..	...C.C.TC.	..G....A..	.T..CT						
T11at	....	..C.....T	A...-.....	G..-......	...C......	A..C.CCT..	.TG....A..	....CT						
T12at	....	..C....C..	T...-....C	G..-.T....	...C......	.T.C.CCTC.	.......A..	...TCT						
T13at	....	..C....C..	....-....C	G..-.T....	...C......	...C.CCTC.	.......A..	....CT						
T14at	....	..C.....	....-..C..	G..-......	....	A..C..CTC.	..G....A..	.TC.AT						
T15at	....	....	....-.....	.........G	....	....	....	......						
T16at	....	....	....-.....	....	....	....	........AG.	......						
T17at	....	..C.....	....C.....	...G..T....	....	.......CT.	.......A..	.T....						
T1cn	....	..C.....	....-.....	G..-.T....	....	T..C......	.......AG.	....C.			1			
T2cn	....	..C....C.T	....-....C	G..-.T....	...C......	...C.CCTCT	.......A..	.T..TCT			1			
T3cn	....	..C....C..	....-....C	G..-.T....	...C....T.	...C.CCT..	.T.....A..	...TCT		1	1			
T4cn	....	..C....C..	....-C...C	G..-.T....	...C....T.	...C.CCT..	.T.....A..	...TCT	1					
T5cn	....	..C....C..	....-....C	G..-.TG...	...C....T.	...C.CCTC.	....C..A..	...TCT			1			
T6cn	.....G..	..C.....	...T-.....	GA.-.T...T	...C..T.T.C	...CCC.T..	.......A..	.....T			1			
T1	....	..C.....	....-.....	G..-......	...C......	A..C..CTC.	..G....A..	.TC.CT						
T2	....	..C....C..	....-....C	G..-.T....	.......T..	...C.CCTC.	.......A..	....CT			1			
T3	....	..C.....	....-.....	GA.-.T....	...C....T.C	...CCC.T..	.......A..	.....T						
T4	....	..C.....	....-..C..	G..-......	....	A..C..CTC.	..G....A..	.TCTAT						
T5	....	..C....C..	....-....C	G..-.TG...	...C....TA.	...C.CCTC.	....C..A..	...TCT						
T6	....	..C.....	....-.....	G..-.T....	....	T..C.....T	.......AG.	....C.						
T7	....	..C....C.T	....-....C	G..-.T....	....	...C.CCTCT	.......A..	...TCT						
T8	....	..C....CGT	.C..-....C	G..-.T....	...C......	...C.CCTCT	.......A..	...TCT						
T9	....	..C....C..	....-....C	G..-.TG...	...C....T.	...C.CCTC.	....CC.A..	...TCT						
T10	....	..C....C.T	....-....C	G..-.T....	...C......	...C.CCTCT	.......A..	...TCT		3	3		1	
T11	....	..C....CG.	....-....C	G..-.T....	...C....TA.	...C.CCT..	.......A..	...TCT						
T12	....	..C.....G.	....-.....	GA.-.T....	...C....T.C	...CCC.T..	.......A..	.....T						
T13	....	..C....C..	....-....C	G..-.T....	.......T..	....C..CTC.	.......A..	....CT					1	
T14	....	..C....CG.	....-....C	G..-.T....	.......T..	...C.CCTC.	.......A..	....CT						
A1au	??????????	??????..T	..A.-.CC..	.A.-.T.C..	..CTT.....	G.T-....C.	C.GC...A..	.T..CT						
A2au	??????????	??????..T	..A.-.CC..	.A.-.T....	.TCTT.....	G.T-....C.	C.GC...A..	.T..CT						
A3au	??????????	??????..T	....-.CC..	.A.-.T.C..	.TCTT.....	G.T-....C.	C..C...A..	.T..CT						
A4au	??????????	??????..T	....-.CC..	.A.-.T.C..	..CTT.....	G.T-....C.	C.....A..	.T..CT						
A1cn	.....A....	..C.....	T...-.CC..	.A.-.T.C..	.TCTT.....	G.T-....C.	C.G....A..	.TC.CT					1	
A2cn	.....A....	..C.....	T...-.CC..	.A.-.T.C..	..CTT.....	G.T-....C.	C..C...A..	.T...T					1	
A3cn	.....A....	..C.....	T...-.CC..	.A.-.T.C..	..CTT.....	G.T-.....	C.....A..	.T...T					1	
A4cn	.....A....	..C.....	T...-.CC..	.A.-.T.C..	..CT...T..	G.T-.....	C.....A.T	.T.TCT					1	
A5cn	.....A....	..C.....	T...-.CC..	.A.-.T.C..	..CT.....	G.T-.....	C.....A..	.T..CT					1	
A6cn	.....A....	..C.....	T...-.CC..	....-.T....	.TCTT.....	G.T-....C.	C.G....A.T	.TC.C.						1
A7cn	.....A....	..C.....	T...-.CC..	....-.T....	.TCTT.....	G.T-....C.	C.G....A..	.T..C.				1		
A1	.....A....	..C.....	T...-.CC..	....-.T....	.TCTT.....	G.T-....C.	C.G....A..	.TC.C.				1	1	2
A2	.....A....	..C.....	T...-.CC..	.A.-.T.C..	..CTT.....	G.T-.....	C..C...A..	.T..CT						
A3	.....A....	..C.....	T...-.CC..	.A.-CT....	..CTT.....	G.T-....C.	...C...A..	.T..CT						
A4	.....A....	..C.....	T...-.CC..	....-.T....	.TCTT.....	G.T-....C.	C.....A..	.TC.C.						
A5	.....A....	..C.....	T...-.CC.C	.A.-.T.CA.	.TCTT.....	G.T-....C.	C..C...A..	.T..CT						
A6	.....A....	..C.....	T...-.CC..	.A.-.T.C..	.TCTT.....	GTT-....C.	...C...A..	.T..CT						
A7	.....A....	..C.....	T...-.CC..	.A.-.T.C..	..CTT.....	G.T-.....	C..C...A..	.T...T					1	
A8	.....A....	..C.....	T...-.CC..	.A.-.T.C..	..CT...T..	G.T-.....	C.....A..	.T..CT					2	
A9	.....A....	..C.....	T...-.CC..	....-.T....	.TCTT..T..	G.T-....C.	C.G....A..	....C.						
A10	.....A....	..C.....	T...-.CC..	.A.-CT....	..CTT.....	G.T-....C.	C..C...A..	.T..CT						
A11	.....A....	..C.....	T...-.CC..	.A.-.T.C..	..CTT.....	G.T-.....	...C...A..	.T...T						

