Morphological and Genetic Differentiation of Two Loliginid Squids in Asia

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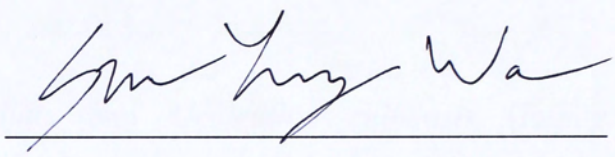

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Morphological and Genetic Differentiation of Two Loliginid Squids in Asia

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Abstract

The squids *Uroteuthis edulis* and *Uroteuthis chinensis* (family Loliginidae) are commercially important fishery species in many coastal regions of Asia. As they are under strong fishing pressure, basic biological knowledge of the squids is needed for formulating appropriate fishery management strategies. The occurrence of cryptic species in the Family Loliginidae has been reported. Cryptic species can show little or no morphological variation but are genetically distinct. The widely distributed *U. chinensis* and *U. edulis* are believed to comprise several cryptic species. The morphology of *U. edulis* and *U. chinensis* are very similar, and identification based on morphology has been inadequate. In this study, the taxonomic status of the two species was elucidated by performing morphological and genetic analyses.

A total of 27 individuals of *U. chinensis* from Hong Kong (China) and Xiamen (China) and 69 individuals of *U. edulis* from Yamaguchi (Japan) and Shanghai (China) were collected in this study. In the first part of this thesis study, morphometic analysis was performed to elucidate the morphometric relationships of the two species, and to determine the relative efficiency of different morphometric variables to discriminate *U. chinensis* and *U. edulis*.

Multivariate morphometric analysis of 27 morphometric indices reveals no new morphological character for taxonomic identification of the two taxa, which could be distinguished by the teeth shape and number of arm sucker rings and the percentage of hectocotylized part of left arm IV of male individuals. Difference in morphometrics between individuals from the two localities was found in *U. edulis*, which is most probably caused by difference in the composition of maturity stages of individuals from the localities.

In the second part of the thesis study, genetic analyses was performed to verify whether *U. edulis* and *U. chinensis* in East Asia are (1) conspecifics exhibiting clinal variations, (2) two distinct species, or (3) more than two species with the presence of cryptic species. The mitochondrial COI and 16S rRNA genes reveal high divergence of 15.5% and 7.7% respectively, indicating that *U. edulis* and *U. chinensis* are distinct species. The applicability of the two mitochondrial genes as effective markers for investigated population structure of *U. edulis* and *U. chinensis* was also tested, but this cannot be ascertained in the present study. As microsatellite markers exhibit a high level of polymorphism and give finer resolution in population study, isolation of microsatellite loci from *U. edulis* and *U. chinensis* was attempted. Two microsatellite loci isolated from *U. chinensis* gave consistent polymorphic products in *U. chinensis* but not in *U. edulis*. A microsatellite locus isolated previously from *Loligo forbesi* gave consistent polymorphic products in both *U. chinensis* and *U. edulis*.

兩種槍烏賊在亞洲的形態和遺傳判別分析研究

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摘要

槍烏賊科的劍尖槍烏賊 (*Uroteuthis edulis*) 和中國槍烏賊 (*Uroteuthis chinensis*) 是亞洲許多沿海地區的重要漁業物種，由於他們受到強大的捕撈壓力，促使我們更有需要去了解他們的基本生物學知識及制訂適當的漁業管理策略。 隱藏種是指在形態上很少或根本沒有差別但基因截然不同的物種，報告顯示頭足類與槍烏賊科擁有不少隱藏種，廣泛分佈的 *Uroteuthis edulis* 與 *Uroteuthis chinensis* 亦可能包含了幾個隱藏種。由於 *Uroteuthis edulis* 與 *Uroteuthis chinensis* 在形態上十分相似，故現存的基於形態上的鑑定系統是不足夠去區分他們的。

這項研究共收集了27個來自香港(中國)和廈門(中國) *Uroteuthis chinensis* 與69個來自山口縣(日本)和上海(中國) *Uroteuthis edulis* 的標本。本研究的第一部分運用形態性狀分析，以澄清兩個物種在形態上的關係，並嘗試檢驗不同形態特徵對判別兩個物種的效率。形態的多元分析顯示兩個物種可以由腕吸盤角質環

尖齒的形狀和數目及雄性左側第４腕莖化程度來區分，但是沒有發現可有效地判別兩個物種的新形態特徵。不同地方的 *Uroteuthis edulis* 存有形態上之差異，這極有可能是由於他們正處於不同的成熟階段而造成的結果。

本研究的第二部分嘗試從遺傳分析闡明在東亞地區的 *Uroteuthis edulis* 與 *Uroteuthis chinensis* 是否：（1）同種展示的漸變現象，（2）兩種截然不同的物種，或（3）包含了隱藏種的兩個以上的物種。分析表明線粒體細胞色素C氧化酶I(COI)基因及16 S rRNA基因擁有高度的遺傳分化，分別為15.5％和7.7％，顯示 *Uroteuthis edulis* 與 *Uroteuthis chinensis* 是截然不同的物種。本研究亦檢驗兩個線粒體基因能否作為有效的標記基因去揭示 *Uroteuthis edulis* 與 *Uroteuthis chinensis* 的種群結構，但研究結果並不足以確定兩個基因的變異性是否合適。微衛星標記具有較高的多態性，並能給予種群結構研究更精細的結果。因此本研究嘗試從 *Uroteuthis edulis* 與 *Uroteuthis chinensis* 中篩選微衛星位點，分析表明兩個從 *Uroteuthis chinensis* 中篩選的微衛星位點能夠在 *Uroteuthis chinensis* 擴增多態性位點結果，一個從福氏槍烏賊(*Loligo forbesi*)中篩選的微衛星位點能夠在 *Uroteuthis edulis* 與 *Uroteuthis chinensis* 擴增多態性位點結果。

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Chapter 1

General introduction

1.1 Introduction to loliginid squids and the target species of this study

1.1.1 Cephalopod taxonomy and fishery

The systematics of cephalopods is currently in an unsettled state due to a number of analytical difficulties. First, the sampling of cephalopod specimens remains very limited. The sampling techniques are inadequate to capture all of the species as most cephalopods have good vision and are fast swimmers. Secondly, many genera and species have been named based on different life stages, such as some early life-history stages that are easier to catch but are often different from the adults, and these differences between life stages may cause confusion (Voss 1977a). Thirdly, cephalopods are mostly constituted of soft tissues that are easily damaged during capture. Hence there are also fewer reliable taxonomic characters in preserved specimens than live specimens as soft tissues may change shape and size during preservation. So the systematics of cephalopods, which is based on analysis of morphological characters, could be greatly affected. Furthermore, conventional description of taxonomic characters is often insufficient to discriminate species (Ogden et al. 1998), and a fully integrated approach was recommended (Nesis 1998). Given the above problems, the systematics of cephalopods is still an on-going process. New species have continuously been discovered (Allcock et al. 2001), and more discoveries are expected to come. There is usually a lack of

taxonomic knowledge in the fisheries community, and people sometimes do not know what species they are actually exploiting (Voss and Ramirez 1966; Smith et al. 1981). As less than 60% of the world cephalopod catches are identified to species (FAO 2001). This poor state of knowledge should be tackled in order to carry out proper fishery management.

The world annual catch of cephalopods increased from 2.4 to 3.4 million tonnes between 1990 and 1999, and the contribution of cephalopod production to total fishery catch increased by about 42% (FAO 2001). It has been proposed that since groundfish stocks are overexploited and diminished, more cephalopods are exploited instead (Caddy and Rodhouse 1998). The consumption of cephalopods is highest in Asian countries, such as China, Japan, Korea and Thailand, which predominate in the commercial fisheries for cephalopods. The high global demand for cephalopods also increases the landings, mostly of squid, to over three million tonnes annually. As they are a group of animals with rapid growth and short lifespan, which leads to little overlap between generations, they are more vulnerable to overfishing (Caddy 1983). This, together with the increase in exploitation, raises the need of scientific knowledge for fishery management purpose.

1.1.2 Family Loliginidae

The class Cephalopoda includes the squid, octopus, cuttlefish, vampire squid and *Nautilus*. The loss of external shell occurrs in the subclass Coleoidea, but remain in another subclass, Nautiloidea. A simple and widely adopted classification scheme (Voss 1977b) divides the Coleoidea into four orders, which are Teuthoidea,

Sepioidea, Octopoda and Vampyromorpha. The order Teuthoidea is the most diverse order with over 20 families. Teuthoidea contains all the neritic and pelagic squids, from oceans all over the world including shallow to deep waters, and polar to tropic regions. In squids, the ancestral shell is reduced during evolution to a supporting structure called the gladius, which lies inside the dorsal surface of mantle. The teuthoids are divided into two suborders, the Myopsida and Oegopsida. The oegopsids are oceanic squids that have eyes open to water, paired gonoducts, and may have suckers modified into hooks. The myopsids are neritic squids that have a transparent cornea covering their eyes, single gonoduct and generally have suckers on the buccal lappets around the mouth (Roper et al. 1969). The suborder contains two families, the Loliginidae and the Pickfordiateuthidae. It has been proposed that the family Pickfordiateuthidae should be integrated into the Loliginidae (Brachionecki 1996).

There are 41-50 species in the family Loliginidae. Most loliginid squids inhabit continental shelf, with a few sometimes descending to the upper bathyal. They usually live near the bottom, but are also able to ascend into midwater and the surface. They are active and agile. Many species of Loliginidae are important commercial fishery species.

There are many confusions in the systematics of Loliginidae (Vecchione et al. 1998). In addition to the two systems of genus-level classification used in the past decades, there has been three separate revisions (Natsukari 1984a; Brakoniecki 1986; Alexeyev 1991) each of which has a different conclusion of the generic-level classification from the previous systems. The differences between the five

conflicting systems were caused by disagreements in the morphological characters used to classify genera. Effort has been paid trying to resolve the differences of generic-level classification of the Loliginidae between the systems, and to stabilize the taxonomy at the generic level (Vecchione et al. 1998). Five genera and four subgenera are assigned to the family Loliginidae according to Vecchione et al. (1998).

1.1.3 Genus *Uroteuthis*

According to Vecchione et al. (1998), the genus *Uroteuthis* Rehder, 1945, consists of twelve species. All the *Uroteuthis* species are distributed within the Indo-West Pacific region, suggesting that they are closely related (Vecchione et al. 1998). The strongest indication of being an *Uroteuthis* species is the presence of photophores on the ventral surface of the ink sac, together with similar hectocotylus and arm sucker dentition. The genus *Photololigo* Natsukari, 1984 was established for Indo-West Pacific *Loligo* or *Doryteuthis* species with photophores. However, *Uroteuthis bartschi* Rehder, 1945, has all generic characters of *Photololigo*, and hence priority goes to *Uroteuthis* as the name of the genus, instead of *Photololigo*. Two subgenera, *Uroteuthis* and *Photololigo*, were established under the genus *Uroteuthis* (Vecchione et al. 1998). *Uroteuthis* (*Uroteuthis*) contains only one species, *U. bartschi*, which has a tail-like elongation of posterior mantle extending beyond lateral fins. This mantle elongation is so extreme that it warrants separate taxonomic status inside *Uroteuthis* (Vecchione et al. 1998). The other subgenus *Uroteuthis* (*Photololigo*) contains all the remaining eleven species, including *U. chinensis* and *U. edulis*.

The biology of squids in this genus is mostly obscure, and it is common that the species identity of the fishery species is unknown (Patterson 1988). Since there is increasing exploitation, the fundamental knowledge of the squids is important for deciding fishery management strategies.

1.1.4 *Uroteuthis chinensis* and *Uroteuthis edulis*

Uroteuthis chinensis (Gray 1849) and *Uroteuthis edulis* (Hoyle 1885) were formerly classified under the genus *Loligo* Lamarck, 1798, as *L. chinensis* and *L. edulis*. Natsukari (1984b) subdivided the genus *Loligo* and established *Photololigo*. Since *L. chinensis* and *L. edulis* both have two photophores on the ventral side of the ink sac, they were designated as *P. chinensis* and *P. edulis* by Natsukari (1984b). With similar generic characters to *Uroteuthis* Rehder, 1945, *Uroteuthis* was used as the genus name instead of *Photololigo*. Under the generic classification system of Vecchione *et al.* (1998), with the establishment of subgenus *Photololigo* under *Uroteuthis*, the two species are named as *Uroteuthis* (*Photololigo*) *chinensis* and *Uroteuthis* (*Photololigo*) *edulis*.

Synonyms of *U. chinensis* include *Loligo etheridgei* Berry 1918, and *Loligo formosana* Sasaki, 1929. For *U. edulis*, there are two forms of unclear taxonomic relationships in the northwestern Pacific. They are *U. edulis* f. *edulis* and *U. edulis* f. *kensaki* Wakiya & Ishikawa, 1921. *Loligo budo* Wakiya & Ishikawa, 1921, is synonymous with *U. edulis* (Sasaki 1929).

In *U. chinensis* (Fig. 1.1), the rings of arm suckers are distally with 10-18 sharp and conical teeth (Gray 1849). The hectocotylized part of the arm occupies 33-40% of arm length. The maximum mantle length is up to 38 cm. In *U. edulis* (Fig. 1.2), the arm sucker rings are distally with 6-12, more often 6-8, obtuse teeth (Hoyle 1885). There is a longitudinal cutaneous ridge along the middle of ventral mantle side in mature males. The length of hectocotylized part of arm is equal to 50-67% of arm length. The mantle length is 20-45 cm. However, the presence of longitudinal ridge is regarded as a character that is too variable to be used in generic systematics (Vecchione et al. 1998). So the most useful characters for identification of the two species are the teeth of arm sucker rings and the percentage length of hectocotylized part/total arm length (Voss and Williamson 1971). Nevertheless, *U. chinensis* and *U. edulis* are still highly similar in their morphology.

The common morphological characters of *U. chinensis* and *U. edulis* include: slender-bodied with rhombic fins extending along sides to posterior tip; a paired photophore on ink sac; proximal suckers on hectocotylus unmodified; hectocotylus with two rows of papillae; club suckers larger than arm suckers; central suckers larger than marginal suckers; distal part of club suckers arranged in four rows; arms longer than 25% of mantle length; teeth of large central club suckers very unequal in length; large club sucker teeth alternated with small inconspicuous teeth; and suckers on the third arm slightly larger than those on other arms (Gray 1849; Hoyle 1885; Voss and Williamson 1971; Vecchione et al. 1998).

It is not surprising that *U. chinensis* (English name: Mitre squid) and *U. edulis* (English name: Swordtip squid) are sometimes regarded as the same species

Fig. 1.1 Dorsal view of *Uroteuthis chinensis*. Scale bar 5 cm.

Fig. 1.2 Dorsal view of *Uroteuthis edulis*. Scale bar 5 cm.

as they are morphologically very similar. In fact both species share the same Chinese name (拖 魷 魚, (Tor Yau Yue) meaning "Trawl Softfish"). During the description of species, Voss & Williamson (1971) used the same figure to illustrate both species. They also proposed that *U. chinensis* and *U. edulis* might be synonymous. In their description of the two species, the size of both species is similar (maximum size 30 cm in mantle length in Hong Kong record), the colour is the same (colourless translucent, bright red or any intermediate shade), and both are important to commercial fishery (Voss and Williamson 1971). In Hong Kong, both species are widespread and common between 30-170 m depth contours and occur in small numbers in shallow water during April-November. There is an annual peak of abundance during July-September in Hong Kong, which is largely composed of squid immigrant from the south. They appear to swim at all depths, from the surface to the bottom (Voss and Williamson 1971). The worldwide geographical distributions of *U. chinensis* and *U. edulis* are highly overlapped (Fig. 1.3). They are both dominant squid species on the continental shelf between Taiwan and Hainan. *U. edulis* is distributed from central Japan, northern South China Sea, Philippine islands, and northern Australia, occurring in 30 to 170 m depth. It overwinters in deeper water and migrates inshore during spring and summer to form large aggregations and spawn on sandy bottom in 30-40 m depth (Voss and Williamson 1971; Roper et al. 1984). *U. chinensis* distributes from the South China Sea, East China Sea to Japan, Arafuru Sea, northeastern Australia to New South Wales, occurring from 15-170 m depth. Peaks of spawning are observed in spring (February to May) and in fall (August to November) (Voss and Williamson 1971; Roper et al. 1984). However, the widely distributed *U. chinensis* and *U. edulis*

Fig. 1.3 Distribution map of *U. chinensis* and *U. edulis*. The known geographical distribution of *U. chinensis* is shown in light grey, and that of *U. edulis* is shown as parallel diagonal lines.

probably comprise several allopatric cryptic species, and some of them may be endemic to Australia (Yeatman and Benzie 1994).

U. chinensis and *U. edulis* support a very important commercial fishery in mainland China and Taiwan on the continental shelf off Guangdong, southern Fujian and around the Pescadore Islands in the Taiwan Strait (Voss and Williamson 1971). The two species are also the most commercially important cephalopods around Hong Kong, as there are about 2000 metric tons landed annually in Hong Kong, which constitute 50% of all cephalopod landings. They are caught by trawlers mostly in summer with August being the peak month (Voss and Williamson 1971). *U. chinensis* accounts for up to 90% of the loliginid catch in several parts of China. It also accounts for 15-40% of the trawl catch in the Gulf of Thailand. *U. chinensis* is also caught in north Australian waters and it is believed to occur in small quantities in Indonesian, Malaysian and Philippine catches (Roper et al. 1984). The annual landing of *U. edulis* reached 6100 metric tons in 1979. *U. edulis* also supports local fisheries in western Japan, the Philippines, and probably in Alas Straits, Indonesia. Catches in Australian waters are also reported (Roper et al. 1984).

1.2 Introduction to morphological differentiation methods in cephalopods

Although there are some difficulties in collecting morphological data from soft-bodied animals, morphometric studies have been applied to squids for many years, such as the morphometric measurements employed to characterize *Loligo*

pealei and *Lolliguncula brevis* (Haefner 1964). Morphometry has also been employed to identify differences between subspecies or species in some squids (Augustyn and Grant 1989; Sanchez et al. 1996; Barón and Ré 2002a; Martinez et al. 2002). The population structure of *Loligo gahi* in Falkland waters was determined using morphomerics together with biochemical genetic studies (Carvalho and Pitcher 1989). Populations of other loliginid species were also identified by morphometric studies (Cohen 1976; Kashiwada and Recksiek 1978). The use of morphometric measurement is an important tool for identification of species in the digestive contents of many predators (dosSantos and Haimovici 1998).

The application of multivariate analysis of morphometric data is a well established method to study geographical variation and taxonomy in teleosts and invertebrates (Bembo et al. 1996; Wolf et al. 1998; Rincon 2000). Regression can be used to check for possible relationship (Barón and Ré 2002a; Arkhipkin 2003), but is practical only for analyzing few variables. The use of indices in morphometry of squid is common (Voss 1956; Haefner 1964; Sanchez et al. 1996; Pineda et al. 1998). The multivariate discriminant analysis has indicated variations and allow diagnosis of squids (Augustyn and Grant 1988; Barón and Ré 2002a).

1.3 Introduction to genetic differentiation methods

1.3.1 Molecular markers

In the last 20 years, DNA-based markers have made great contribution to many fields of biology, including population biology, phylogenetics and systematics. Until 10 years ago their input to cephalopod biology was still limited (Shaw 2002). Traditional approaches to the study of cephalopods can be difficult as they are highly morphological plastic animals that respond readily to environmental conditions and have few hard body parts (Shaw 2002). So DNA-based method have great potential in contributing to future studies of cephalopods.

1.3.1.1 Animal mitochondrial DNA

Animal mitochondrial DNA (mtDNA) is an important genetic marker in evolutionary and population biology. Analysis of animal mtDNA is the most popular and powerful approach among all molecular approaches in ecology and evolution studies (Randi 2000). The studies of gene arrangement, tRNA and rRNA structures, restriction fragment length polymorphism (RFLP), and direct sequencing of individual genes or complete genome of mtDNA provide insights in phylogeny, population structure, and biogeography.

The general structure and genetic basis of variation in animal mtDNA molecular have been well known for many years (Attardi 1985; Wolstenholme 1992). Animal mtDNA is a closed circular DNA molecule, 15-20 kilobases in

length. It is composed of 37 genes, coding for 2 rRNAs (12S rRNA and 16S rRNA), 22 tRNAs, and 13 protein coding genes (ATP6 and ATP8, CoI-III, Cyt b, ND1-6 and 4L), and a control region that initiates replication and transcription.

The most important property for mtDNA to act as genetic marker is that mtDNA is maternally transmitted in most species (Birky 1995). This uniparental inheritant characteristic limits the recombination between mtDNA molecules. So this permits clear trace of maternal genealogies and discrimination between common ancestry and convergence (Harrison 1989). Animal mtDNA evolves rapidly at the sequence level relative to nuclear DNA, since its mutation repair mechanism is inefficient (Brown et al. 1979). Different genes of mtDNA evolve at different rates. The rate of substitution is faster in the control region and slower in rRNA genes (Moritz et al. 1987). So mtDNA allows analyses for population structure, and phylogenetic researches at different taxonomic levels.

1.3.1.2 Microsatellite DNA

Microsatellites are short tandem repeats of DNA consisting of repeat units of 1-6 bp in length. They seldom include more than 70 repeat units and are interspersed throughout the genome. They are highly abundant in eukaryotic genomes, and are tandemly arrayed at particular chromosomal location (Hamada et al. 1984). The variation in repeat number of microsatellites often gives rise to a large amount of distinguishable alleles within a population. Microsatellites have become popular for inferring population structure and dynamics, as they are powerful markers for their high level of polymorphism, mode of Mendelian

inheritance and simple mode of evolution (Zhang and Hewitt 2003). Microsatellites exhibit high level of polymorphism even in species showing low levels of variability with other genetic markers (Hughes and Queller 1993; O'Connell and Wright 1997), thus giving finer resolution in population study. This type of marker has been used extensively to detect population genetic structure, to test parentage and relatedness, to assess genetic diversity, and to study recent population history. The development and application of microsatelite DNA markers is a rapidly developing area in cephalopod population genetics (Shaw 2002).

1.3.2 Systematic studies of cephalopods using molecular markers

The use of molecular markers is a new tool for taxonomy, and molecular systematics is a relatively young science, particularly in cephalopods. Some sequence analyses have been done in the cephalopods to determine phylogenetic relationships. Nucleotide sequence data from partial 16S rRNA gene was used for phylogenetic analysis of decapod cephalopods, and showed promise for determining phylogenetic relationships at infra-family level (Bonnaud et al. 1994). The molecular evidence provides an approach to review the taxonomy based on morphological data. Phylogenetic relationships among the cirrate octopods was also investigated using partial sequences of the mitochondrial 16S rRNA gene (Allcock and Piertney 2002; Piertney et al. 2003). The reconstructed phylogeny is in agreement with the result from morphological data, and supports the traditional separation of cirrate families (Piertney et al. 2003). The results also suggest some revisions in the systematic classification of the cirrates, such as uniting of genera and placement of genera in another family. Sequence analysis of another

mitochondrial gene, cytochrome oxidase III (COIII), has also been used to infer phylogeny of cephalopods (Bonnaud et al. 1996; Bonnaud et al. 1997). It was suggested that the order Sepioidea, which is not monophyletic, should be abandoned. The COIII gene was also used to elaborate the phylogenetic relationships between five species of Pacific octopuses (Barriga-Sosa et al. 1995). Genetic differentiation between *Octopus vulgaris* and *O. mimus* was demonstrated by randomly amplified polymorphic DNA (RAPD), supporting the taxonomic separation of the two species (Warnke et al. 2000).

DNA sequences from the cytochrome *c* oxidase subunit I (COI) and 16S rRNA genes from 19 loliginid species and several outgroups have been used to examine phylogeny of loliginid squids (Anderson 2000b). The result supports monophyly of Loliginidae and reveals four clades in accordance with geographical difference. Another study analyzed sequences from three mitochondrial genes, 12S rRNA, 16S rRNA and COI, from 14 gonatid squids and 6 outgroup cephalopods, to determine the evolutionary relationships among squids of the family Gonatidae (Lindgren et al. 2005). The combined analysis of the three genes confirms the separation of gonatid groups established by morphological characteristics. To resolve the deep relationship among coleoid cephalopod families, more genes are needed to provide enough resolution. So the higher level phylogeny of the coleoid cephalopods was investigated using three mitochondrial genes (12S rRNA, 16S rRNA, and COI) and three nuclear genes (*octopine dehydrogenase, pax-6,* and *rhodopsin*) from 35 species including representatives from each of the higher taxa (Strugnell et al. 2005). The use of multiple genes could increase the resolution as

different genes can evolve at different rates and exhibit different evolutionary properties (Ticher and Graur 1989; Mouchiroud et al. 1995).

The presence of cryptic species is common in cephalopods. Analysis of the mitochondrial COIII gene indicated the distinctiveness of *Octopus mimus* from *Octopus vulgaris*, and also revealed the existence of cryptic species among *O. vulgaris*-like specimens (Soller et al. 2000). Many of the previous biochemical genetic studies carried out on teuthoid squids have shown the presence of cryptic species (Carvalho et al. 1992; Brierley et al. 1993a). This is also common in *Loligo* species (Augustyn and Grant 1989; Brierley et al. 1993b; Yeatman and Benzie 1993; 1994). Furthermore, species misidentification (Smith et al. 1981) is another problem due to the difficulty discerning the differences between and within species. This suggests that there are problems on identification based on current morphological criteria, which are insufficient for discrimination of species (Yeatman and Benzie 1994).

The separation of *U. edulis* and *U. chinensis* from the genus *Loligo* was supported by a biochemical genetic study using allozyme electrophoresis, which showed distinct difference of the two species from *Loligo vulgaris* (Brierley et al. 1996). In a genetic study of *Uroteuthis* species from northern Australia, four species were revealed. They comprised two "*U. chinensis*" morphs and two "*U. edulis*" morphs on gross morphology (Yeatman and Benzie 1994). It is believed that the widely distributed *U. chinensis* and *U. edulis* are composed of several cryptic species, and some are probably endemic to Australia.

1.3.3 Population genetic studies of cephalopods using molecular markers

Knowledge on the population structure of commercially important species is needed to develop responsible management and conservation strategies. Population structuring could be revealed in the light of molecular markers. The phylogeography of two loliginid species, *Loligo pealei* and *L. plei*, was examined using PCR-RFLP of the COI gene (Herke and Foltz 2002). It was found that population structuring occurs in both species across the region from the Gulf of Mexico to northwestern Atlantic Ocean. Another kind of molecular marker, microsatellite DNA, was firstly applied in population genetic study of cephalopod in 1999 (Shaw et al. 1999). It was demonstrated that microsatellite markers exhibit greater sensitivity to population differentiation than allozyme and mtDNA markers, and subtle population structure in *Loligo forbesi* throughout Northeast Atlantic was revealed by microsatellite markers (Shaw et al. 1999). Since then the utility of microsatellite DNA in determining population structuring becomes more common. For instance, it was used for determining whether seasonal and/or geographical groups represent distinct populations in a few loliginid squids and cuttlefishes (Reichow and Smith 2001; Garoia et al. 2004; Shaw et al. 2004).

Most *Loligo* species are considered to have broad distribution range (Roper et al. 1984). Yet with the detection of allopatric occurrence of sibling species, the range of the squids are suggested to be more limited (Augustyn and Grant 1989; Brierley et al. 1993b; Yeatman and Benzie 1993; 1994). This is supported by the studies of a high number of endemic Japanese *Loligo* species, each of which has comparatively restricted distribution (Voss 1983; Roper et al. 1984). In addition, the

Loligo species in tropical waters appear to be depth-constrained, which is believed to be an important factor governing speciation especially in topographically complex areas such as the Indo-Pacific region (Yeatman and Benzie 1994).

1.4 Objectives

There are very limited morphological characters that could be used to discriminate *U. chinensis* and *U. edulis*, and no morphometric study has been performed on these two species. The objective of the present study is to elucidate the morphometric relationships of the two species, and to determine the relative efficiency of different morphometric variables to discriminate the *U. chinensis* and *U. edulis*.

The mitochondrial genes, which have been widely used in DNA-based methods of phylogenetic and taxonomic researches, were used in this study. The COI and 16S rRNA genes were used to clarify the taxonomic status of *U. chinensis* and *U. edulis*. The present study also tested whether there is sufficient polymorphism in the chosen mitochondrial gene to permit population-level study in *U. chinensis* and *U. edulis*. Since microsatellite DNA markers are powerful for revealing population structure, microsatellite DNA loci were developed in this study.

Chapter 2

Morphological differentiation of *Uroteuthis chinensis* and *Uroteuthis edulis*

2.1 Introduction

The loliginid squids, *Uroteuthis chinensis* and *U. edulis*, are the dominant species in the cephalopod catch from the South China Sea. Their systematic status have undergone several revisions, and the recently proposed classification is based on current observation on morphological characters among taxa in Loliginidae (Vecchione et al. 1998). Their taxonomic identification is mainly based on the dentition of arm suckers. The teeth on arm suckers are sharp in *U. chinensis* and are blunt in *U. edulis* (Natsukari and Okutani 1975). This character is used for species identification in this study. The percentage of total arm length that is hectocotylized (Gray 1849; Hoyle 1885) is another phenotypic character that is different among the two species, but hectocotylus is only found in mature males. The validity of whether the hectocotylized proportion is an informative character that could be used to distinguish the two species among all morphological characters was tested in the present study. Nevertheless, using these two characters only is insufficient to distinguish between the two species in some situations, such as the loss of squid body parts or the unavailability of microscope for examining the arm sucker teeth in the field. Moreover, the morphometric relationships have not been examined in these two species. Identification of useful morphological characters would prevent taxonomic confusion and benefit fishery management.

Morphometric studies have been employed to identify squid species or subspecies (Augustyn and Grant 1988; Sanchez et al. 1996; Pineda et al. 2002). Morphometric indices have often been extensively used for the analysis of loliginid squids (Sanchez et al. 1996; Pineda et al. 1998; Pineda et al. 2002) to eliminate the effect of size during analysis. These indices were also used in the present study. Multivariate analysis of morphometric data, which is well-established in morphological differentiation analysis (Wernberg et al. 2003; Castilho et al. 2007; Chan et al. 2007), was applied.

The objective of the present study was to elucidate the morphometric relationships of *U. chinensis* and *U. edulis*, and attempt to identify morphological characters that are useful for species identification.

2.2 Materials and methods

2.2.1 Specimens

Specimens of *Uroteuthis chinensis* were collected from Hong Kong (China) and Xiamen (China) (Fig. 2.1). Specimens of *U. edulis* were collected from Yamaguchi (Japan) and Shanghai (China). The specimens were stored at -20°C immediately after collection until analysis. The number of individuals employed in the morphometric analysis is indicated in Table 2.1.

2.2.2 Morphometric characters

The squids were identified to species based on the shape of the arm sucker ring teeth, which are point-ended in *U. chinensis* (Fig. 2.2) and blunt-ended in *U. edulis* (Fig. 2.3) (Natsukari and Okutani 1975). The sex was established by the observation of the reproductive organs. Maturity was determined by the presence of spermatophores in the penis/spermatophoric sac of males or the presence of mature oocytes in females. A five-level maturity stage scale (modified from Boyle and Ngoile (1993) and Jackson (1997)) was used to assess individual maturity:

Stage I: Immature

Female – gonads very small and undeveloped

Male - gonads very small and undeveloped; penis and hectocotylus not developed

Stage II: Maturing

Female – considerable development in the gonads; nidamental glands opaque and ovary translucent; no eggs visible

Male - considerable development in the gonads; penis and testis developed but no spermatophores visible; hectocotylization of fourth left arm begins

Stage III: Mature

Female – ovary with eggs; nidamental glands enlarged and opaque

Male – testis is opaque and ridged; spermatophoric sac without spermatophores; hectocotylized arm clearly recognizable

Stage IV: Mature

Female – eggs present in ovary; oviducal glands creamy white

Male - testis is opaque and ridged; spermatophores present in spermatophoric sac; hectocotylization complete

Stage V: Mature

Female – same as Stage IV, but with eggs present in distal oviduct; spermatheca with sperm

Male – same as Stage IV, but with spermatophores in penis.

Twenty-three morphometric characters and one meristic character, the teeth number of the largest arm sucker ring on arm III, were recorded (Figs. 2.4-2.8; Table 2.2). Measurements were made with a ruler or calliper to the nearest 1 mm. The shape of arm sucker ring teeth was observed under the light microscope. To avoid the effect of size difference, most characters were analysed in proportion to the mantle length. A total of 27 indices were calculated and used for analysis: 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 (see Table 2.2 for abbreviations). Most of these indices have frequently been used in morphometric studies of squid (Haefner 1964; Barón and Ré 2002a).

2.2.3 Multivariate analysis of data

Variations in the morphometric characters between *U. chinensis* and *U.*

edulis were analysed using multivariate analysis (PRIMER 6, Plymouth Routine in Multivariate Analysis) (Clarke 1993). The program PRIMER is principally designed for the study of changes in biotic communities using species data matrices, but it is also applicable to numerical taxonomy, in this case using matrices of individual squids based on morphometric indices. Data were standardized before 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 (Castilho et al. 2007; Chan et al. 2007), indicated by stress values. MDS was conducted to generate two-dimensional plots of the morphometric indices between individuals of the two species. Analysis of Similarity (ANOSIM) 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 equals to 1 if all individuals within a population are more similar to each other than to any individual in another population, and 0 means if there is no difference between populations. Similarity percentages (SIMPER) procedure was used to calculate the percentage contribution of each morphological character to the overall difference between groups.

2.3 Results

The MDS plot based on the 27 morphometric indices (Fig. 2.9) can be considered a good representation of the multivariate information, as the stress <0.1 (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, *Uroteuthis chinensis* and *U. edulis* (ANOSIM, R=0.1, P=0.034) (Fig. 2.9). From the pattern observed in the MDS plot, individuals in each of the species, particularly *U. edulis*, appear to separate into two groups.

According to the above observation, the result is analyzed based on localities since each species was collected from two sampling sites. Fig. 2.10 shows the MDS plot that group the individuals according to their respective localities. There is significant separation among locations (R=0.692, P=0.001). The results of the ANOSIM are summarized in Table 2.3. The pairwise procedure indicates a clear difference between some pairs of localities (R>0.75, P<0.01) (Clarke and Gorley 2001). They are Yamaguchi (Japan) vs. Shanghai (China), and Yamaguchi vs. Xiamen (China). The other locality pairings are also significantly different (0.75>R>0.5, P<0.01), except for Hong Kong and Xiamen that shows no significant difference (R=0.164, P>0.05). The result indicates intraspecific difference between localities in *U. edulis*, but not in *U. chinensis*. The results of SIMPER shows the character that contributed most to the intraspecific differences in *U. edulis* between Yamaguchi vs. Shanghai is the tentacle length to mantle length index. The results show that the morphometric differences between individuals from different localities may explain the separation in *U. edulis*, but not *U. chinensis*.

It was noticed that the samples include individuals at different maturity stages (Table 2.4). In *U. edulis*, >87% of samples from Yamaguchi were at stage V, while >60% of Shanghai samples were at stage I for both sexes. For *U. chinensis*, the compositions of individuals from different maturity stages were more even. Due to such differences, the result was also analyzed based on maturity stages. It was performed separately for the two species, for the male sex. Females were not analyzed because their sample size was too small. There were only four individuals at stage I and two individuals at stage II for *U. edulis*, and three individuals at stage V and two individuals at stage II for *U. chinensis*. Figure 2.11 shows the MDS plot on the morphometric indices of male *U. edulis*. Individuals at different stages are significantly different (Global R value=0.732, P=0.001). The results of the ANOSIM are summarized in Table 2.5. The pairwise procedure indicates significant difference between some pairs of stages (R>0.5, P<0.01): V vs. III, V vs. I, and IV vs. I. The results of SIMPER reveal that the index contributed most to the difference between these maturity stage pairings is the tentacle length to mantle length (all >23%). Figure 2.12 shows the MDS plot on the morphometric indices of male *U. chinensis*. Individuals at different stages are overlapped (Global R value=0.348, P=0.012). The results of the ANOSIM are summarized in Table 2.6. The pairwise procedure indicates significant difference between all pairings that included stage V (R>0.5, P<0.01). The results of SIMPER reveal that the index that most contributed to the difference between these maturity stage pairings is the tentacle length to mantle length (all >33%). This index is higher in individuals of early maturity stage than that in later stages (Table 2.7).

The index that contributed most to the difference between *U. edulis* of the two localities is the tentacle length to dorsal mantle length, which is also the index that contributed most to the difference between maturity stages. The pattern of intraspecific difference on MDS for *U. edulis* and *U. chinensis* (Fig. 2.9) are similar to the MDS patterns showing the difference in maturity stages (Figs. 2.11 and 2.12). Thus the intraspecific difference could be explained by the difference in maturity stages. The separation of *U. edulis* from Yamaguchi and Shanghai is due to the difference in the maturity stage composition. The individuals of *U. chinensis* from Hong Kong and Xiamen are mixed together because they have similar maturity stage composition.

Multivariate analysis between the two species was done with the addition of one sex-specific character, which is the percentage of total length of left arm IV that is hectocotylized (HL%), in mature male individuals (stages II to V). It was not added to the morphometric analysis above because it is only found in mature male individuals, as hectocotylus is a specialized arm for spermatophore transfer. By excluding the individuals with broken hectocotylus, seventeen *U. chinensis* and 36 *U. edulis* are included in this analysis. The MDS plot (Fig. 2.13) in this analysis can be considered a good representation of the multivariate information, as the stress < 0.05. A clear pattern is formed on the MDS plot. The individuals of *U. chinensis* and *U. edulis* are clustered together with individuals of the same species (ANOSIM, R=0.928, P=0.001). SIMPER analysis shows that the character that contributed most to the dissimilarity of the two species is the HL% that accounts for 92.1% dissimilarity of the two species. The mean HL% is 35.7% (range: 25.8-42.0%) in *U. chinensis* (Fig. 2.14), and 59.6% (range: 53.8-67.7%) in *U. edulis* (Fig. 2.15). There

is no significant difference between stage IV and V of *U. edulis* revealed by Mann-Whitney U test (Fig. 2.16). Kruskal-Wallis test also revealed no significant difference between stages II, IV and V of *U. chinensis* (Fig. 2.17). Hence the HL% is not affected by maturity stage. There were only two individuals at stage III for *U. edulis* and *U. chinensis* respectively, and they were not included in the above statistical analyses.

The only meristic character, the teeth number of arm sucker ring, was analyzed by two-tailed t-test. There is significant difference ($P<0.001$) between *U. edulis* and *U. chinensis* (Fig. 2.18). The mean teeth number of arm sucker ring is 11.4 (range: 9-14) for *U. chinensis*, and 9.0 (range: 7-12) for *U. edulis*.

Table 2.1 Sampling locality and sample size of *Uroteuthis chinensis* and *U. edulis* included in morphometric study. Number of samples for multivariate analysis is in parentheses.

Species	Locality	No. of samples	Sex		Total no.
			Male	Female	
Uroteuthis chinensis	Hong Kong (China)	18 (16)	15 (13)	3 (3)	
	Xiamen (China)	9 (7)	7 (5)	2 (2)	27 (23)
Uroteuthis edulis	Yamaguchi (Japan)	42 (33)	42 (33)	0	
	Shanghai (China)	27 (16)	21 (10)	6 (6)	69 (49)

Table 2.2 Morphological characters recorded on *Uroteuthis chinensis* and *U. 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 largest sucker on arm III

Table 2.3 Summary of results from one-way ANOSIM of *U. edulis* and *U. chinensis* in different localities. Value of ANOSIM statistic (*R*) for global test for differences between localities in each matrix, and results of pairwise tests. *R*-values in bold indicate well-separated localities (*R*>0.75: Clarke and Gorley, 2001). Localities 1, 2, 3 and 4 correspond to Yamaguchi (Japan), Hong Kong (China), Xiamen (China) and Shanghai (China) respectively.

Locality		
Global *R*=0.692, P=0.001		
Pairwise test results		
Locality	*R*	P
1 vs. 3	**0.802**	0.001
1 vs. 2	0.512	0.001
1 vs. 4	**0.963**	0.001
3 vs. 2	0.164	0.084
3 vs. 4	0.590	0.001
2 vs. 4	0.703	0.001

Table 2.4 The percentage of samples for different maturity stages in the four localities.