See figure 1 for abbreviations QD, LYG, ZS, XM, DS, and BH.

Fig. 2. Polymorphic sites from 386-bp of mitochondrial control region of bottlenose dolphins determined in the present study in comparison with those *Tursiops* sequences downloaded from GenBank. Sequence identity to reference sequences of first haplotype (T1au) is indicated by dot, and indels indicated by dash. Individual number of each haplotype identified in the present study is also presented

level of nucleotide and haplotype diversities, which were considerably higher than those of *aduncus*-type populations in Australian waters (π=(0.40±0.03)%, h=0.55±0.04 for Port Stephens population, and π=(0.48±0.06)%, h=0.64±0.07 for Jervis Bay population, all were calculated in the present study based on the haplotype sequences and frequencies available in Möller & Beheregaray, 2001). The between-type divergence, which was 5.58%, was much higher than within-type diversity. The net sequence divergence, the between-type divergence corrected by the within population variation, was estimated to be 3.79% between two *Tursiops* types in Chinese waters. Neutrality test indicated that all mutations detected in the control region sequences were selectively neutral (D*=0.12889, P>0.10; F*=0.58513, P>0.10, non-significant).

DISCUSSION

Although nearly 30 haplotypes were added in the present study compared to that of Wang et al. (1999), and addition of new haplotypes decreased the number of diagnostic sites from seven in Wang et al. (1999) to four, the phylogenetic reconstructions using different methods (i.e. NJ, ML, and MJ) generated almost the same result in that the haplotypes were divided into two monophyletic clades, each of which corresponds to *truncatus*-type or *aduncus*-type. The sequence variability comparison also obtained the same result as Wang et al. (1999) that the between-type divergence was much higher than within-type diversities. The findings provided strong support to the full species status of *T. truncatus* and *T. aduncus* for previously recognized *truncatus*- and *aduncus*-type, respectively (Wang et al., 1999; Zhou, 1987). Further, the finding presented by Leduc et al. (1999) that two *Tursiops* species are not monophyletic was supported by the present study. As shown in Fig. 4, *truncatus*-type has a closer relationship to a monophyletic group including *Stenella* and *Delphinus* than to *aduncus*-type.

In addition, the present study also provided some information

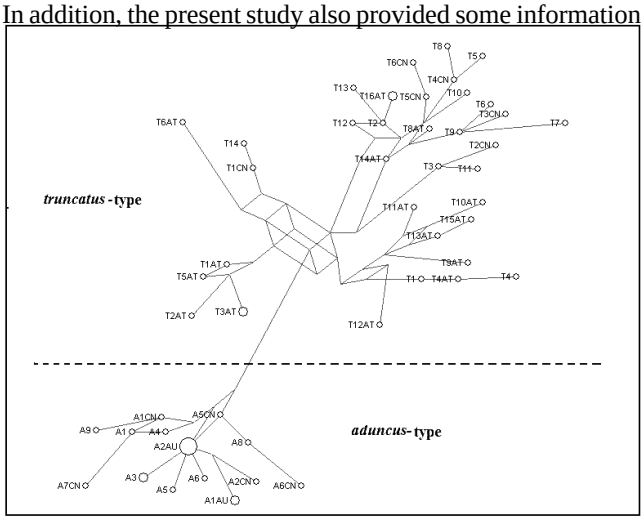

Fig. 3. A median-joining network for *Tursiops* mitochondrial control region haplotypes. Haplotype codes correspond to those in figure 1. The size of the circle is proportional to the number of individuals bearing that haplotype, except for haplotypes from Atlantic waters because of unavailability of any frequency data.