Species	Locality	Sex	n	Maturity stage				
				I	II	III	IV	V
U. 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. 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 2.5 Summary of results from one-way ANOSIM of male *U. edulis*. Value of ANOSIM statistic (*R*) for global test for differences between stages in each matrix, and results of pairwise tests. *R*-values in bold indicate well-separated maturity stages (*R*>0.75: Clarke and Gorley, 2001).

Maturity stage		
Global *R*=0.732, P=0.001		
Pairwise test results		
Maturity stage	*R*	P
V vs. IV	0.38	0.009
V vs. III	**0.995**	0.002
V vs. I	**0.993**	0.001
IV vs. III	0.49	0.036
IV vs. I	0.639	0.006
III vs. I	-0.271	0.857

Table 2.6 Summary of results from one-way ANOSIM of male *U. chinensis* . Value of ANOSIM statistic (*R*) for global test for differences between maturity stages in each matrix, and results of pairwise tests. *R*-values in bold indicate well-separated maturity stages (*R*>0.75: Clarke and Gorley, 2001).

Maturity stage		
Global *R*=0.348, P=0.012		
Pairwise test results		
Maturity stage	*R*	P
V vs. IV	0.525	0.032
V vs. III	0.745	0.048
V vs. II	**0.775**	0.003
IV vs. III	-0.071	0.667
IV vs. II	-0.066	0.667
III vs. II	-0.071	0.611

Table 2.7 TL/DML ratios at different maturity stages of male *U. edulis* and *U. chinensis*.

Maturity stage	Mean value (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) (2)	1.53 (1.45-1.6) (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)

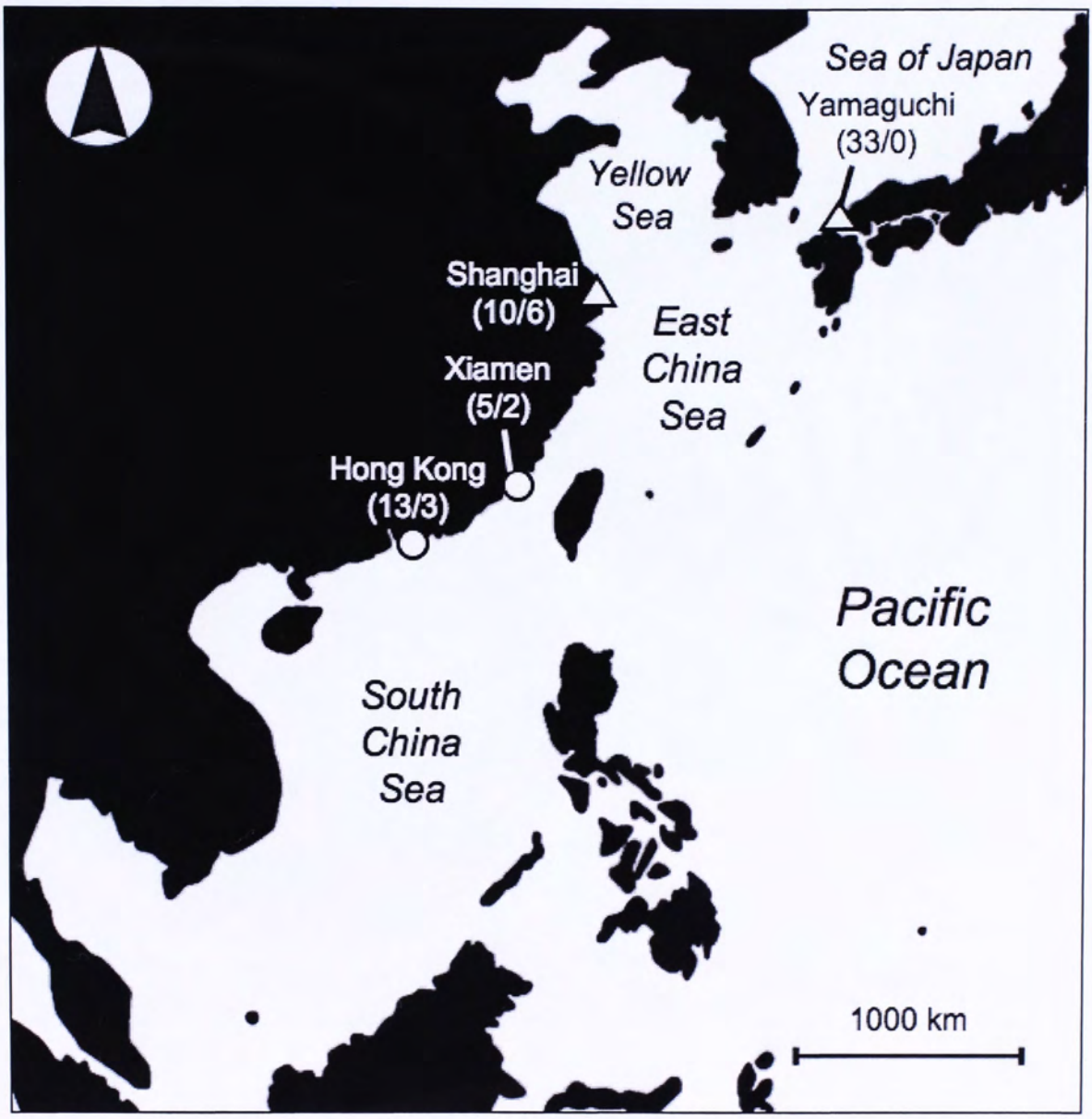

Fig. 2.1 Sampling localities of *Uroteuthis chinensis* and *U. edulis*. Circle indicates *U. chinensis* sampling locality and triangles indicates *U. edulis* sampling locality. Number of samples for morphometric multivariate analyses is shown in parentheses for both sexes (male/female).

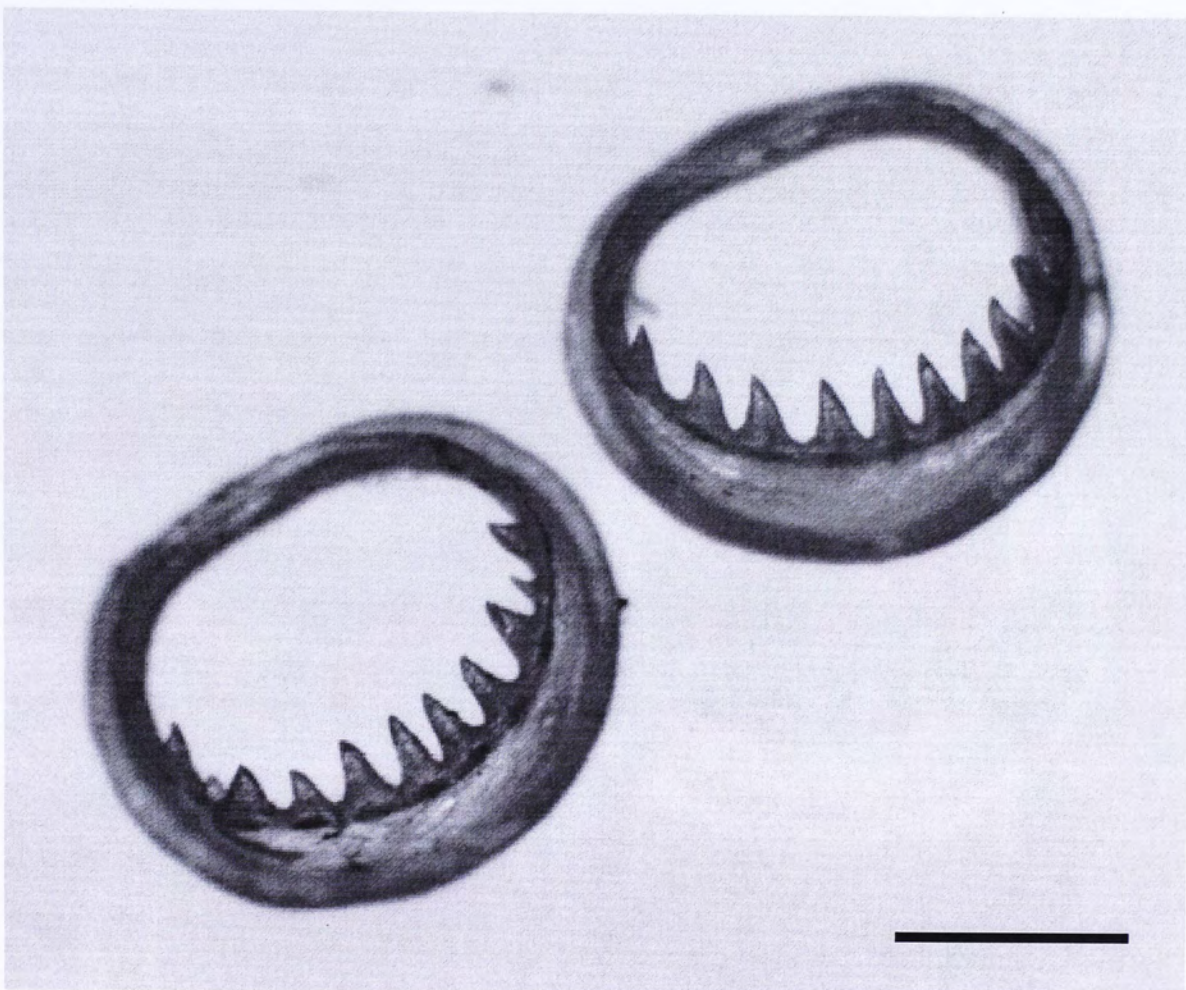

Fig. 2.2 Teeth of arm sucker ring of *Uroteuthis chinensis*. Teeth number: 9-14; teeth shape: sharp; scale bar 1 mm.

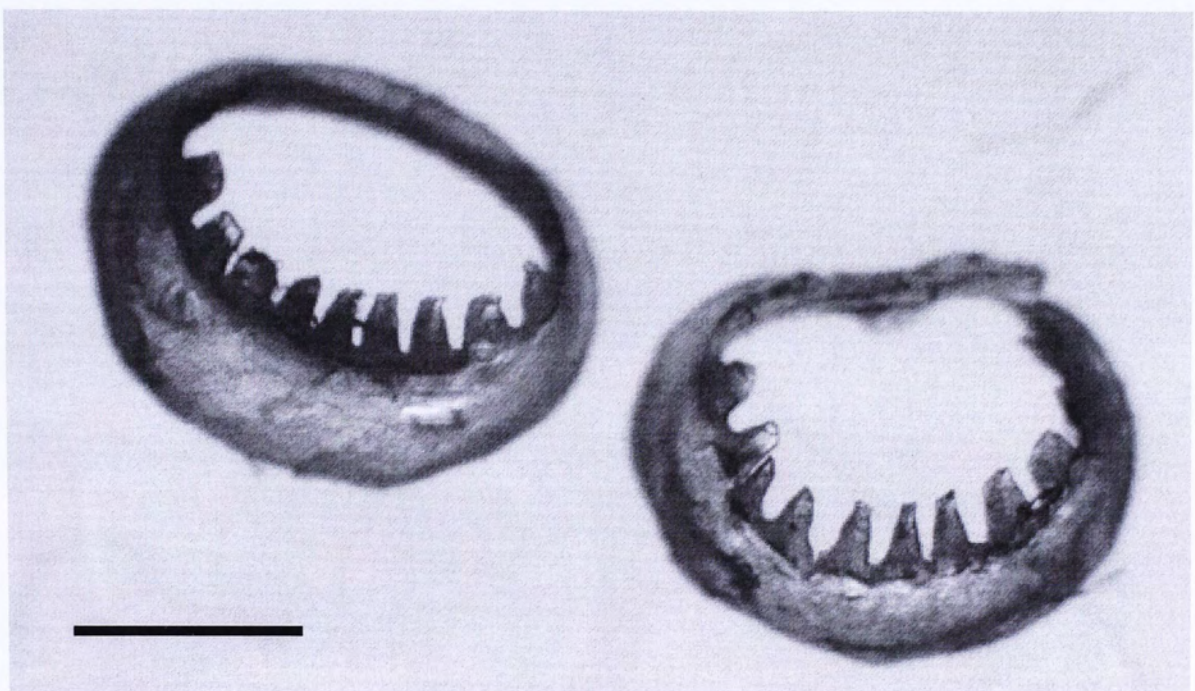

Fig. 2.3 Teeth of arm sucker ring of *Uroteuthis edulis*. Teeth number: 7-12; teeth shape: blunt; scale bar 1 mm.

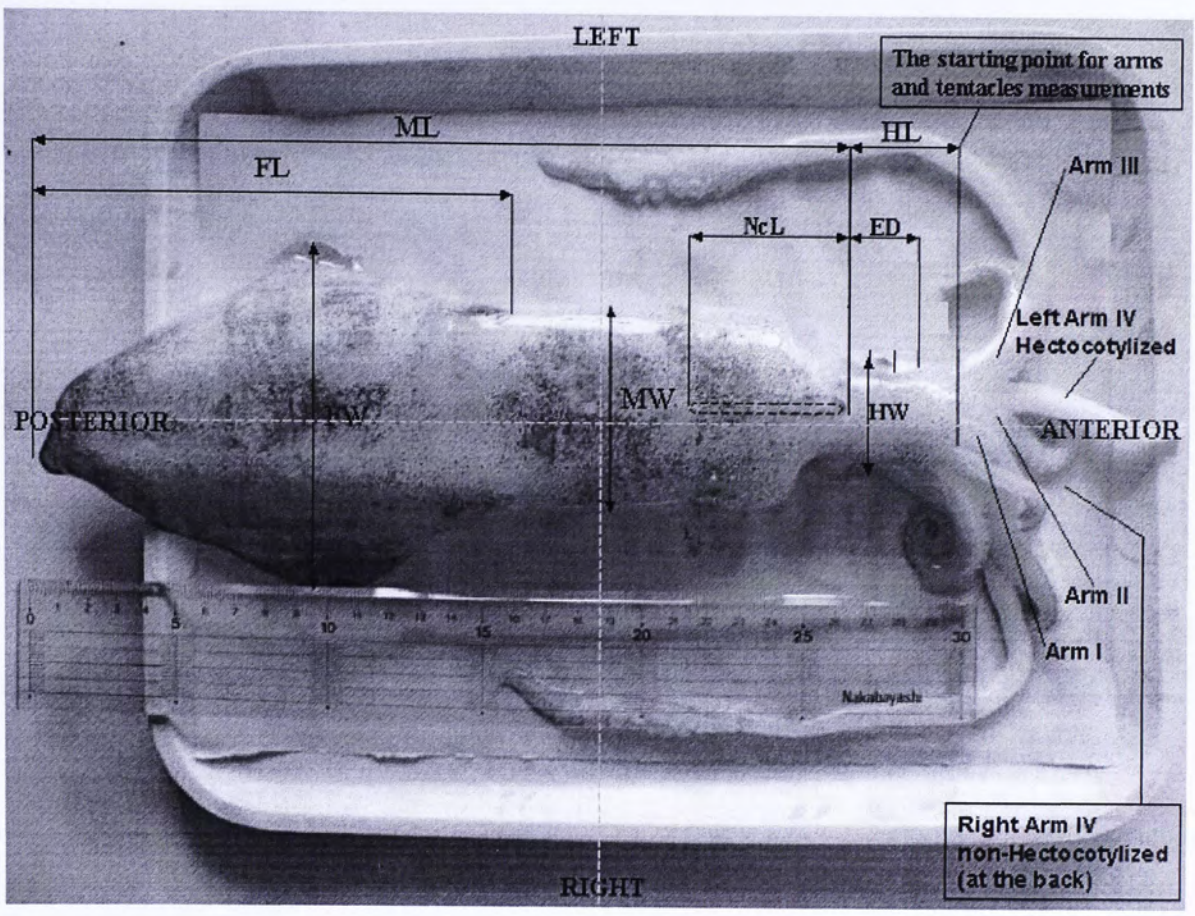

Fig. 2.4 Dorsal view of squid showing 12 of the 23 morphometric characters recorded on *Uroteuthis chinensis* and *U. edulis*. See Table 2.2 for abbreviations.

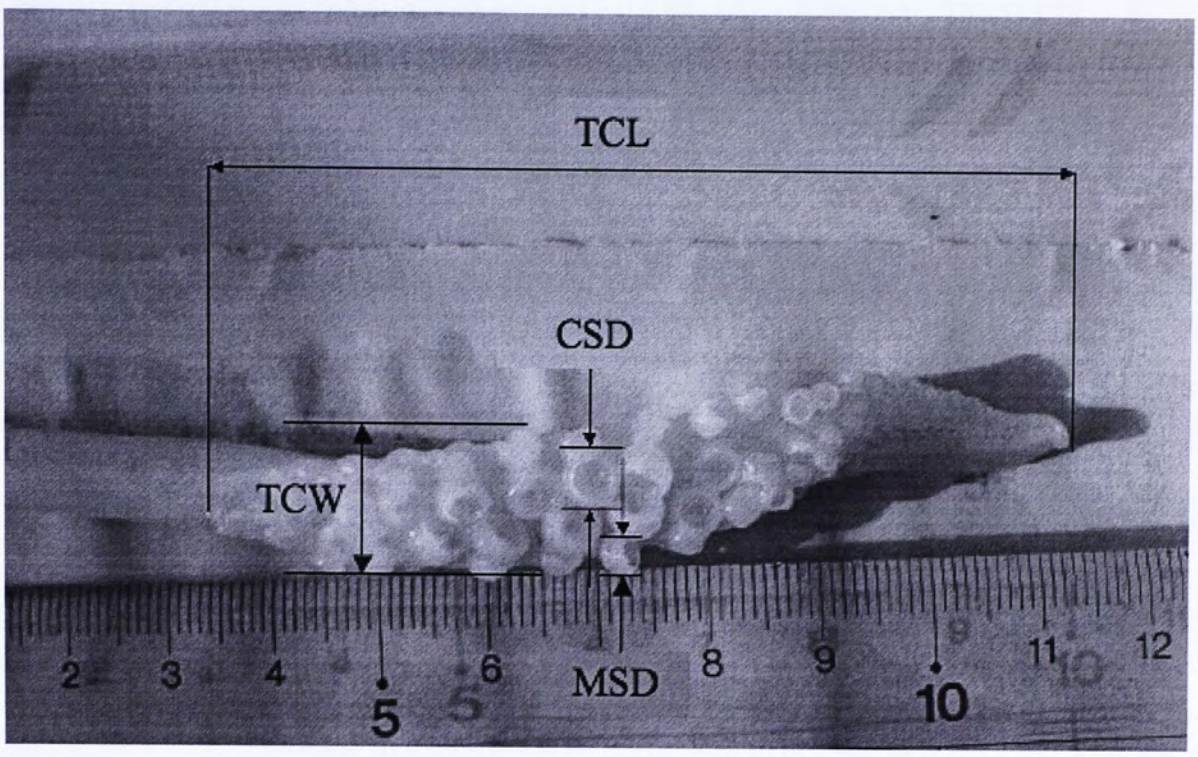

Fig. 2.5 Tentacular club showing four morphometric characters recorded on *Uroteuthis chinensis* and *U. edulis*. See Table 2.2 for abbreviations.