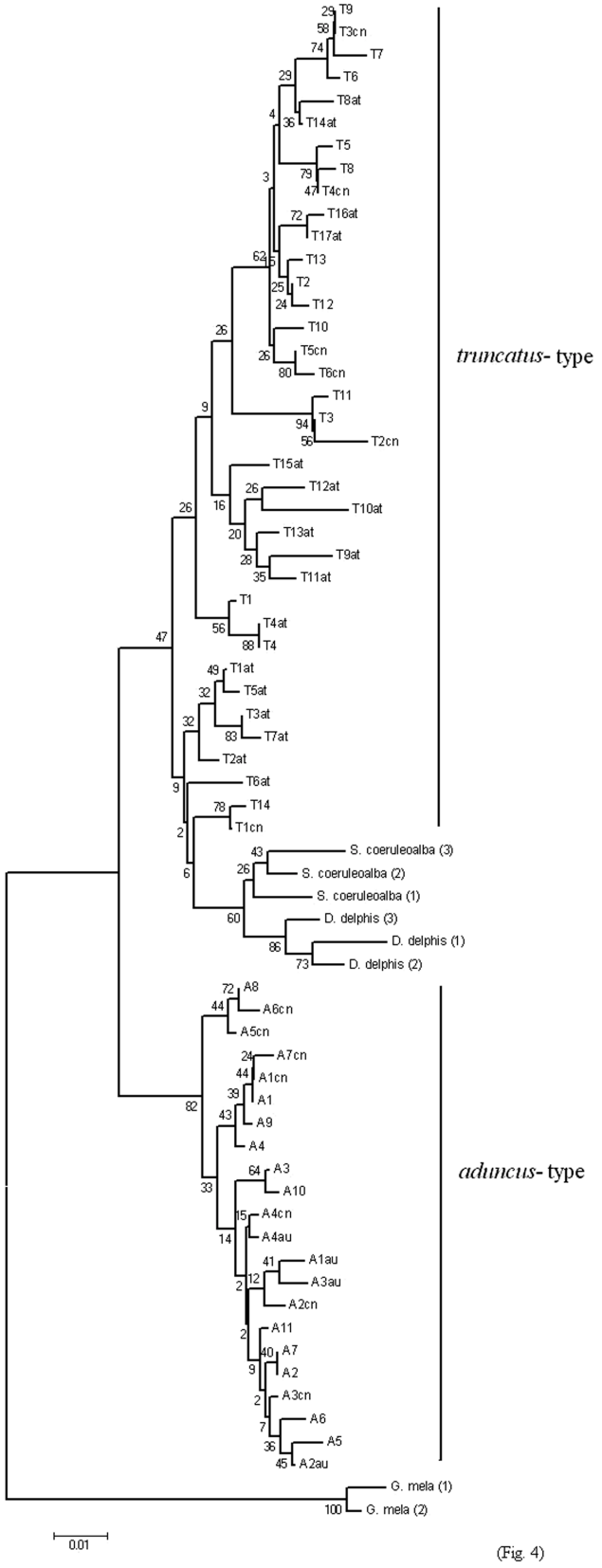

Fig. 4. Plylogenetic reconstruction of *Turisops* mitochondrial control region haplotypes and some haplotypes of striped dolphin *Stenella coeruleoalba* and common dolphin *Delphinus delphis*, reconstructed using the neighbor joining algorithm with short-finned pilot whale *Globicephala macrorhynchu* as outgroup. Bootstrap values from 500 iterations are indicated near branches. Haplotype codes correspond to the codes in figure 1.

relevant to the genetic pattern of two *Tursiops* species in the whole Chinese waters. Completely congruent with the morphological and/or osteological identification, all specimens from the Yellow Sea (Qingdao, Shandong Province, and Lianyuguang, Jiangsu Province) and the northern East China Sea (Zhoushan, Zhejiang Province) were molecularly identified as *T. truncatus* exclusively, whereas samples in the southern East China Sea (Pingtan, Fujian Province), the Taiwan Straits, and the Gulf of Beibuwan were mainly, but not exclusively, identified as *T. aduncus*. Two (NJNU0232 and NJNU-D1) of 13 samples collected from the Taiwan Straits were identified as *T. truncatus*. The overlapping of *truncatus*-type and *aduncus*-type in the Taiwan Straits (and/or adjacent waters) are better supported after adding Wang et al. (1999)'s samples. As to the 29 samples collected in the Taiwan Straits and examined by Wang et al. (1999), 15 and 14 are respectively occupied by *truncatus*-type and *aduncus*-type. All samples from the Gulf of Beibuwan, including samples in Wang et al. (1999) and the present study, were exclusively identified as *T. aduncus*. Although three samples from Hong Kong waters were identified to be *T. truncatus* (Wang et al., 1999), it is premature to conclude that the bottlenose dolphins in those waters are mainly or exclusively *T. truncatus* before more samples are examined. The pattern revealed by the present molecular evidence is quite similar to that inferred by Zhou & Qian (1985) based on morphological data. In a summary of the distribution pattern of *Tursiops*, Zhou & Qian (1985) concluded that *Tursiops* are widely distributed in the China Seas. *Tursiops* was found in the Yellow, Bohai, and East China Seas, whereas *T. aduncus* occurred in the South China Sea and also lived in the eastern border of the East China Sea where the range of the two species overlapped. However, the species boundary for these two *Tursiops* species in Chinese waters needs to be documented in a finer resolution based on more samples and through better sampling programmes. Further, divergence and isolation between two *Tursiops* species should be confirmed by more molecular markers, especially nuclear markers.

Although the distributions of the two *Tursiops* species overlapped in the Taiwan Strait (and maybe adjacent waters), they had significant morphological differences, including osteology and external morphology. Especially, they have no shared haplotypes, four diagnostic sites, large net sequence divergence, and long divergence time. These results lead to the conclusion that there is no genetic interchange between the two species. That is to say, although these two species are sympatric, they are reproductively isolated from each other. This is quite different with that of finless porpoises (*Neophocaena phocaenoides*) which are sympatric with bottlenose dolphins in Chinese waters. The finless porpoises in Chinese waters were assigned to three subspecies or populations (Gao & Zhou, 1995a). It has been shown that obvious external differentiation exists among the three populations. Specimens of each population can be reliably distinguished based on just two external characters (i.e. width of tuberculate region and height of dorsal ridge) (Gao & Zhou, 1995a). Although it is more difficult to distinguish populations on the basis of skeletal morphology, we can clearly distinguish the populations of finless porpoises by stepwise discriminant analysis of skull characters (Gao & Zhou, 1995b; c). However, population genetics analysis based on mitochondrial control region sequences (Yang et al., 2002) showed that finless porpoises, although identified to different populations exclusively by external morphology and skeletal discriminant analysis, have some shared haplotypes. Nearly 30% of the haplotypes were found in more than one population in Chinese waters, and sequence divergence and diversity all were lower than those of *Tursiops*, which suggested genetic exchanges and short-term divergence among finless porpoise populations.

Significant difference in genetic diversity (nucleotide diversity and haplotype diversity) was found between *aduncus* dolphins in Chinese and Australian waters. Further, no shared haplotype was found between Chinese and Australian *aduncus*, or Chinese and Atlantic *truncatus*. This could be regarded as an implication of population structure for bottlenose dolphins in different waters. However, at present, except for USA waters (Curry & Smith, 1997), little is known or investigated about population structure of *Tursiops* in their distribution areas, including Chinese waters. For this reason, although the present study strongly supported the conclusion of Wang et al. (1999) to refer the bottlenose dolphins in Chinese waters to two species, and to manage them as independent management units, it is very urgently needed to investigate intraspecific structure of *Tursiops* species in Chinese waters in the near future. Only by doing so can we design really effective conservation programmes for the two species.

ACKNOWLEDGEMENTS

We are grateful to Anli Gao, Hua Cheng, and Xinrong Xu for many years of hard work in collecting *Tursiops* samples from various parts of Chinese Waters. This study was financially supported by the National Natural Science Foundation Commission (NSFC) of China grants nos. 30270212 and 30070116 to G. Yang, NSFC major project grant no. 39899400 to K. Zhou, and NSFC project for Distinguished Scholars grant no. 30125006 to F. Wei. National "211" Project for the Tenth Five Years to G. Yang, and "Qinglan" Project of Jiangsu Province to G. Yang.

LITERATURES CITED

Bandelt, H. G., P. Forster & A. Röhl, 1999. Median-Joining Networks for Inferring Intraspecific Phylogenies. *Molecular Biology and Evolution*, **16**(1): 37-48.

Curry, B. E. & J. Smith, 1997. Phylogeographic structure of the bottlenose dolphin (*Tursiops truncatus*): stock identification and implication for management. In: Dizon, A. E., Chivers, S. J. & Perrin, W. F. (eds.), *Molecular Genetics of Marine Mammals*. The Society for Marine Mammalogy, Special Publication 3. Allen Press, Lawrence. Pp. 227-247.

Duffield, D. A., S. H. Ridgway & L. H. Cornell, 1983. Hematology

distinguishes coastal and offshore forms of dolphins (*Tursiops*). *Canadian Journal of Zoology*, **61**: 930-933.

Excoffier, L. & P. E. Smouse, 1994. Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. *Genetics*, **136**: 343-359.

Fu, Y. X. & W. H. Li, 1993. Statistical tests of neutrality of mutations. *Genetics*, **133**: 693-709.

Gao, A. & K. Zhou, 1995a. Geographical variation of external measurements and three subspecies of *Neophocaena phocaenoides* in Chinese waters. *Acta Theriologica Sinica*, **15**(2): 81-92. (In Chinese with English summary)

Gao, A. & K. Zhou, 1995b. Geographical variation of skull among the populations of *Neophocaena phocaenoides* in Chinese waters. *Acta Theriologica Sinica*, **15**(3): 161-169. (In Chinese with English summary).

Gao, A. & K. Zhou, 1995c. Geographical variation of postcranial skeleton among the population *Neophocaena phocaenoides* in Chinese waters. *Acta Theriologica Sinica*, **15**(4): 246-253. (In Chinese with English summary).

Gao, A., K. Zhou & Y. Wang, 1995. Geographical variation in morphology of bottlenose dolphins (*Tursiops* sp.) in Chinese waters. *Aquatic Mammals*, **21**: 121-135.

Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins & T. J. Gibson, 1998. Multiple sequence alignment with Clustal X. *Trends in Biochemical Science*, **23**: 403-405.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo, F. X. Villablanca, & A. C. Wilson, 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, USA, **86**: 6196-6200.

Kumar, S., K. Tamura, I. Jackobsen & M. Nei, 2001. MEGA: molecular evolutionary genetics analysis software, ver 2.0. *Bioinformatics*, **17**: 1244-1245.

Leduc, R. G., W. F. Perrin & A. E. Dizon, 1999. Phylogenetic relationships among the delphinid cetaceans based on full cytochrome b sequences. *Marine Mammal Science*, **15**(3): 619-648.

Möller, L. M., & L. B. Beheregaray, 2001. Coastal bottlenose dolphins from southern Australia are *Tursiops aduncus* according to sequences of the mitochondrial DNA control region. *Marine Mammal Science*, **17**(2) : 249-263.