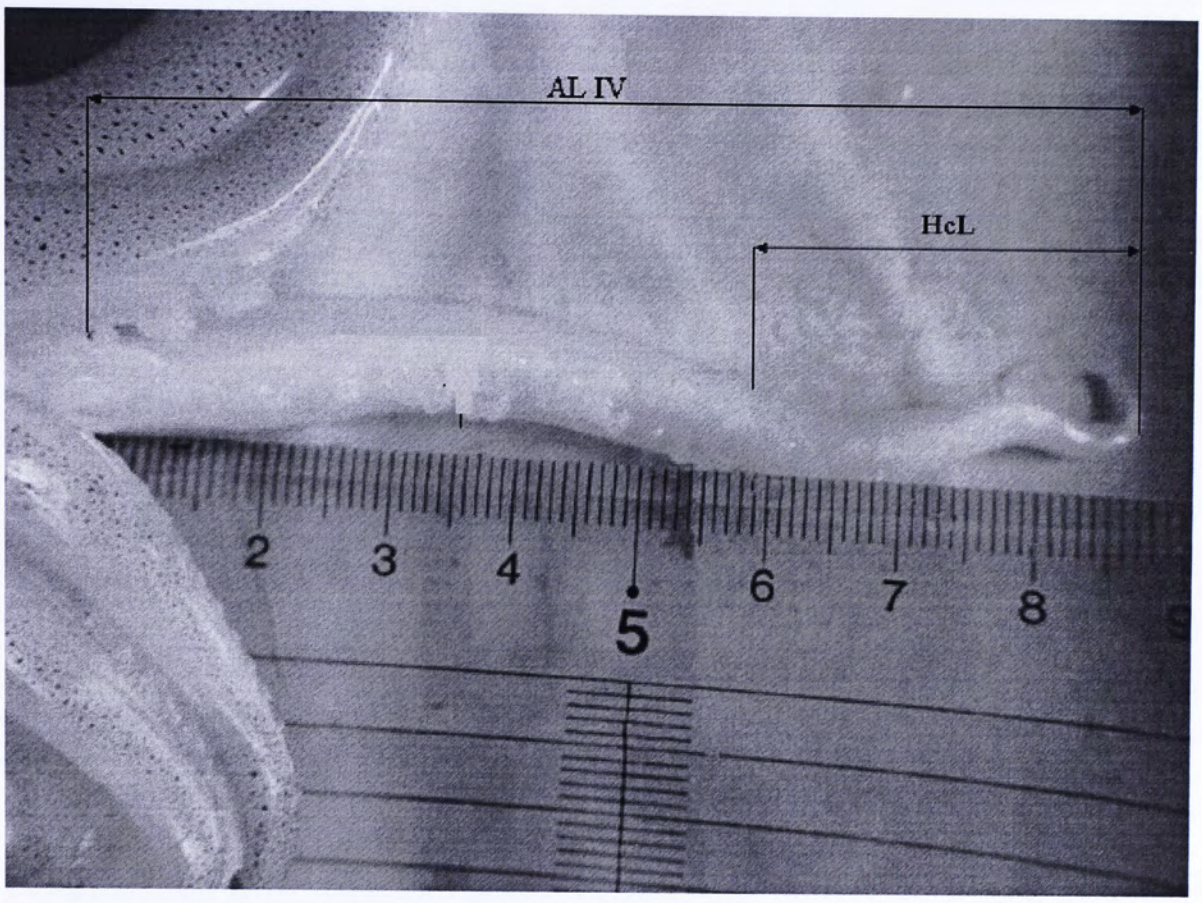

Fig. 2.6 Hectocotylus showing two morphometric characters recorded on *Uroteuthis chinensis* and *U. edulis*. See Table 2.2 for abbreviations.

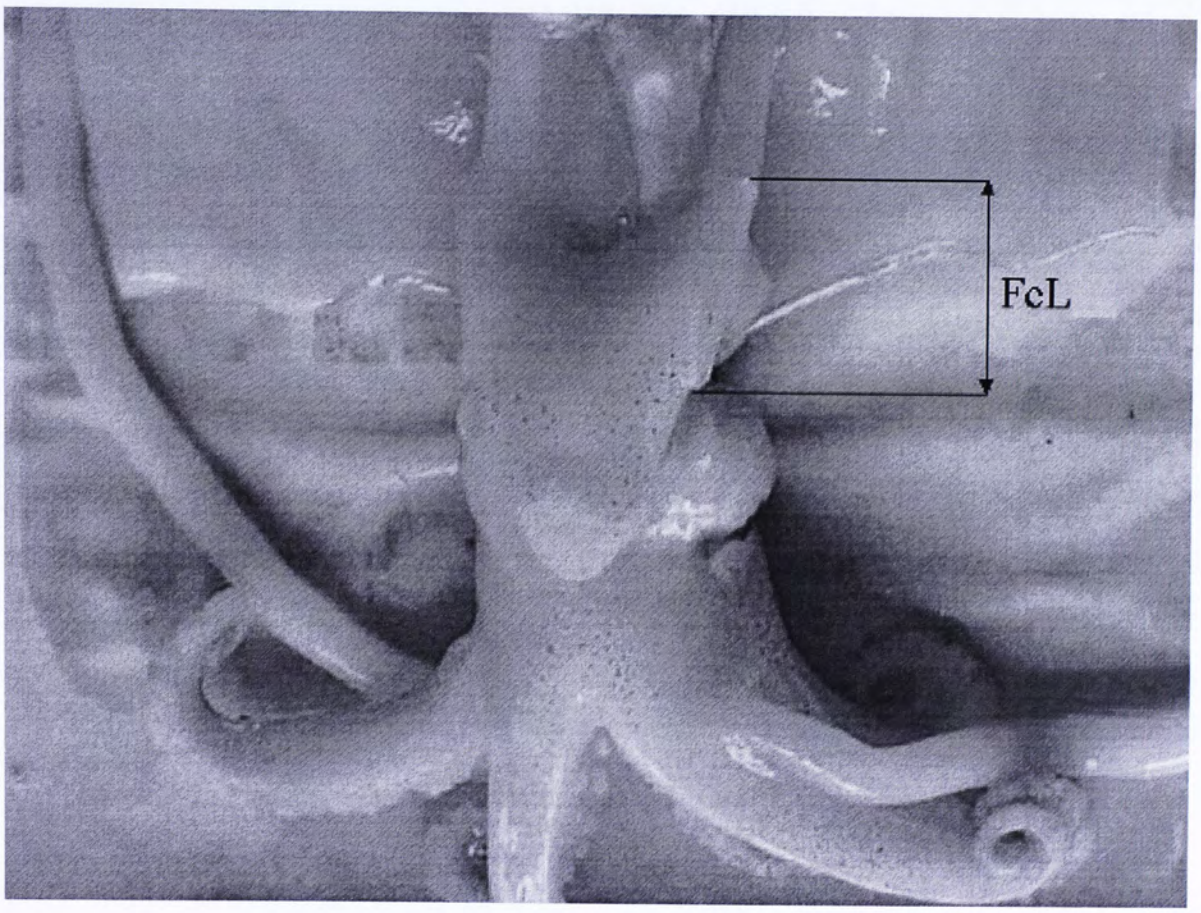

Fig. 2.7 Funnel locking cartilage length recorded on *Uroteuthis chinensis* and *U. edulis*. See Table 2.2 for abbreviations.

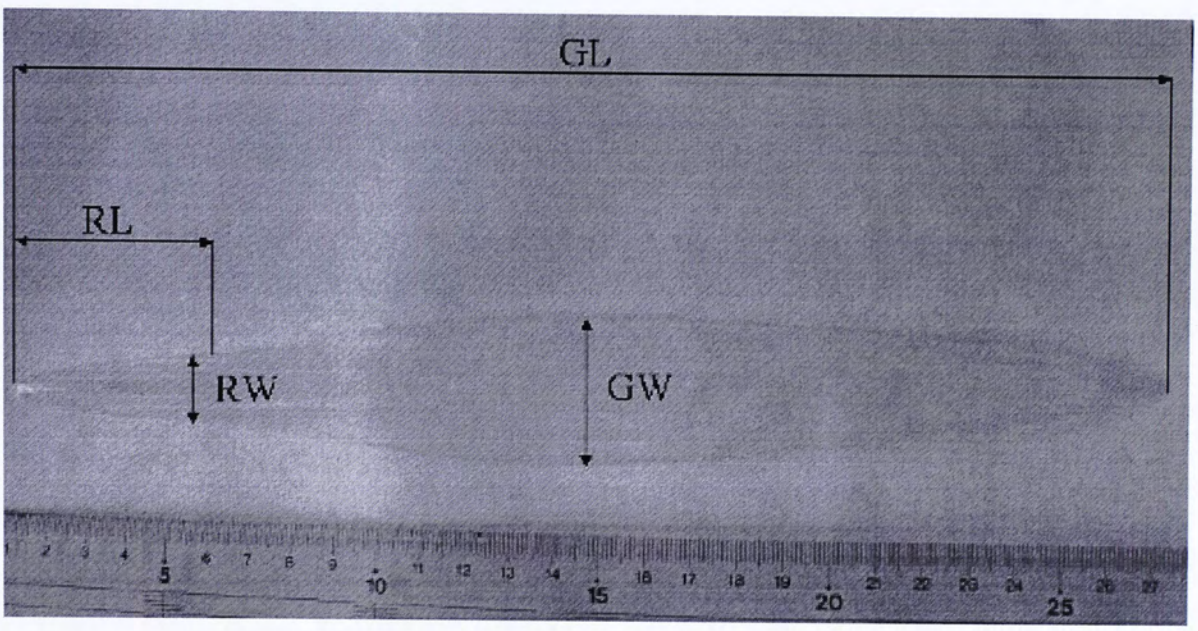

Fig. 2.8 Gladius showing four morphometric characters recorded on *Uroteuthis chinensis* and *U. edulis*. See Table 2.2 for abbreviations.

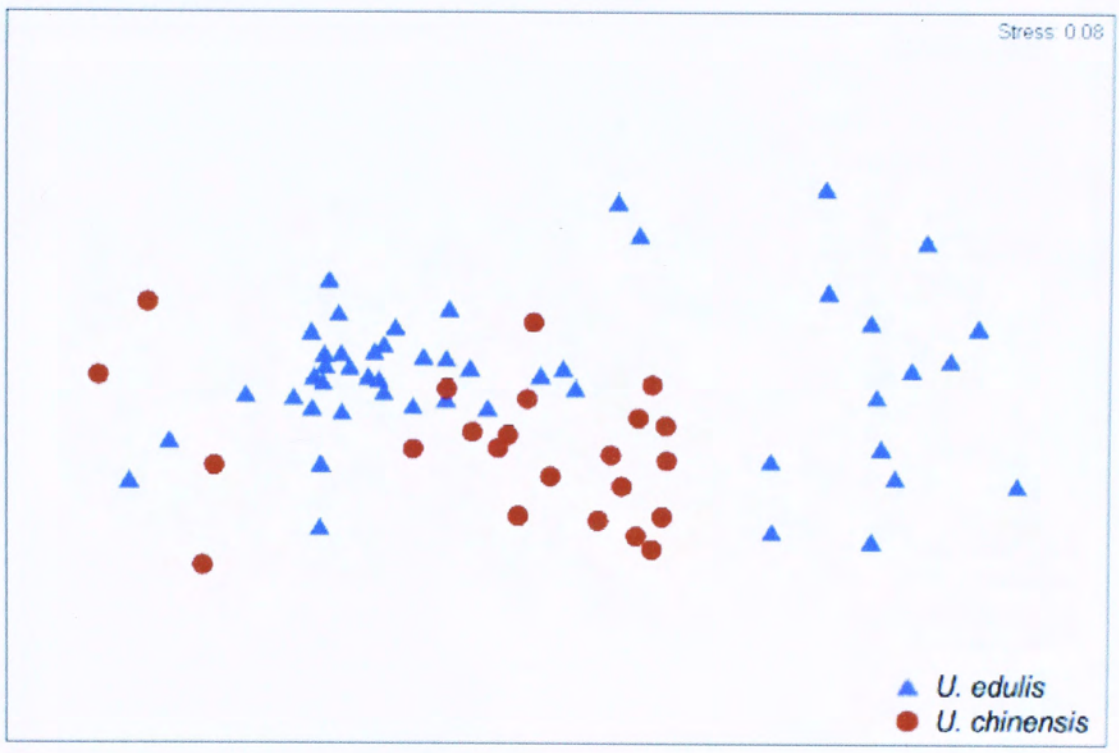

Fig. 2.9 Multidimensional scaling (MDS) plot on the morphometric indices of *Uroteuthis chinensis* and *U. edulis*

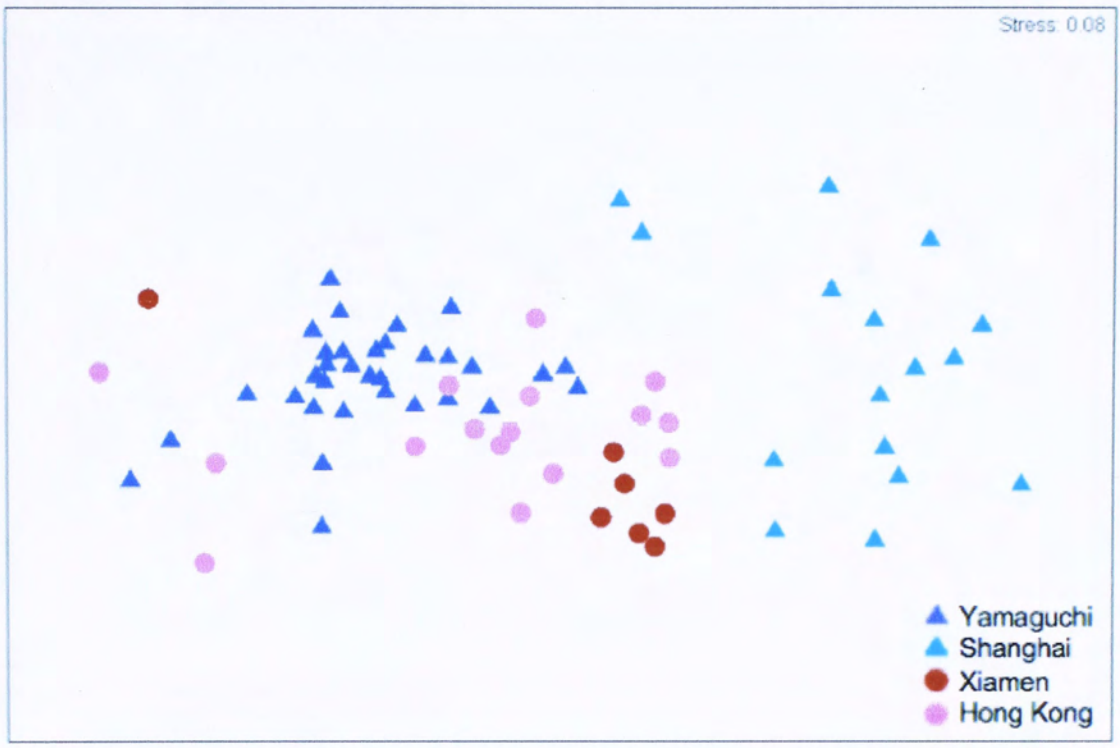

Fig. 2.10 Multidimensional scaling (MDS) plot on the morphometric indices of *Uroteuthis chinensis* and *U. edulis*, grouped according to locality. Triangles indicate *U. edulis* and circle indicate *U. chinensis*.

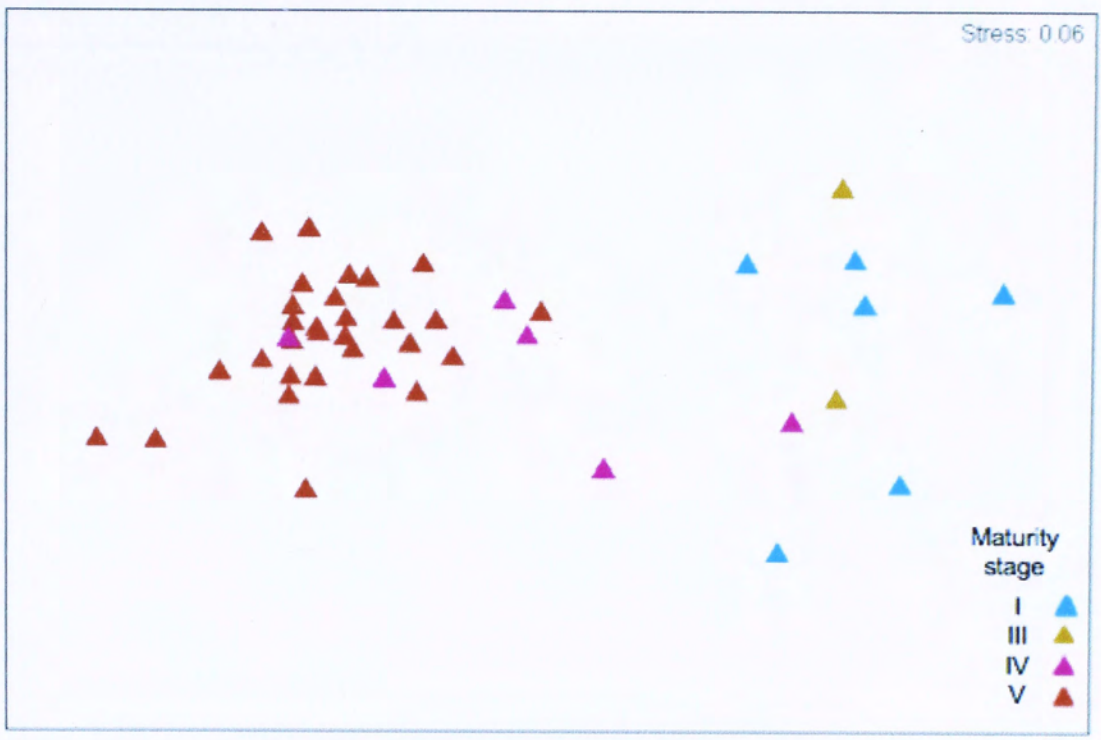

Fig. 2.11 Multidimensional scaling (MDS) plots on the morphometric indices of male *U. edulis*, grouped according to maturity stage.

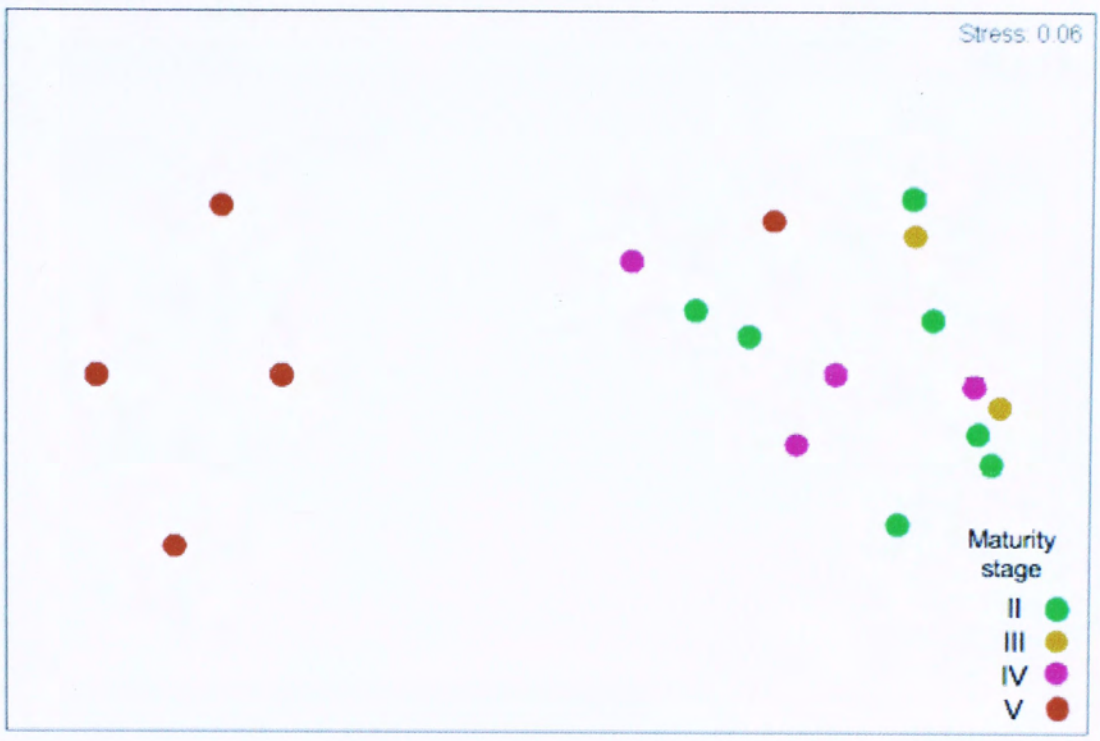

Fig. 2.12 Multidimensional scaling (MDS) plots on the morphometric indices of male *Uroteuthis chinensis*, grouped according to maturity stage.