Murray, B. W., R. A. McClymount & C. Strobeck, 1995. Forensic identification of ungulate species using restriction digests of PCR amplified mitochondrial DNA. *Journal of Forensic Science*, **40**: 943-951.

Nei, M.,1987. Molecular Evolutionary Genetics. Columbia University Press, New York, 512 pp.

Parsons, K. M., J. F. Dallas, D. E. Claridge, J. W. Durban, K. C. Balcomb, P. M. Thompson, & L. R. Noble, 1999. Amplifying dolphin mitochondrial DNA from faecal plumes. *Molecular Ecology*, **8**(10): 1766-1768.

Rice, D.W., 1998. Marine Mammals of the World: Systematics and Distribution. Marine Mammal Society, Special Issue 4. *The Society for Marine Mammalogy*, Lawrence, K. S., 231 pp.

Rosel, P. E., A. E. Dizon & J. E. Heyning, 1994. Genetic analysis of sympatric morphotypes of common dolphins (genus *Delphinus*). *Marine Biology*, **119**: 159-167.

Ross, G. J. B., 1977. The taxonomy of bottlenose dolphins *Tursiops* species in South African waters, with notes on their biology, *Annals of the Cape Provincial Museums (Natural History)*, **11**: 135-194.

Ross, G. J. B. & V. G. Cockcroft, 1990. Comments on Australian bottlenose dolphins and the taxonomic status of *Tursiops* aduncus (Ehrenberg, 1832), In: *the Bottlenose Dolphin* Academic Press, San Diego. Pp. 101-128.

Rozas, J. & R. Rozas, 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**: 174-175.

Sambrook, J., E. Fitch & T. Maniatis, 1989. *Molecular cloning, a laboratory manual. Second edition*. Cold Spring Harbour Laboratory Press.

Siemann, L. A., 1994. *Mithochondrial DNA sequence variation in North Atlantic long-finned pilot whales, Globicephala melas*. Ph. D dissertation, Woods Hole Oceanogphic Institution, Woods Hole, MA. 164 pp.

Swofford, D. L. 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10*. Sinauer Associates, Sunderland, USA.

Walker, W. A., 1981. *Geographical variation in morphology and biology of bottlenose dolphins (Tursiops) in the eastern North Pacific*. National Oceanic and Atmospheric Administration, US Department of Commerce, Administrative Report LJ-81-03C. National Marine Fisheries Service, Southwest Fisheries Center, La Jolla, California.

Wang, J. Y., L. S. Chou & B. N. White, 1999. Mitochondrial DNA analysis of sympatric morphotypes of bottlenose dolphins (genus: *Tursiops*) in Chinese waters. *Molecular Ecology*, **8**: 1603-1612.

Wang, J. Y., L. S. Chou & B. N. White, 2000a. Differences in the external morphology of two sympatric species of bottlenose dolphins (genus: *Tursiops*) in waters of China. *Journal of Mammalogy*, **81**(4): 1157-1165.

Wang, J. Y., L. S. Chou & B. N. White, 2000b. Osteological differences between two sympatric species of bottlenose dolphins (genus: *Tursiops*) in Chinese waters. *Journal of Zoology* (London), **252**: 147-162.

Xia, X. H., 2000. *Data Analysis in Molecular Biology and Evolution*. Kluwer Academic Pub, Boston.

Yang, G., W. Ren, K. Zhou, S. Liu, G. Ji & J. Yan, 2002. Population genetic structure of finless porpoises, *Neophocaena phocaenoides*, in Chinese waters, inferred from mitochondrial control region sequences. *Marine Mammal Science*, **18**(2): 336-347.

Yang, G., W. Ren, M. Niu & K. Zhou, 2002. Variability of the complete mitochondrial control region of striped dolphins (*Stenella coeruleoalba*). *Acta Zoologica Sinica*, **48**(1): 131-134.

Yang, H. C., 1976. Studies on the whales, porpoises, and dolphins of Taiwan. *Annual Report of Science, Taiwan Museum* (Taipei), **19**: 131-178.

Zhou, K., 1987. Notes on two species of dolphins of genus *Tursiops* in Chinese waters. *Acta Theriologica Sinica*, **7**(4): 246-254. (În Chinese with English summary).

Zhou, K. & W. Qian, 1985. Distribution of the dolphins of the genus *Tursiops* in the China Seas. *Aquatic Mammals*, **1**: 16-19.