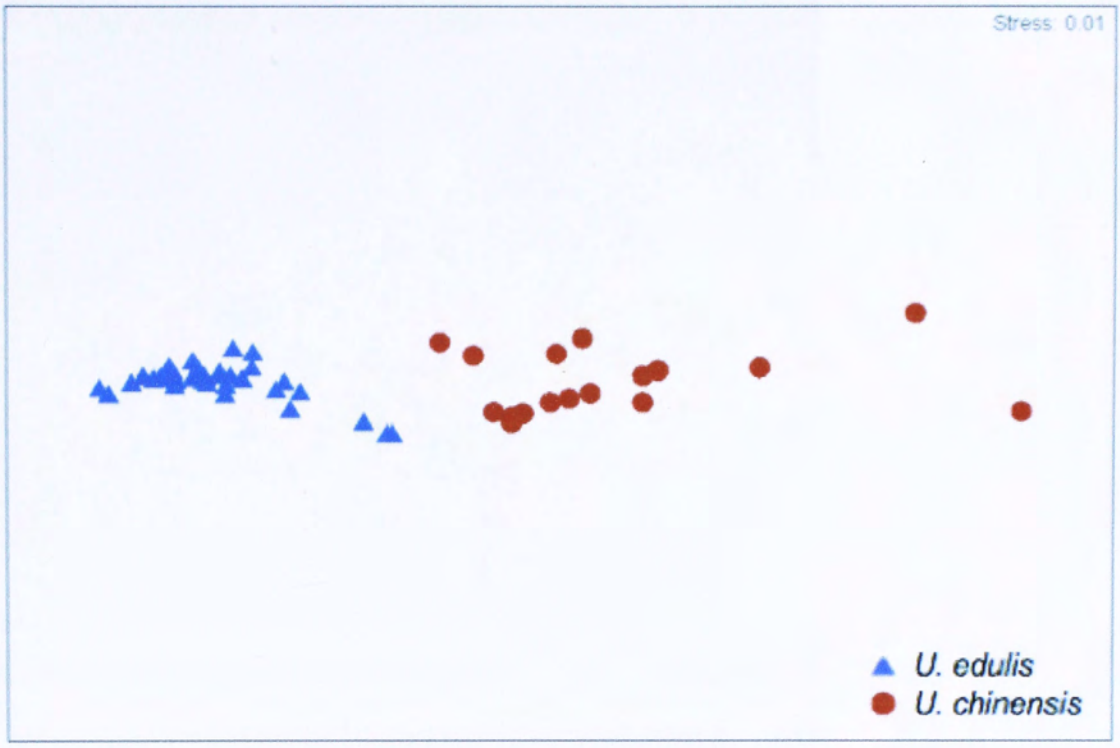

Fig. 2.13 Multidimensional scaling (MDS) plots on the morphometric indices, including the percentage of hectocotylus, of mature male *Uroteuthis chinensis* and *U. edulis*

Fig. 2.14 Hectocotylus of *Uroteuthis chinensis*. The black bar indicates the HL% of left arm IV.

Fig. 2.15 Hectocotylus of *Uroteuthis edulis*. The black bar indicates the HL% of left arm IV.

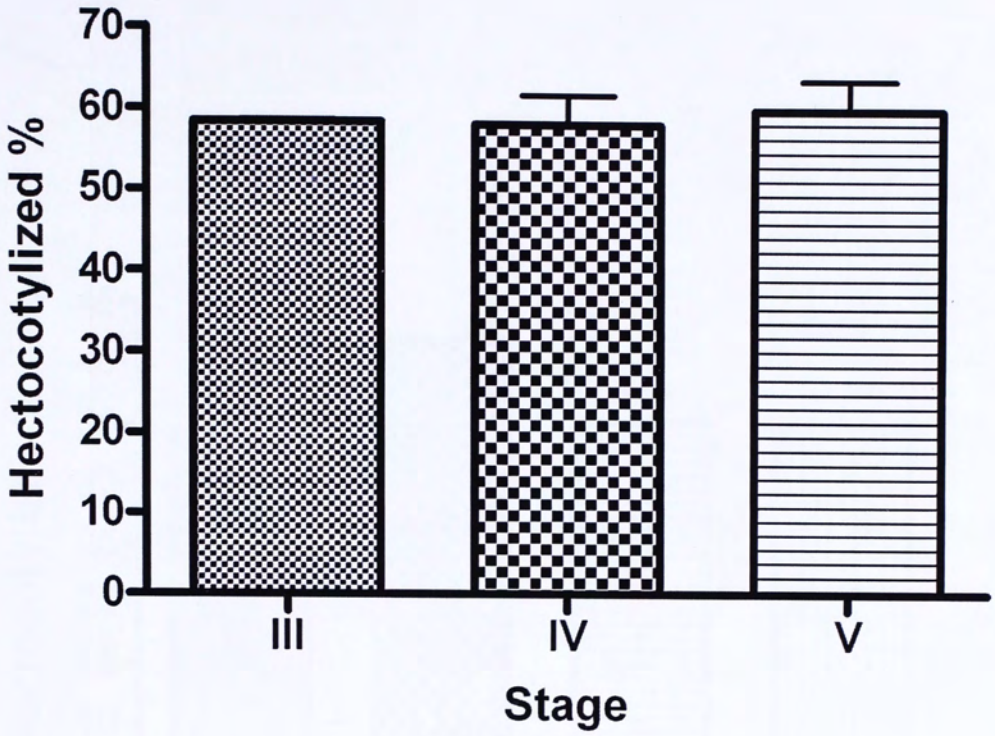

Fig. 2.16 HL% for stage III (n=2), stage IV (n=6) and stage V (n=37) of *U. edulis*

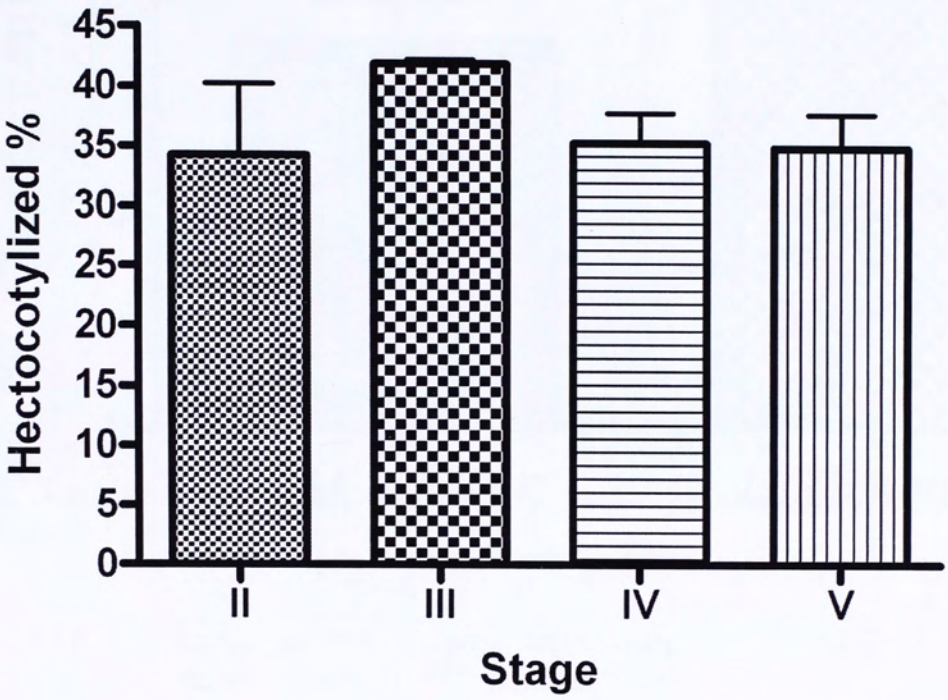

Fig. 2.17 HL% for stage II (n=8), stage III (n=2), stage IV (n=4) and stage V (n=6) of *U. chinensis*

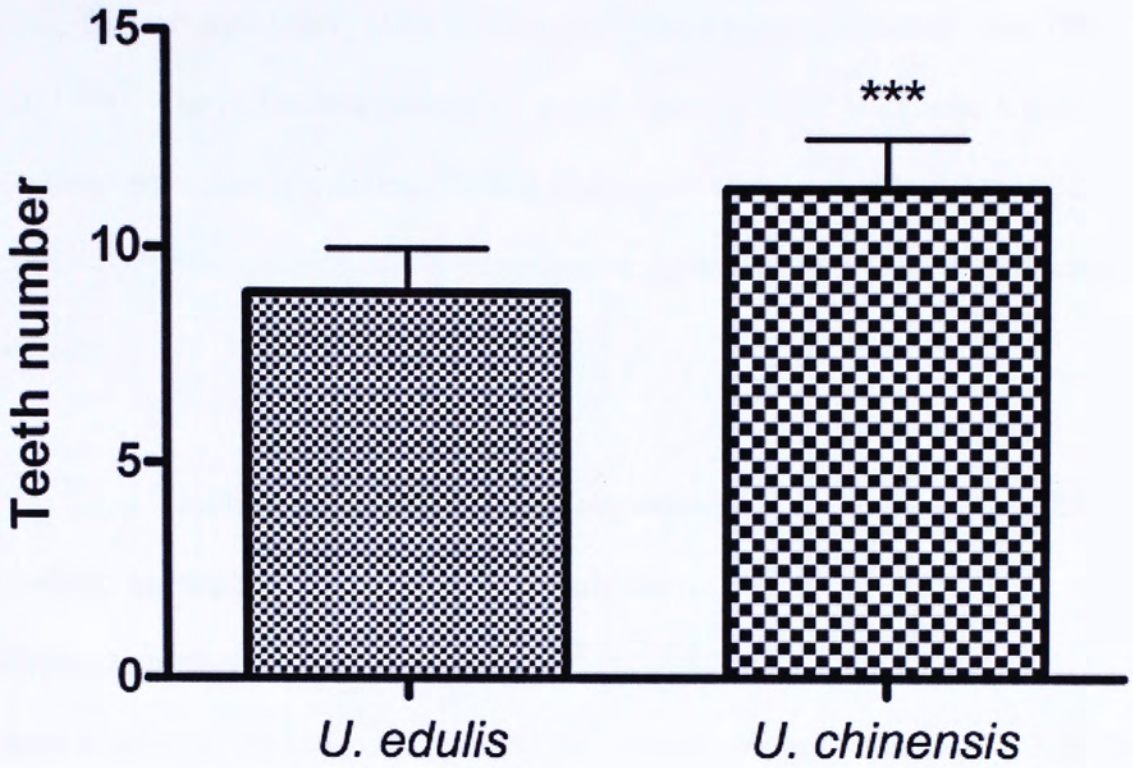

Fig. 2.18 Teeth number on arm sucker ring, for *U. edulis* (n=66) and *U. chinensis* (n=25). *** indicates P value < 0.001.

2.4 Discussion

The soft squid body leads to some difficulties in morphometric study (Pierce et al. 1994). The difficulties include the easily damaged squid body, which may lose their body parts during trawling. In this study some samples had lost their tentacles or other body parts, which lead to reduction in the sample size used in multivariate analysis.

In this multivariate study, the two loliginid squids, *Uroteuthis chinensis* and *U. edulis*, are highly similar in their morphometric indices as deduced by visual interpretation of the MDS plot (Fig. 2.9) and ANOSIM. The 27 morphometric indices cannot be used to distinguish the two species. Thus it is not able to use this method to differentiate the two species.

The analysis shows that the morphometric indices are affected by maturity stages, indicating that the squids grow allometrically. It was reported that the size composition of the octopus samples could affect morphological indices (Voight 1991; 1994), and some indices are highly correlated with size in loliginid squids (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 to capture more food for growth. At late maturity stage, the energy resources would switch to the expense of reproduction. As the squid matures, the mantle length

becomes larger, relative to the tentacles. It has been documented in other loliginid squids that the reproductive organs developed quickly at the onset of maturity (Barón and Ré 2002b).

While it is common in cephalopods that morphometric characters are geographically variable (Carvalho and Nigmatullin 1998), it is also found that this variability could be insignificant among distant populations of some loliginid squids (Cohen 1976). In this study, the difference between squids of different localities could be explained by their difference in the composition of individuals at various maturity stages from different localities. The *U. chinensis* samples from Hong Kong and Xiamen are of similar maturity stage composition (Table 2.4), and hence no separation is revealed for individuals from these two places. For *U. edulis*, most individuals are at stage V for samples from Yamaguchi, and most individuals are at stage I for samples from Shanghai. So the observed difference between these two localities is most probably due to the difference in maturity stage composition. This is supported by the index that contributes most to the difference between stages is the index that contributed most to the difference between localities.

With the addition of the percentage of length of hectocotylized part to total length of left arm IV (HL%) of mature male into the analysis, the two species are clearly separated from each other. It shows that only this index is informative for species identification of *U. chinensis* and *U. edulis*, among all indices under this study. This result validates previous observation of the HL% to be 33-40% in *U. chinensis* (Gray 1849) and 50-67% in *U. edulis* (Hoyle 1885), as the HL% of *U. chinensis* (mean: 35.7%) and *U. edulis* (mean: 59.6%) in this study are within the

reported ranges. The HL% is not affected by maturity. The limitation of using this morphological character to identify the two species is that it cannot be used to identify females and immature males. Another character previously used to distinguish the two species is the teeth number of arm sucker ring, which ranges 6-12 in *U. edulis* (Hoyle 1885) and 10-18 in *U. chinensis* (Gray 1849). The values, 7-12 in *U. edulis* and 9-14 in *U. chinensis*, determined in the present study fall 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. In addition, meristic characters are highly sensitive to environment variations during their formation (Barlow 1961), which also makes the teeth number not a good character for species identification.

In conclusion, the HL% of male individuals, the teeth shape and number of arm sucker ring are informative characters in distinguishing between *Uroteuthis chinensis* and *U. edulis*. Other morphometric indices included in the present study are not informative to distinguish the two species, and hence no new characters could be identified for species identification. The results show that there is difference in morphometrics between individuals of different localities in *U. edulis*, which is most probably caused by difference in maturity stages composition. However, to determine the morphometric differences in the two species between different localities and among maturity stages has not been the main aim in the present study. Therefore results are preliminary for revealing the morphometric relationship between localities and maturity stages because sample size in some localities and/or stages is low.

Chapter 3

Genetic Differentiation of *Uroteuthis chinensis* and *Uroteuthis edulis* in Asia Based on Mitochondrial DNA Sequence Data

3.1 Introduction

The loliginid squids, *U. chinensis* and *U. edulis*, are two commercially important species. They are difficult to distinguish as they are very similar in morphology and their geographical range overlaps. The identification of *U. chinensis* and *U. edulis* is based mainly on the shape of the teeth on arm sucker ring (Natsukari and Okutani 1975) and hectocotylus length that can only be applied to mature males, since other morphological characters are not useful in discriminating the two species as stated in chapter 2. There is also no information available to identify the paralarval or early juvenile stages. In addition, it is believed that the widely distributed *U. chinensis* and *U. edulis* are composed of several cryptic species (Yeatman and Benzie 1994), which is common in squids (Augustyn and Grant 1988; Brierley et al. 1993a; Yeatman and Benzie 1993; 1994). However there has been no study on the population structure of *U. chinensis* and *U. edulis* along their distribution range.

In this study we aim to clarify the taxonomic identification of the two species by determining whether these two species are conspecific exhibiting clinal variation,

two distinct species, or more than two species with the presence of cryptic taxa. The mitochondrial genes, which have been used widely in addressing such issues (Triantafillos et al. 2004), were used in this study. Herke and Foltz (2002) used COI as the marker for population structure study in two loliginid squid species, *Loligo pealei* and *Loligo plei.* The COI (cytochrome *c* oxidase subunit I) and 16S rRNA genes, that have been used to reveal loliginid phylogeny (Anderson 2000b; a), were used in this study to clarify the taxonomic status of *U. chinensis* and *U. edulis*. In the present study we also tested if there was sufficient polymorphism in the mitochondrial genes to permit population-level studies in *U. chinensis* and *U. edulis*, so as to study their variation across their distribution range. The loliginid squids, *U. chinensis* and *U. edulis*, could be a single homogeneous population with extensive genetic exchange across their distribution range, or constitute localized populations with limited genetic exchange. The two mitochondrial genes sequenced in this study could contribute to the identification of unidentified samples by comparing the DNA sequences.

3.2 Materials and methods

3.2.1 Collection of specimens

Specimens of *U. chinensis* were collected from Hong Kong (China) and Xiamen (China) (Fig. 2.2, Table 3.1). Specimens of *U. edulis* were collected from Yamaguchi (Japan) and Shanghai (China). *Uroteuthis duvauceli* collected from Hong Kong, Xiamen and Shanghai was included in the analysis for comparison.

Specimens of *Sepioteuthis lessoniana* collected from Hong Kong were used as the outgroup for phylogenetic analysis.

The *U. chinensis* and *U. edulis* samples in this study were discriminated based on the shape of arm sucker ring teeth. The specimens were stored at -20°C immediately after collection. Tissues were taken from arm and mantle of each specimen and preserved in 95% ethanol before DNA extraction.

3.2.2 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 rRNA. The 16S rRNA 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. The PCR amplifications for the two genes were performed in 30 µl reaction mixture containing 1.5 µl of DNA extract, 0.4 µM of each primer, 0.2 µM of dNTPs (Qiagen, Hilden, Germany), 1 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), 1.5 mM of magnesium chloride (Invitrogen, Carlsbad, CA, USA), and 1X Mg^{2+} free PCR buffer. Thermal cycling for COI and16S rRNA gene

amplification were performed as follows: initial denaturation for 3 minutes at 94°C, followed by 32 cycles of 30 seconds at 94°C, 30 seconds at 52°C, and 30 seconds at 72°C, with a final extension at 72°C for 3 minutes.

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) following the manufacturer's instructions. The COI and 16S rRNA gene segments were sequenced using the same forward and reverse primers for PCR amplification. The 20 µl cycle sequencing mix contained 8 µl of ABI Prism dRodamine terminator (Applied Biosystems, Foster City, CA, USA), 3-6 µl of purified PCR products, 0.16 µM of primer, and ddH_2O to make up to 20 µl. The cycling profile involved 1 minute at 96°C, followed by 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C, and 4 minutes at 60°C. The products were purified with an ethanol-sodium acetate precipitation protocol to remove unincorporated primers and dNTPs. The purified products were loaded onto ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) for analysis. The sequences were confirmed by reference to both strands. All sequences were aligned and analyzed using ABI SeqEd version 1.0.3.

3.2.3 Sequence data analysis

Alignments of the data sets were conducted using Clustal W (Thompson et al. 1994) with default gap weighting parameters (gap opening penalty: 15; gap extension penalty: 6.66), and then adjusted by eye.

A total of 50 and 33 individuals of the squid samples were sequenced for COI and 16S rRNA gene respectively (Table 3.1). Four COI sequences and eight 16S rRNA sequences were downloaded from GenBank and incorporated in the analysis (Table 3.1). Some of the sequences from GenBank were based on samples that were classified as the genus *Loligo* (*L. chinensis*, *L. edulis* and *L. duvauceli*). Four methods were used to infer phylogenetic relationships: distance (BIO neighbor-joining, BIONJ), maximum parsimony (MP), and maximum likelihood (ML) performed in PAUP 4.0b10 (Swofford 2002), and Bayesian inference (BI) by using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Analyses were conducted for each data set separately (COI, 54 sequences; 16S rRNA, 41 sequences) and for the combined data set (29 sequences).

Prior to analyzing the combined data set, a test of partition homogeneity (incongruence length difference (ILD) test of Farris et al., 1995) was performed with PAUP 4.0b10 (Swofford 2002) to examine possible incongruence between genes. One thousand replicates of the ILD test were implemented. No evidence was presented for phylogenetic conflict between COI and 16S rRNA gene partitions (P=0.789), therefore justifying a combined data approach. The best-fit model of nucleotide substitution used for BIONJ, ML and BI analyses was determined in Modeltest version 3.5 (Posada and Crandall 1998) using the hierarchical likelihood ratio test (hLRT, Huelsenbeck and Crandall, 1997).

Heuristic MP and ML searches were executed with all characters unordered and equally weighted, and using tree bisection reconnection (TBR) branch swapping with as-is stepwise addition. Starting tree for branch-swapping was obtained by

stepwise addition. Gaps were treated as missing. Bootstrap analysis, based on full heuristic search of 1000 and 500 pseudoreplicates using TBR branch-swapping and as-is stepwise addition, was carried out to determine the MP and ML branch support respectively. One thousand bootstrap replicates were performed in BIONJ analysis to assess the confidence level at each branch. Bayesian inference analysis was performed with specific models and parameters assigned separately to the individual and combined data set. A Markov chain Monte Carlo search was run for 500,000 generations with a sampling frequency of 100 generations. A consensus tree was calculated after omitting the first 20% trees as burn-in.

A haplotype network was conducted using the 95% parsimony criterion as implemented by the program TCS version 1.13 (Clement et al. 2000) to determine the genealogical relationship among haplotypes.

3.3 Results

3.3.1 Sequence data set

Both COI and 16S rRNA gene segments were successfully amplified from 50 and 33 individuals respectively of the four species studied (including outgroup taxa) (Table 3.1). The aligned COI segments consisted of 587 bp. There are 190 variable sites of which 163 are parsimony-informative. The nucleotide composition is 29.2% A, 35.1% T, 20.2% C, and 15.6% G (A+T = 55.3%). With an aligned length of 522 bp in the 16S rRNA sequences, there are 135 variable sites of which 93 are

parsimony-informative. The nucleotide composition is 32.3% A, 38.2% T, 10.5% C, and 19.0% G (A+T = 70.8%).

The pairwise Kimura's 2-parameter distances between sequences of each species for the two gene segments are listed in tables 3.2 and 3.3. For each species pair, the divergence is always higher in COI than in 16S rRNA. For instance, the sequence divergences between *U. chinensis* and *U. edulis* are 15.50 % (14.7%-16.2%, excluding AY185505) and 7.71 % (7.5%-7.9%, excluding AF369956, AJ000103 and AF369955) for COI and 16S rRNA, respectively.

3.3.2 Phylogenetic analysis

Results of the homogeneity test supports combined analysis of COI and 16S rRNA genes (P = 0.789). In the combined data set analysis, the best-fit DNA substitution model is General Time Reversible incorporating rate variation among sites (GTR+G with base frequencies A=0.310, C=0.157, G=0.169, T=0.365; R(A-G)=10.201, R(C-T)=20.276, R(A-C)=2.825, R(A-T)=4.478, R(C-G)=0.000, R(G-T)=1.000; I=0; G=0.158) (Rodriquez et al. 1990). In COI analysis, the best-fit DNA substitution model is General Time Reversible incorporating rate variation among sites (GTR+G with base frequencies A=0.286, C=0.213, G=0.154, T=0.347; R(A-G)=49.106, R(C-T)=133.275, R(A-C)=17.028, R(A-T)=35.645, R(C-G)=0.000, R(G-T)=1; I=0; G=0.230). The transversion model incorporating rate variation among sites (TVM+G) with base frequencies A=0335, C=0.095, G=0.186, T=0.384; R(A-G)=3.136, R(C-T)=3.136, R(A-C)=0.245, R(A-T)=1.407, R(C-G)=0.000, R(G-T)=1; I=0; G=0.176) is most appropriate for the 16S rRNA data set. MP analysis of

the combined analysis gave 22 most parsimonious trees, with 377 steps, a consistency index (CI) of 0.862 and a retention index (RI) of 0.971. MP analysis of the COI and 16S rRNA data sets analysis gave 136 and 4 most parsimonious trees, with 360 and 205 steps, CI of 0.708 and 0.824, and RI of 0.955 and 0.962, respectively.

Neighbor-joining trees of mitochondrial COI gene sequences of 54 individuals were constructed. Sequence data of the COI gene reveal that *Uroteuthis chinensis*, *U. edulis*, and *U. duvauceli*, together with the outgroup *Sepioteuthis lessoniana*, each form a distinct clade (Fig. 3.1). The clustering of all *U. chinensis* (except *Loligo chinensis* (AY185505)) is highly supported (≥98% bootstrap (BP) support in BIONJ, MP and ML analyses; Bayesian posterior probabilities (BPP)=1.00). The grouping of all *U. edulis* is also well supported (BP values 100% in BIONJ, MP and ML analyses; BPP=1.00). The *U. duvauceli* (BP values ≥99% in BIONJ, MP and ML analyses; BPP=1.00) and outgroup *S. lessoniana* (BP values 100% in BIONJ, MP and ML analyses; BPP=1.00) each also form a well-supported clade. In the COI gene tree, the grouping of the three *Uroteuthis* species is supported (BP values ≥79% in BIONJ, MP and ML analyses; BPP=1.00). *U. chinensis* is more closely related to *U. duvauceli* than to *U edulis*, but with a very weak support (BP values ≥40% in BIONJ, MP and ML analyses; BPP=0.63). The outgroup, *Sepioteuthis lessoniana*, is distantly related to *U. chinensis* and *U. edulis*, with nucleotide divergence 20.3-23.4%.

Neighbor-joining trees of mitochondrial 16S rRNA gene sequences of 41 individuals were also constructed. Sequence data of the 16S rRNA gene also reveal

that the four species each form a distinct clade (Fig. 3.2). The clustering of all *U. chinensis* (except *Loligo chinensis* (AF369955)) is highly supported (BP values 100% in BIONJ, MP and ML analyses; BPP=1.00). The grouping of all *U. edulis* (except *L. edulis* (AJ000103 and AF369956)) is also well supported (BP values ≥99%% in BIONJ, MP and ML analyses; BPP=0.95). The clade of *U. duvauceli* (include *L. edulis* (AJ000103)) is supported (BP values ≥82% in BIONJ, MP and ML analyses; BPP=0.9). The outgroup, *S. lessoniana* (BP values 100% in BIONJ, MP and ML analyses; BPP=1.00) also form a well-supported clade. In the 16S rRNA gene tree, the grouping of the three *Uroteuthis* genus is supported (BP values ≥74% in BIONJ, MP and ML analyses; BPP=0.85). *U. chinensis* were more closely related to *U. edulis*, with a weak support (BP values ≥51% in BIONJ, MP and ML analyses; BPP=0.83). The nucleotide divergence between *U. chinensis* and *U. edulis* was 7.5-7.9% (Table 3.3). The outgroup, *Sepioteuthis lessoniana*, was distantly related to *U. chinensis* and *U. edulis*, with nucleotide divergence 8.4-10.8%.

In the combined gene tree, there were ten and eight individuals of *U. chinensis* and *U. edulis* respectively. The four species each form a distinct clade (Fig. 3.3). The grouping of *U. chinensis* (BP values 100% in BIONJ, MP and ML analyses; BPP=1.00) and *U. edulis* (BP values 100% in BIONJ, MP and ML analyses; BPP=1.00) is strongly supported. *U. duvauceli* also forms a well-supported clade (BP values ≥97% in BIONJ, MP and ML analyses; BPP=0.98). The grouping of the three *Uroteuthis* species is highly supported (BP values 100% in BIONJ, MP and ML analyses; BPP=1.00), with *U. chinensis* and *U. edulis* more closely related (BP values ≥56% in BIONJ, MP and ML analyses; BPP=0.98).

For the four COI sequences from GenBank, AF075394 (*U. chinensis*), AF075398 (*U. duvauceli*) and AF075400 (*U. duvauceli*) are clustered with the sequences of the corresponding species determined in the present study in the phylogenetic tree. Yet AY185505 (*L. chinensis*) is not grouped with the sequences of *U. chinensis* and is very divergent from them (Fig. 3.1). It is not inside the *Uroteuthis* clade and Basic Local Alignment Search Tool (BLAST) result shows that it is more similar to sequence of *Loligo opalescens* (99%). The sequence of *U. chinensis* (AF075394) from the Gulf of Thailand is a little divergent from the grouping of Hong Kong and Xiamen samples. The sequences of *U. duvauceli* (AF075398 and AF075400) from the Andaman Sea are divergent from the grouping of Hong Kong, Xiamen and Shanghai samples. For the eight 16S rRNA sequences from GenBank, AF110091 (*U. chinensis*), AJ000105 (*L. chinensis*), AF110093 (*U. duvauceli*), AF110092 (*U. duvauceli*), and AJ000101 (*L. duvauceli*) are clustered with sequences of the corresponding species in the phylogenetic tree, while AJ000103 (*L. edulis*), AF369956 (*L. edulis*) and AF369955 (*L. chinensis*) are not grouped with the sequences of the same species. 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 showed that they are most similar to *Sepia robsoni* (100%) and *Loligo opalescens* (99%) respectively. The sequence of *U. chinensis* (AF110091) from the Gulf of Thailand is divergent from the grouping of Hong Kong and Xiamen samples. The sequence of *U. duvauceli* (AF110093 and AF110092) from the Gulf of Thailand and Andaman Sea are divergent from the grouping of Hong Kong samples and each other. The four

sequences that are not grouped with the sequences of the same species come from two research groups, and they are not included in the haplotype analysis.

3.3.3 Sequence divergence within species

The intraspecific sequence divergences in COI are 0-1.1% within *U. chinensis* (excluding AY185505) and 0-0.3% within *U. edulis*, and the values for 16S rRNA are 0-2.2% within *U. chinensis* (excluding AF369955) and 0% within *U. edulis* (excluding AJ000103 and AF369956).

In *U. duvauceli*, the intraspecific sequence divergences in COI are 0-0.5% within Hong Kong-Xiamen-Shanghai grouping, and 5.9-6.4% between Andaman Sea individuals and the Hong Kong-Xiamen-Shanghai grouping (Table 3.2). The COI sequences of Andaman Sea individuals are clearly divergent from the Hong Kong-Xiamen-Shanghai grouping (Fig. 3.1). The intraspecific sequence divergences values for 16S rRNA are 0-0.2% within Hong Kong grouping, 0.7-1.5% between Gulf of Thailand individual and Hong Kong grouping, and 1-1.2% between Andaman Sea individual and Hong Kong grouping (Table 3.3). But the divergence between sequence of Gulf of Thailand and Andaman Sea is just 0.2%. The Gulf of Thailand and Andaman Sea individuals are divergent from the Hong Kong grouping (Fig. 3.2).

Eighteen fixed nucleotide substitutions (Ts: 13, Tv: 5) are found within *U. chinensis* (Table 3.4) of the 19 COI sequences analyzed. The frequency distribution of the haplotypes in each locality is shown in figure 3.4. Four fixed nucleotide

substitutions (Ts: 3, Tv: 1) are found within *U. edulis* (Table 3.5) of the 16 COI sequences analyzed. The frequency distribution of the haplotypes in each locality is shown in figure 3.5. The present results yielded 13 and 5 distinct haplotypes for *U. chinensis* (Fig. 3.6, Table 3.6) and *U. edulis* (Fig. 3.7, Table 3.7) respectively, with two haplotypes shared by individuals from both localities for each of the two species. Haplotype networks for COI sequences of *U. chinensis* and *U. edulis* (Figs. 3.6, 3.7) reveal that the individual haplotypes are randomly distributed within the network without any clustering with respect to localities. Many haplotypes are only found in a single individual. In *U. chinensis*, one haplotype (A) is shared by two individuals from Hong Kong and two from Xiamen, while another haplotype (B) is shared by one individual from Hong Kong and two from Xiamen (Fig. 3.6). The other haplotypes are found in single individual. The haplotype of the GenBank sequence from individual in Gulf of Thailand differs from the most common haplotype (A) by three transversions. In *U. edulis*, eight individuals from Yamaguchi and two from Shanghai share one haplotype (A) (Fig. 3.7), and two individuals from Yamaguchi and one from Shanghai share another haplotype (B). The other haplotypes are found in single individual.

Nine fixed nucleotide substitutions (Ts: 5, Tv: 4) are found in the 16 16S rRNA sequences of *U. chinensis* (Table 3.8). The frequency distribution of the haplotypes in each locality is shown in figure 3.8. There are five distinct haplotypes (Table 3.9), with one haplotype shared by individuals from the two localities (nine from Hong Kong and three from Xiamen). The other four haplotypes are only found in single individuals, which are from localities different from each others. The 16S rRNA haplotype network (Fig. 3.9) revealed no clustering with respect to localities.

The sequence of Thailand sample from GenBank differs from the most common haplotype by two transitions and one transversion (Fig. 3.9). The sequence of a sample of unknown locality differs from a haplotype of Xiamen sample (C) by one transition and three transversions. This sample may come from a locality that is far away from Hong Kong and Xiamen, and even farther away from the Gulf of Thailand, such as some southeast Asian countries, e.g. the Philippines.

The frequency distribution of COI and 16S rRNA haplotypes revealed no population structure among localities. This was also revealed in an analysis of F_{ST} based on COI sequences (Table 3.10), which showed that there was no significant genetic differentiation detected among populations in different localities.

Table 3.1 Sample localities of *Uroteuthis chinensis, U. edulis, U. duvauceli* and *Sepioteuthis lessoniana*. The number of individuals studied for COI and 16S rRNA sequences. Asterisk indicates GenBank sequence. [a, b, c] indicate sequence from different research groups. [a] Anderson, 2000; [b] Lin *et al.* (2004) or the same research group; [c] A research group involved Liu, L., Hudelot, C., Boucher-Rodoni, R., Lu, C. and Bonnaud, L. # indicates sequences that are derived from misidentified individuals.

Species	Sample locality	Sequence no.	
		COI	16S rRNA
Uroteuthis chinensis	Hong Kong (China)	10	10
	Xiamen (China)	8	4
	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 edulis	Yamaguchi (Japan)	10	5
	Shanghai (China)	6	4
'*Loligo edulis*'	unknown	--	2* (AF369956 [b] #, AJ000103 [c] #)
Uroteuthis duvauceli	Hong Kong (China)	7	6
	Shanghai (China)	3	--
	Xiamen (China)	2	--
	Gulf of Thailand	--	1* (AF110093 [a])
	Andamen Sea	2* (AF075398 [a], AF075400 [a])	1* (AF110092 [a])
'*Loligo duvauceli*'	unknown	--	1* (AJ000101 [c])
Sepioteuthis lessoniana	Hong Kong (China)	4	4
Total		54	41

Table 3.2 Kimura's 2-parameter distances of the COI genes between and within *U. chinensis*, *U. edulis* and the outgroups.
1-10: *U. chinensis* (Hong Kong), 11-18: *U. chinensis* (Xiamen), 19: *U. chinensis* (Gulf of Thailand, AF075394), 20: *L. chinensis* (AY185505), 21-30: *U. edulis* (Yamaguchi), 31-36: *U. edulis* (Shanghai), 37-43: *U. duvauceli* (Hong Kong), 44-45: *U. duvauceli* (Xiamen), 46-48: *U. duvauceli* (Shanghai), 49-50: *U. duvauceli* (Andaman Sea, AF075398, AF075400), 51-54: *Sepioteuthis lessoniana* (Hong Kong)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
[1]																																																							
[2]	0.002																																																					
[3]	0.005	0.003																																																				
[4]	0.003	0.002	0.005																																																		
[5]	0.003	0.002	0.005	0.003																																																
[6]	0.003	0.002	0.002	0.003	0.003																																															
[7]	0.003	0.002	0.005	0.003	0.003	0.003																																														
[8]	0.003	0.002	0.002	0.003	0.003	0.000	0.003																																													
[9]	0.002	0.000	0.003	0.002	0.002	0.002	0.002	0.002																																												
[10]	0.003	0.002	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.002																																									
[11]	0.005	0.003	0.007	0.005	0.005	0.005	0.005	0.002	0.005	0.003	0.005																																								
[12]	0.002	0.000	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.003																																							
[13]	0.007	0.005	0.005	0.007	0.007	0.003	0.007	0.003	0.005	0.007	0.009	0.005																																							
[14]	0.002	0.000	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.003	0.000	0.005																																						
[15]	0.005	0.003	0.007	0.005	0.005	0.005	0.005	0.005	0.063	0.005	0.007	0.003	0.009	0.003																																						
[16]	0.003	0.002	0.005	0.003	0.003	0.003	0.003	0.000	0.003	0.002	0.003	0.002	0.002	0.007	0.002	0.005																																						
[17]	0.003	0.002	0.005	0.003	0.003	0.003	0.000	0.003	0.002	0.003	0.002	0.002	0.007	0.002	0.005	0.000																																						
[18]	0.007	0.005	0.009	0.007	0.007	0.007	0.007	0.007	0.005	0.007	0.009	0.005	0.010	0.005	0.009	0.007	0.007																																					
[19]	0.007	0.005	0.009	0.007	0.007	0.007	0.007	0.007	0.005	0.007	0.009	0.005	0.011	0.005	0.009	0.007	0.007	0.011																																					
[20]	0.198	0.198	0.203	0.201	0.201	0.201	0.201	0.201	0.198	0.196	0.201	0.198	0.198	0.198	0.196	0.201	0.201	0.198	0.196																																					
[21]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188																																					
[22]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000																																				
[23]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000	0.000																																				
[24]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000	0.000	0.000																																				
[25]	0.155	0.153	0.153	0.151	0.155	0.151	0.155	0.151	0.153	0.151	0.158	0.153	0.155	0.153	0.151	0.155	0.155	0.152	0.149	0.185	0.002	0.002	0.002	0.002																																				
[26]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000	0.000	0.000	0.000	0.002																																			
[27]	0.155	0.153	0.153	0.151	0.155	0.151	0.155	0.151	0.153	0.151	0.158	0.153	0.155	0.153	0.151	0.155	0.155	0.152	0.149	0.185	0.002	0.002	0.002	0.002	0.000	0.002																																			
[28]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000	0.000	0.000	0.000	0.002	0.000	0.002																																			
[29]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000																																			
[30]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000	0.000																																			
[31]	0.160	0.157	0.157	0.155	0.160	0.155	0.160	0.155	0.157	0.155	0.162	0.157	0.160	0.157	0.155	0.160	0.160	0.157	0.153	0.192	0.002	0.002	0.002	0.002	0.004	0.002	0.004	0.002	0.002	0.002																																			
[32]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.003	0.003	0.003	0.003	0.002	0.003	0.002	0.003	0.003	0.003	0.005																																			
[33]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.002	0.003																																			
[34]	0.153	0.151	0.151	0.149	0.153	0.149	0.153	0.149	0.151	0.149	0.155	0.151	0.153	0.151	0.149	0.153	0.153	0.150	0.147	0.183	0.003	0.003	0.003	0.003	0.002	0.003	0.002	0.003	0.003	0.003	0.005	0.003	0.003																																		
[35]	0.158	0.155	0.155	0.153	0.158	0.153	0.158	0.153	0.155	0.153	0.160	0.155	0.158	0.155	0.153	0.158	0.158	0.155	0.152	0.188	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.002	0.003	0.000	0.003																																		
[36]	0.156	0.153	0.153	0.151	0.156	0.151	0.156	0.151	0.153	0.151	0.158	0.153	0.156	0.153	0.151	0.156	0.156	0.153	0.149	0.186	0.002	0.002	0.002	0.002	0.000	0.002	0.000	0.002	0.002	0.002	0.004	0.002	0.002	0.002																																		
[37]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.218	0.147	0.147	0.147	0.147	0.145	0.147	0.145	0.147	0.147	0.147	0.144	0.147	0.147	0.143	0.147	0.146																																		
[38]	0.157	0.155	0.159	0.157	0.153	0.157	0.157	0.157	0.155	0.157	0.159	0.155	0.161	0.155	0.155	0.157	0.157	0.152	0.150	0.218	0.146	0.146	0.146	0.146	0.143	0.146	0.143	0.146	0.146	0.146	0.142	0.146	0.146	0.141	0.146	0.144	0.000																																		
[39]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.218	0.147	0.147	0.147	0.147	0.145	0.147	0.145	0.147	0.147	0.147	0.144	0.147	0.147	0.143	0.147	0.146	0.000	0.000																																		
[40]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.218	0.147	0.147	0.147	0.147	0.145	0.147	0.145	0.147	0.147	0.147	0.144	0.147	0.147	0.143	0.147	0.146	0.000	0.000	0.000																																		
[41]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.213	0.152	0.152	0.152	0.152	0.150	0.152	0.150	0.152	0.152	0.152	0.149	0.152	0.152	0.147	0.152	0.150	0.003	0.003	0.003	0.003																																		
[42]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.218	0.147	0.147	0.147	0.147	0.145	0.147	0.145	0.147	0.147	0.147	0.144	0.147	0.147	0.143	0.147	0.146	0.000	0.000	0.000	0.000	0.003																																		
[43]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.218	0.147	0.147	0.147	0.147	0.145	0.147	0.145	0.147	0.147	0.147	0.144	0.147	0.147	0.143	0.147	0.146	0.000	0.000	0.000	0.000	0.003	0.000																																		
[44]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.218	0.147	0.147	0.147	0.147	0.145	0.147	0.145	0.147	0.147	0.147	0.144	0.147	0.147	0.143	0.147	0.146	0.000	0.000	0.000	0.000	0.003	0.000	0.000																																		
[45]	0.154	0.152	0.157	0.154	0.150	0.154	0.154	0.154	0.152	0.154	0.157	0.152	0.159	0.152	0.152	0.154	0.154	0.149	0.148	0.215	0.145	0.145	0.145	0.145	0.143	0.145	0.143	0.145	0.145	0.145	0.142	0.145	0.145	0.141	0.145	0.143	0.002	0.002	0.002	0.002	0.005	0.002	0.002	0.002																																		
[46]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.218	0.147	0.147	0.147	0.147	0.145	0.147	0.145	0.147	0.147	0.147	0.144	0.147	0.147	0.143	0.147	0.146	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.002																																		
[47]	0.159	0.157	0.161	0.159	0.154	0.159	0.159	0.159	0.157	0.159	0.161	0.157	0.163	0.157	0.157	0.159	0.159	0.154	0.153	0.220	0.150	0.150	0.150	0.150	0.147	0.150	0.147	0.150	0.150	0.150	0.147	0.150	0.150	0.145	0.150	0.148	0.002	0.002	0.002	0.002	0.005	0.002	0.002	0.002	0.003	0.002																																		
[48]	0.157	0.154	0.159	0.157	0.152	0.157	0.157	0.157	0.154	0.157	0.159	0.154	0.161	0.154	0.154	0.157	0.157	0.152	0.150	0.218	0.147	0.147	0.147	0.147	0.145	0.147	0.145	0.147	0.147	0.147	0.144	0.147	0.147	0.143	0.147	0.146	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.002	0.000	0.002																																		
[49]	0.153	0.150	0.150	0.153	0.148	0.148	0.153	0.148	0.150	0.148	0.155	0.150	0.153	0.150	0.145	0.153	0.153	0.153	0.151	0.224	0.166	0.166	0.166	0.166	0.164	0.166	0.164	0.166	0.166	0.166	0.169	0.166	0.166	0.161	0.166	0.164	0.064	0.064	0.064	0.064	0.062	0.064	0.066	0.064																																		
[50]	0.172	0.170	0.170	0.172	0.168	0.168	0.168	0.168	0.170	0.172	0.170	0.170	0.172	0.170	0.170	0.168	0.168	0.166	0.166	0.219	0.159	0.159	0.159	0.159	0.157	0.159	0.157	0.159	0.159	0.159	0.155	0.159	0.157	0.059	0.059	0.059	0.059	0.063	0.059	0.059	0.059	0.061	0.059	0.061	0.059	0.072																																		
[51]	0.221	0.221	0.221	0.219	0.223	0.219	0.219	0.219	0.221	0.223	0.221	0.221	0.221	0.221	0.221	0.219	0.219	0.219	0.217	0.208	0.208	0.208	0.208	0.208	0.205	0.208	0.205	0.208	0.208	0.208	0.213	0.203	0.208	0.203	0.208	0.206	0.198	0.199	0.198	0.198	0.203	0.198	0.198	0.198	0.201	0.198	0.201	0.198	0.218	0.209																																		
[52]	0.221	0.221	0.221	0.219	0.223	0.219	0.219	0.219	0.221	0.223	0.221	0.221	0.221	0.221	0.221	0.219	0.219	0.219	0.217	0.208	0.208	0.208	0.208	0.208	0.205	0.208	0.205	0.208	0.208	0.208	0.213	0.203	0.208	0.203	0.208	0.206	0.198	0.199	0.198	0.198	0.203	0.198	0.198	0.198	0.201	0.198	0.201	0.198	0.218	0.209	0.000																																		
[53]	0.223	0.223	0.223	0.221	0.226	0.221	0.221	0.221	0.223	0.226	0.219	0.223	0.223	0.223	0.223	0.221	0.221	0.219	0.217	0.215	0.229	0.229	0.229	0.229	0.227	0.229	0.227	0.229	0.229	0.229	0.234	0.224	0.229	0.224	0.229	0.227	0.212	0.210	0.212	0.212	0.217	0.212	0.212	0.212	0.215	0.212	0.215	0.212	0.231	0.226	0.024	0.024																																		
[54]	0.221	0.221	0.221	0.219	0.223	0.219	0.219	0.219	0.221	0.223	0.221	0.221	0.221	0.221	0.221	0.219	0.219	0.219	0.217	0.208	0.208	0.208	0.208	0.208	0.205	0.208	0.205	0.208	0.208	0.208	0.213	0.203	0.208	0.203	0.208	0.206	0.198	0.199	0.198	0.198	0.203	0.198	0.198	0.198	0.201	0.198	0.201	0.198	0.218	0.209	0.000	0.000	0.024																																		

Table 3.3 Kimura's 2-parameter distances of the 16S rRNA genes between and within *U. chinensis*, *U. edulis* and the outgroups.
1-10: *U. chinensis* (Hong Kong), 11-14: *U. chinensis* (Xiamen), 15: *U. chinensis* (Gulf of Thailand, AF110091), 16: *L. chinensis* (AF369955), 17: *L. chinensis* (AJ000105), 18-22: *U. edulis* (Yamaguchi), 23-26: *U. edulis* (Shanghai), 27: *L. edulis* (AJ000103), 28: *L. edulis* (AF369956), 29-34: *U. duvauceli* (Hong Kong), 35: *L. duvauceli* (AJ000101), 36: *U. duvauceli* (Gulf of Thailand, AF110093), 37: *U. duvauceli* (Andaman Sea, AF110092), 38-41: *Sepioteuthis lessoniana* (Hong Kong)

[1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41]
[1]
[2] 0.000
[3] 0.000 0.000
[4] 0.002 0.002 0.002
[5] 0.000 0.000 0.000 0.002
[6] 0.000 0.000 0.000 0.002 0.000
[7] 0.000 0.000 0.000 0.002 0.000 0.000
[8] 0.000 0.000 0.000 0.002 0.000 0.000 0.000
[9] 0.000 0.000 0.000 0.002 0.000 0.000 0.000 0.000
[10] 0.000 0.000 0.000 0.002 0.000 0.000 0.000 0.000 0.000
[11] 0.000 0.000 0.000 0.002 0.000 0.000 0.000 0.000 0.000 0.000
[12] 0.002 0.002 0.002 0.004 0.002 0.002 0.002 0.002 0.002 0.002 0.002
[13] 0.000 0.000 0.000 0.002 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.002
[14] 0.000 0.000 0.000 0.002 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.002 0.000
[15] 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.011 0.008 0.008
[16] 0.097 0.097 0.097 0.097 0.099 0.099 0.099 0.099 0.099 0.099 0.099 0.097 0.099 0.099 0.049
[17] 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.008 0.010 0.010 0.022 0.103
[18] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.080 0.079
[19] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.080 0.079 0.000
[20] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.080 0.079 0.000 0.000
[21] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.080 0.079 0.000 0.000 0.000
[22] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.080 0.079 0.000 0.000 0.000 0.000
[23] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.078 0.079 0.000 0.000 0.000 0.000 0.000
[24] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.078 0.079 0.000 0.000 0.000 0.000 0.000 0.000
[25] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.078 0.079 0.000 0.000 0.000 0.000 0.000 0.000 0.000
[26] 0.077 0.077 0.077 0.079 0.077 0.077 0.077 0.077 0.077 0.077 0.077 0.075 0.077 0.077 0.021 0.078 0.079 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000
[27] 0.117 0.117 0.117 0.119 0.119 0.119 0.119 0.119 0.119 0.119 0.119 0.116 0.119 0.119 0.052 0.118 0.115 0.096 0.096 0.096 0.096 0.096 0.093 0.093 0.093 0.093
[28] 0.132 0.132 0.132 0.132 0.132 0.132 0.132 0.132 0.132 0.132 0.132 0.130 0.132 0.132 0.088 0.120 0.134 0.102 0.102 0.102 0.102 0.102 0.102 0.102 0.102 0.102 0.139
[29] 0.091 0.091 0.091 0.089 0.093 0.093 0.093 0.093 0.093 0.093 0.093 0.091 0.093 0.093 0.030 0.084 0.087 0.068 0.068 0.068 0.068 0.068 0.066 0.066 0.066 0.066 0.068 0.111
[30] 0.091 0.091 0.091 0.089 0.093 0.093 0.093 0.093 0.093 0.093 0.093 0.091 0.093 0.093 0.030 0.084 0.087 0.068 0.068 0.068 0.068 0.068 0.066 0.066 0.066 0.066 0.068 0.111 0.000
[31] 0.091 0.091 0.091 0.088 0.093 0.093 0.093 0.093 0.093 0.093 0.093 0.090 0.093 0.093 0.030 0.084 0.086 0.068 0.068 0.068 0.068 0.068 0.066 0.066 0.066 0.066 0.068 0.111 0.000 0.000
[32] 0.091 0.091 0.091 0.088 0.093 0.093 0.093 0.093 0.093 0.093 0.093 0.090 0.093 0.093 0.030 0.084 0.086 0.068 0.068 0.068 0.068 0.068 0.066 0.066 0.066 0.066 0.068 0.111 0.000 0.000 0.000
[33] 0.091 0.091 0.091 0.088 0.093 0.093 0.093 0.093 0.093 0.093 0.093 0.090 0.093 0.093 0.030 0.084 0.086 0.068 0.068 0.068 0.068 0.068 0.066 0.066 0.066 0.066 0.068 0.111 0.000 0.000 0.000 0.000
[34] 0.093 0.093 0.093 0.091 0.095 0.095 0.095 0.095 0.095 0.095 0.095 0.093 0.095 0.095 0.032 0.086 0.089 0.070 0.070 0.070 0.070 0.070 0.068 0.068 0.068 0.068 0.070 0.113 0.002 0.002 0.002 0.002 0.002
[35] 0.108 0.108 0.108 0.110 0.110 0.110 0.110 0.110 0.110 0.110 0.110 0.107 0.110 0.110 0.041 0.106 0.106 0.087 0.087 0.087 0.087 0.087 0.085 0.085 0.085 0.085 0.018 0.126 0.060 0.060 0.059 0.059 0.059 0.059
[36] 0.027 0.027 0.027 0.027 0.030 0.030 0.030 0.030 0.030 0.030 0.030 0.027 0.030 0.030 0.024 0.034 0.037 0.015 0.015 0.015 0.015 0.015 0.012 0.012 0.012 0.012 0.022 0.069 0.007 0.007 0.007 0.007 0.007 0.010 0.015
[37] 0.030 0.030 0.030 0.030 0.032 0.032 0.032 0.032 0.032 0.032 0.032 0.030 0.032 0.032 0.027 0.037 0.040 0.017 0.017 0.017 0.017 0.017 0.015 0.015 0.015 0.015 0.020 0.072 0.010 0.010 0.010 0.010 0.010 0.012 0.012 0.002
[38] 0.105 0.105 0.105 0.105 0.108 0.108 0.108 0.108 0.108 0.108 0.108 0.105 0.108 0.108 0.061 0.069 0.105 0.086 0.086 0.086 0.086 0.086 0.084 0.084 0.084 0.084 0.124 0.103 0.090 0.090 0.090 0.090 0.090 0.092 0.117 0.050 0.053
[39] 0.105 0.105 0.105 0.105 0.108 0.108 0.108 0.108 0.108 0.108 0.108 0.105 0.108 0.108 0.061 0.069 0.105 0.086 0.086 0.086 0.086 0.086 0.084 0.084 0.084 0.084 0.124 0.103 0.090 0.090 0.090 0.090 0.090 0.092 0.117 0.050 0.053 0.000
[40] 0.106 0.106 0.106 0.106 0.108 0.108 0.108 0.108 0.108 0.108 0.108 0.105 0.108 0.108 0.064 0.070 0.105 0.087 0.087 0.087 0.087 0.087 0.084 0.084 0.084 0.084 0.125 0.105 0.090 0.090 0.090 0.090 0.090 0.092 0.117 0.053 0.056 0.002 0.002
[41] 0.106 0.106 0.106 0.106 0.108 0.108 0.108 0.108 0.108 0.108 0.108 0.105 0.108 0.108 0.061 0.070 0.105 0.086 0.086 0.086 0.086 0.086 0.084 0.084 0.084 0.084 0.122 0.100 0.088 0.088 0.088 0.088 0.088 0.090 0.115 0.050 0.053 0.000 0.000 0.002

Table 3.4 Haplotypes and variable sites from 587 bp sequences of mitochondrial COI gene in *U. chinensis*. · nucleotide at the site is the same as that of the first haplotype (sequence). The number of individuals with a particular haplotype from a certain locality are shown inside parentheses. HK: Hong Kong, Xm: Xiamen, GT: Gulf of Thailand.

	Nucleotide position																	
Haplotype	41	43	44	65	128	209	222	224	326	428	488	497	515	524	539	542	554	569
COI-A (HK:2, Xm:2)	A	C	A	T	T	T	T	A	T	T	A	G	A	A	C	A	T	T
COI-B (HK:1, Xm:2)	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·
COI-C (HK:2)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·
COI-D (HK:1)	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·
COI-E (HK:1)	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·
COI-F (HK:1)	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·
COI-G (HK:1)	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
COI-H (HK:1)	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	C	·
COI-I (Xm:1)	·	·	·	·	·	C	·	·	·	·	G	·	·	·	·	·	C	·
COI-J (Xm:1)	·	·	·	·	·	·	·	G	·	·	·	·	·	G	·	·	·	·
COI-K (Xm:1)	·	·	·	·	·	·	·	·	·	·	·	A	G	·	·	·	·	·
COI-L (Xm:1)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	T	·	A
COI-M (GT:1)	C	A	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

Table 3.5 Haplotypes and variable sites from 587 bp sequences of mitochondrial COI gene in *U. edulis*. · nucleotide at the site is the same as that of the first haplotype (sequence). The number of individuals with a particular haplotype from a certain locality are shown inside parentheses. Jp: Japan, Sh: Shanghai.

Haplotype	Nucleotide position			
	126	338	353	389
COI-A (Jp:8, Sh:2)	C	G	T	G
COI-B (Jp:2, Sh:1)	·	·	·	A
COI-C (Sh:1)	A	·	·	·
COI-D (Sh:1)	·	·	C	A
COI-E (Sh:1)	·	A	·	A

Table 3.6 COI haplotype distribution, gene diversity and sample size in three populations of *U. chinensis*

Locality	Haplotype													Gene diversity (SD)	n
	COI-A	COI-B	COI-C	COI-D	COI-E	COI-F	COI-G	COI-H	COI-I	COI-J	COI-K	COI-L	COI-M		
HK	2	1	2	1	1	1	1	1						0.956 (0.059)	10
Xiamen	2	2							1	1	1	1		0.929 (0.084)	8
Thailand													1	--	1
Total	4	3	2	1	1	1	1	1	1	1	1	1	1		19

Table 3.7 COI haplotype distribution, gene diversity and sample size in two populations of *U. edulis*

Locality	Haplotype					Gene diversity (SD)	n
	COI-A	COI-B	COI-C	COI-D	COI-E		
Japan	8	2				0.356 (0.159)	10
Shanghai	2	1	1	1	1	0.933 (0.122)	6
Total	10	3	1	1	1		16

Table 3.8 Haplotypes and variable sites from 522 bp sequences of mitochondrial 16S rRNA gene in *U. chinensis*. · nucleotide at the site is the same as that of the first haplotype (sequence). The number of individuals with a particular haplotype from a certain locality are shown inside parentheses. HK: Hong Kong, Xm: Xiamen, GT: Gulf of Thailand.

Haplotype	Nucleotide position								
	4	26	174	175	177	253	255	345	346
16S-A (HK:9, Xm:3)	T	T	C	T	A	A	T	A	T
16S-B (HK:1)	·	·	·	·	·	·	·	G	·
16S-C (Xm:1)	·	·	T	·	·	·	·	·	·
16S-D (GT:1)	A	C	·	·	·	·	·	·	C
16S-E (unknown:1)	·	·	T	C	T	C	A	·	·

Table 3.9 16S rRNA haplotype distribution, gene diversity and sample size in *U. chinensis*

Locality	Haplotype					Gene diversity (SD)	n
	16S-A	16S-B	16S-C	16S-D	16S-E		
Hong Kong	9	1				0.2 (0.154)	10
Xiamen	3		1			0.5 (0.265)	4
Thailand				1		--	1
unknown					1	--	1
Total	12	1	1	1	1		16

Table 3.10 Pairwise F_{ST}-values for populations of *U. chinensis* (Hong Kong, Xiamen and Gulf of Thailand) and *U. edulis* (Japan and Shanghai) based on COI. *P* value > 0.05 for all comparisons.

	Hong Kong	Xiamen	Yamaguchi
Xiamen	-0.01881		
Gulf of Thailand	0.04444	0.07143	
Shanghai			0.11940

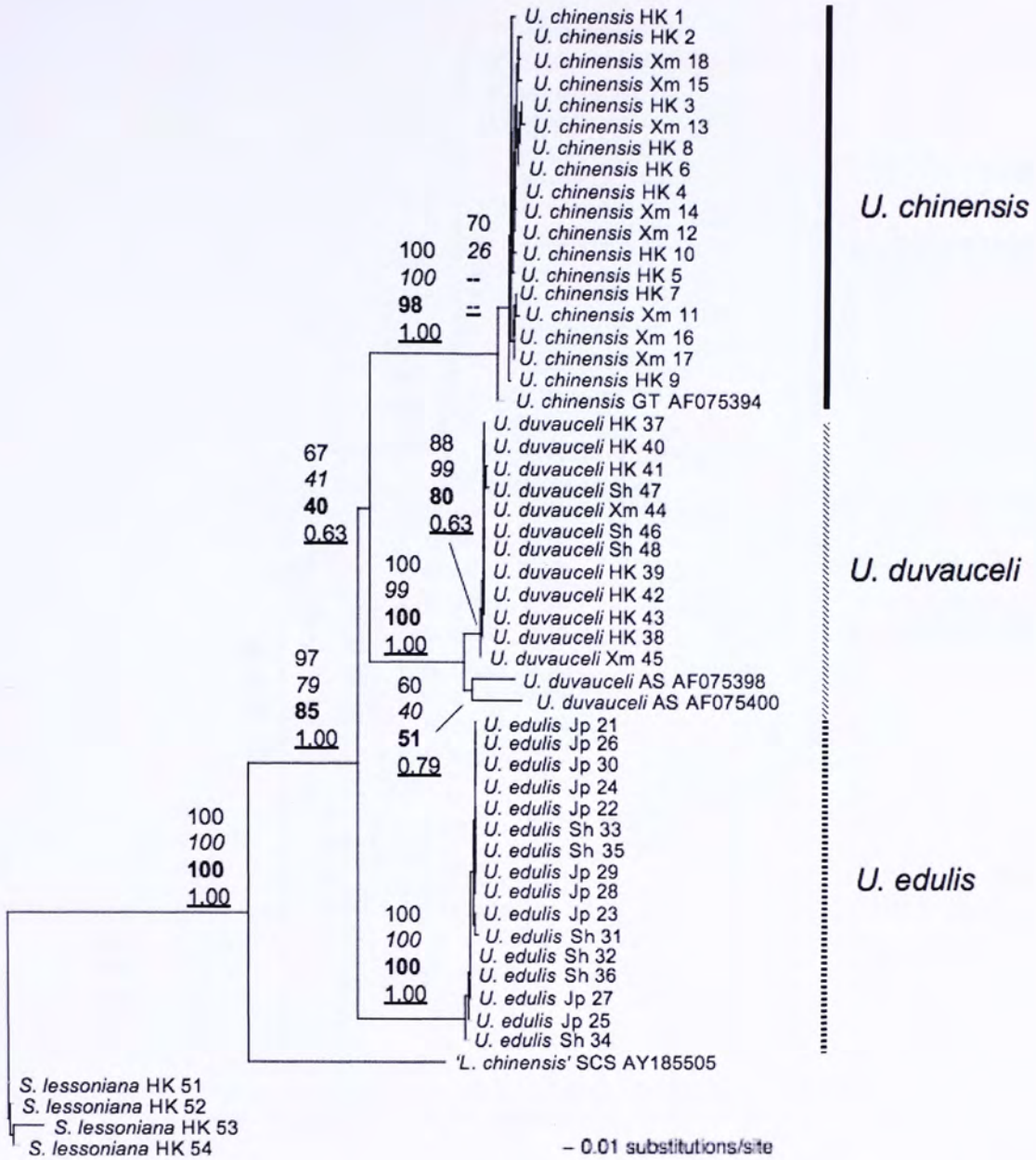

Fig. 3.1 Phylogenetic tree resolved by neighbor-joining analysis based on COI sequences. Percentage levels of support are indicated on each branch for BIO neighbor-joining (in normal), parsimony (in italics), maximum likelihood (in bold), and Bayesian posterior probabilities (underlined). HK: Hong Kong (China), Xm: Xiamen (China), Jp: Yamaguchi (Japan), Sh: Shanghai (China), GT: Gulf of Thailand, AS: Andaman Sea, SCS: South China Sea. Accession number is shown for sequences from GenBank. Sequences believed to be derived from misidentified samples are shown in quotation marks.

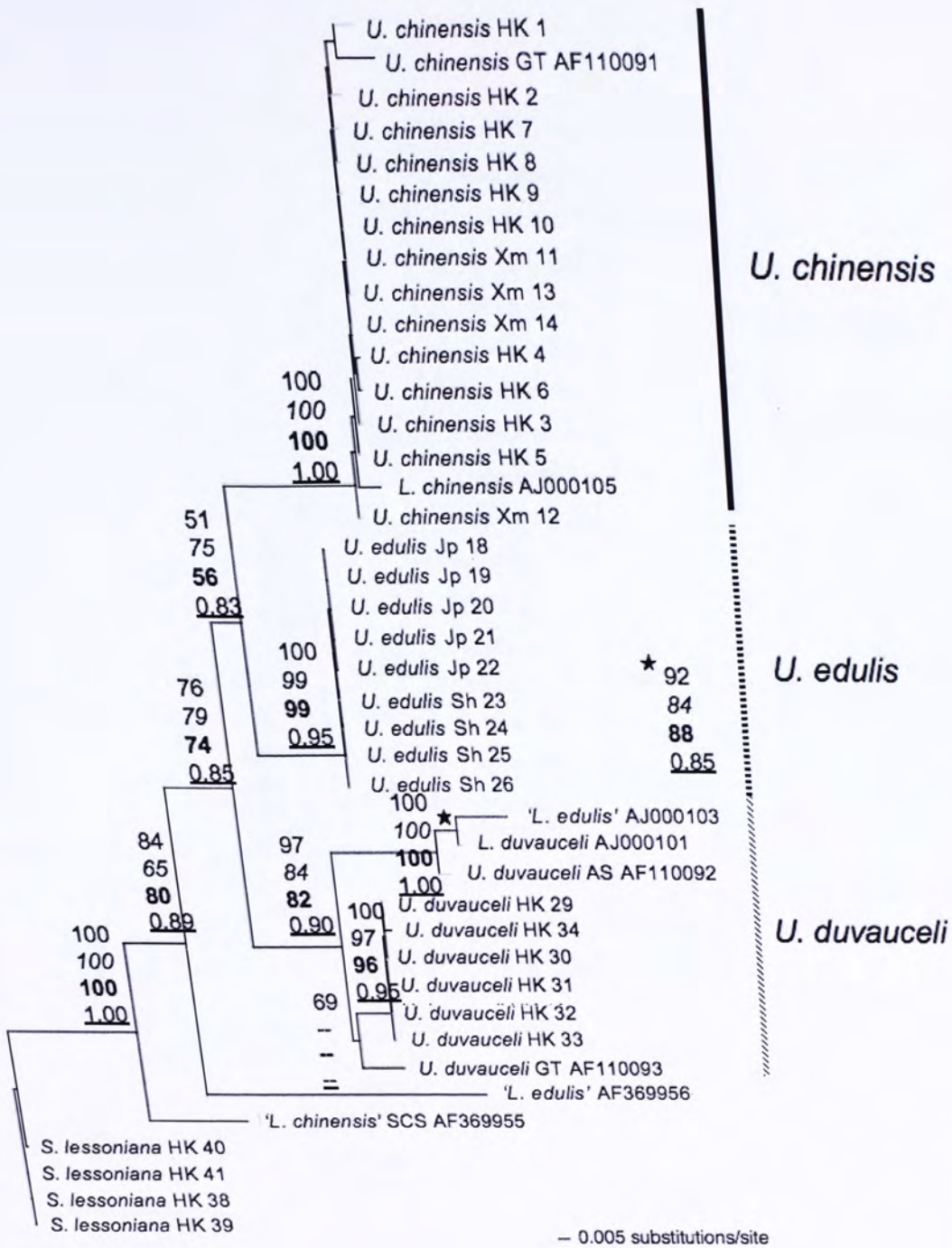

Fig. 3.2 Phylogenetic tree resolved by neighbor-joining analysis based on 16S rRNA sequences. Percentage levels of support are indicated on each branch for BIO neighbor-joining (in normal), parsimony (in italics), maximum likelihood (in bold), and Bayesian posterior probabilities (underlined). HK: Hong Kong (China), Xm: Xiamen (China), Jp: Yamaguchi (Japan), Sh: Shanghai (China), GT: Gulf of Thailand, AS: Andaman Sea, SCS: South China Sea, unknown locality is not shown in the graph. Accession number is shown for sequences from GenBank. Sequences believed to be derived from misidentified samples are shown in quotation marks.

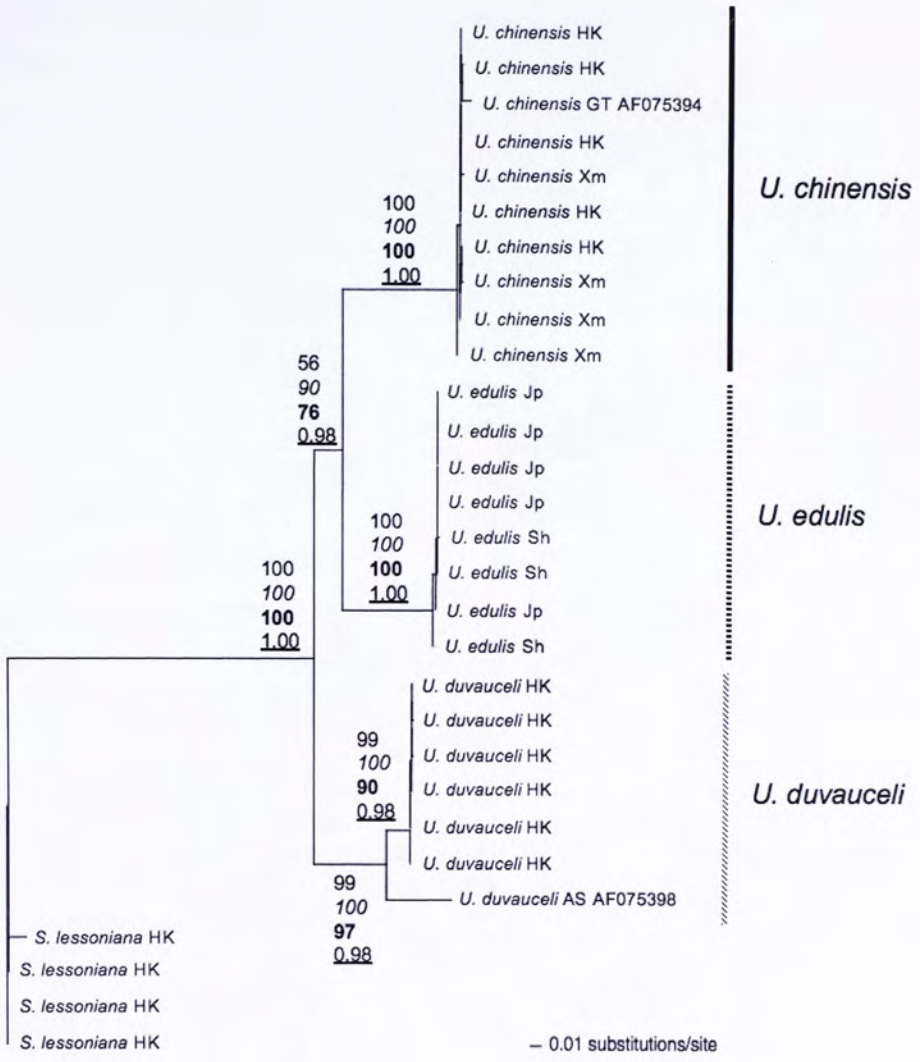

Fig. 3.3 Phylogenetic tree resolved by neighbor-joining analysis based on combined DNA sequences of COI and 16S rRNA. Percentage levels of support are indicated on each branch for BIO neighbor-joining (in normal), parsimony (in italics), maximum likelihood (in bold), and Bayesian posterior probabilities (underlined). HK: Hong Kong (China), Xm: Xiamen (China), Jp: Yamaguchi (Japan), Sh: Shanghai (China), GT: Gulf of Thailand, AS: Andaman Sea. Accession number is shown for sequences from GenBank.

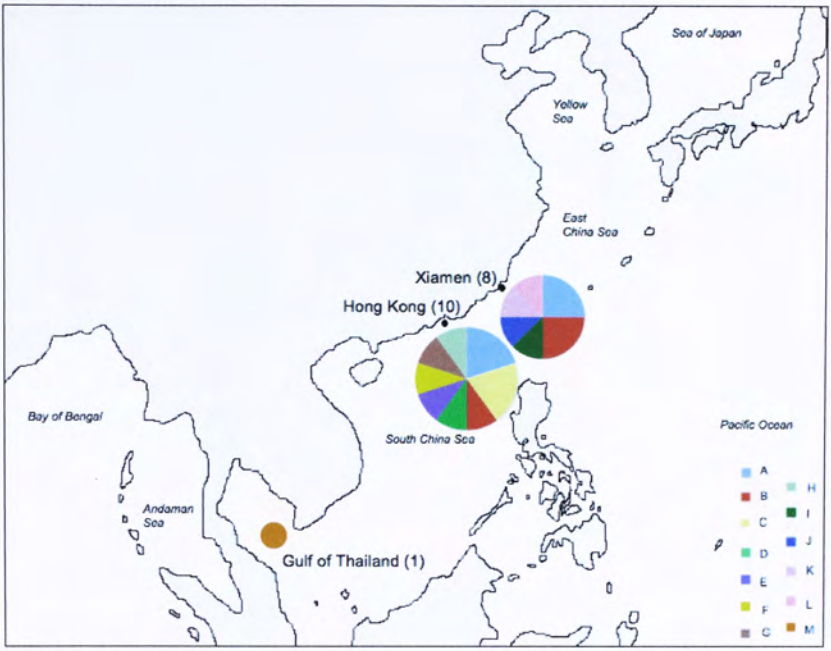

Fig. 3.4 Frequency distribution of haplotypes for COI in *U. chinensis* collected from Hong Kong and Xiamen. The haplotype names correspond to those in Table 3.4. The total number of samples in a population is in parentheses.

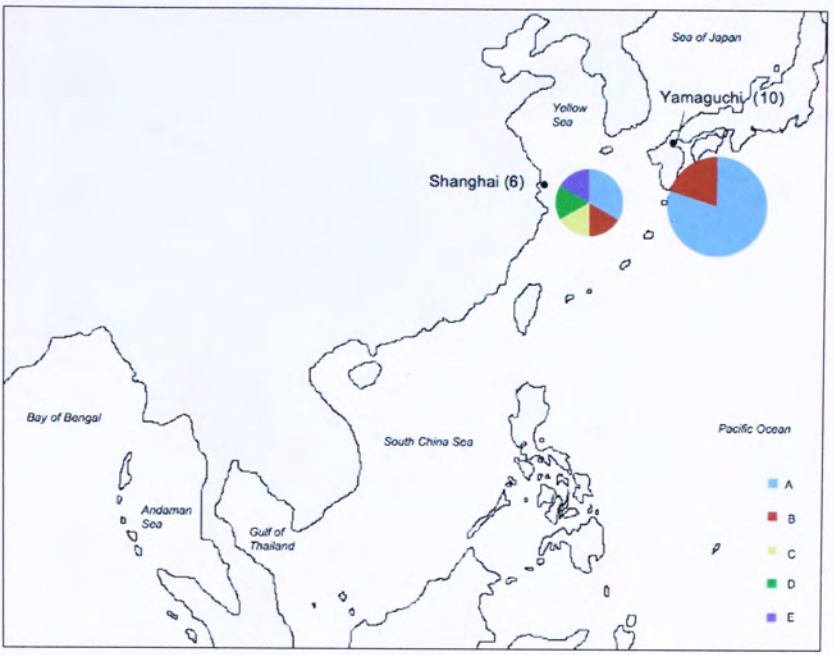

Fig. 3.5 Frequency distribution of haplotypes for COI in *U. edulis* collected from Japan and Shanghai respectively. The haplotype names correspond to those in Table 3.5. The total number of samples in a population is in parentheses.

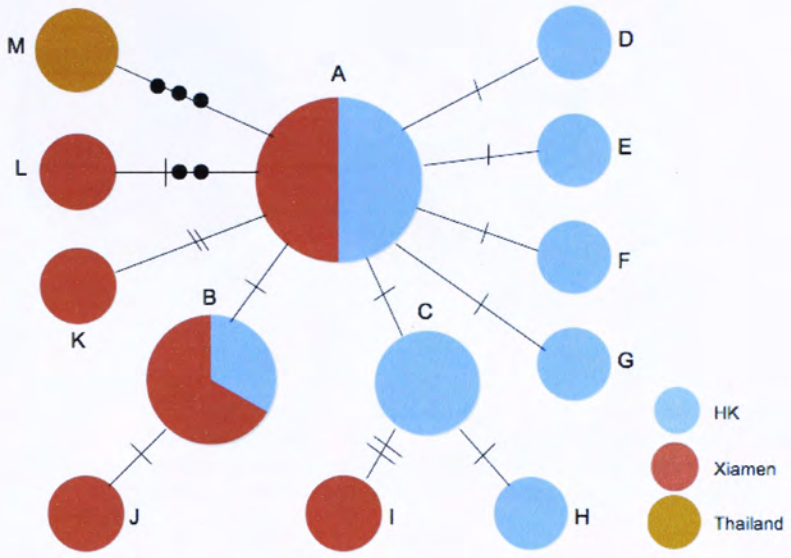

Fig. 3.6 COI haplotype network of *U. chinensis*. Cross-bars indicate inferred transitions; solid circles indicate inferred transversions. Colours within the circles denote locality. The area of the circles corresponds to the number of the individuals matching the particular haplotype.

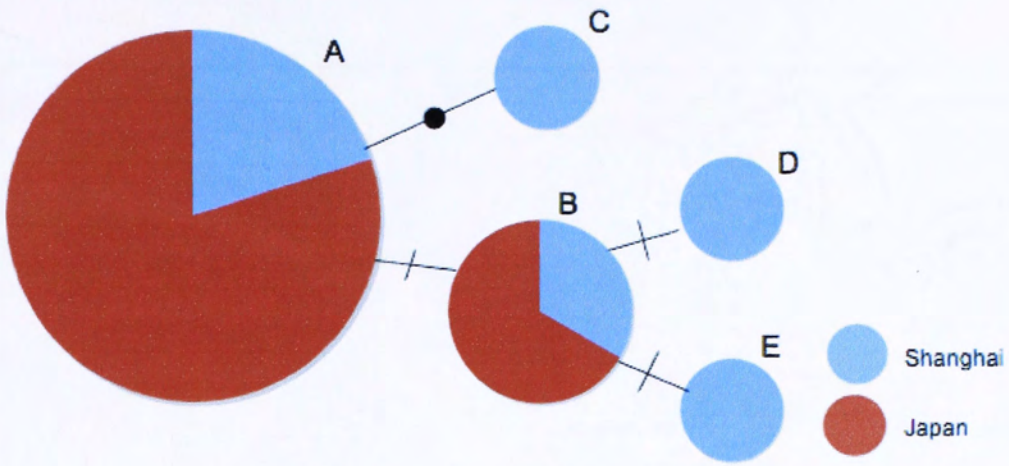

Fig. 3.7 COI haplotype network of *U. edulis*. Cross-bars indicate inferred transitions; solid circles indicate inferred transversions. Colours within the circles denote locality. The area of the circles corresponds to the number of the individuals matching the particular haplotype.

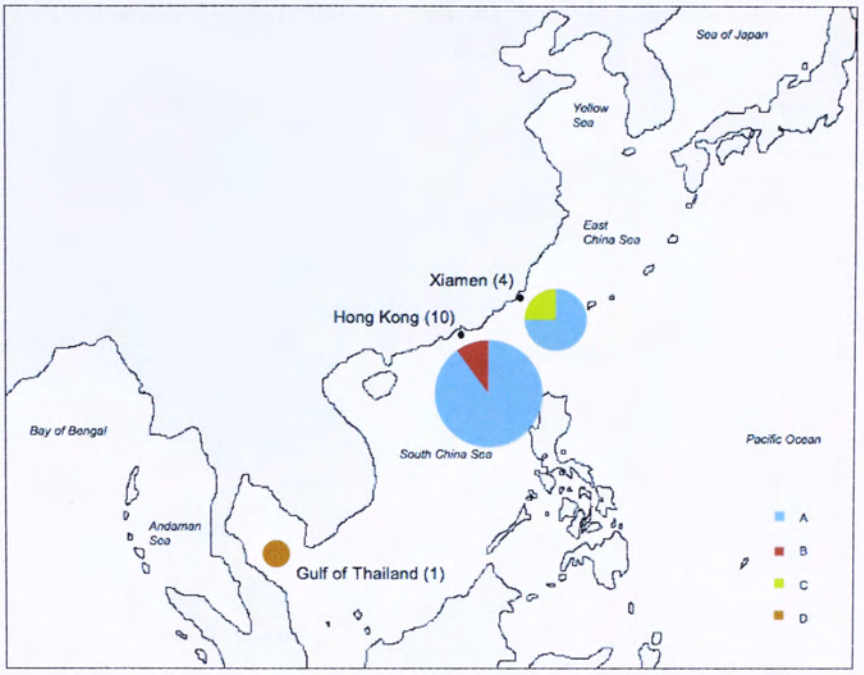

Fig. 3.8 Frequency distribution of haplotypes for 16S rRNA in *U. chinensis* collected from Hong Kong and Xiamen. The haplotype names correspond to those in Table 3.8. The total number of samples in a population is in parentheses.

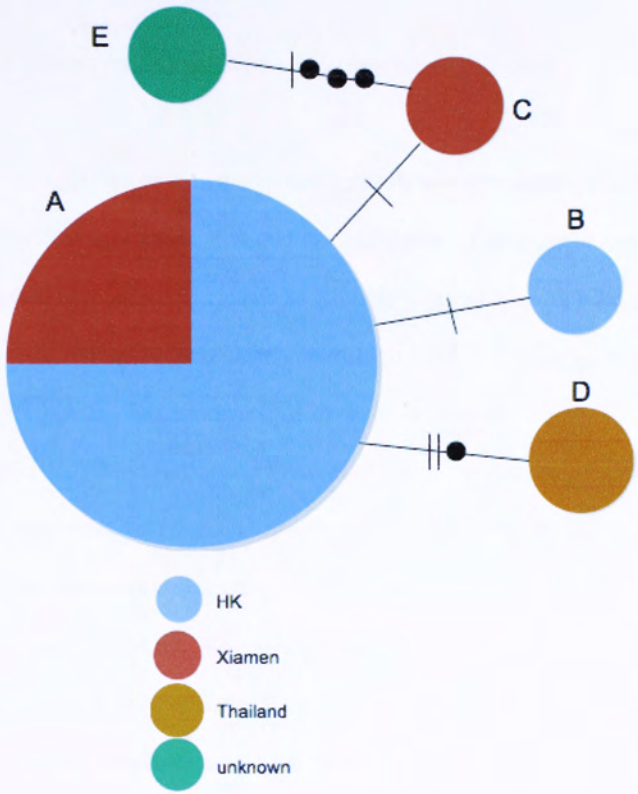

Fig. 3.9 16S rRNA haplotype network of *U. chinensis*. Cross-bars indicate inferred transitions; solid circles indicate inferred transversions. Colours within the circles denote locality. The area of the circles corresponds to the number of the individuals matching the particular haplotype.

3.4 Discussion

3.4.1 Genetic differentiation of *U. chinensis* and *U. edulis*

Analyses based on sequences of two mitochondrial genes clearly separate *U. chinensis* and *U. edulis* into two distinct clades. The aggregation of each of the two loliginid species into a clade was strongly supported by high bootstrap values. The two mitochondrial genes clearly show high level of divergence between *U. chinensis* and *U. edulis*. The average COI divergence between *Loligo* species is about 18% (range: 11-22%) (Herke and Foltz 2002), and the divergence between *U. chinensis* and *U. edulis* (average: 15.5%, range: 14.7-16.2%) is within this range. This provides further evidence that they are two distinct species, but do not represent a clinal variation of the same species along the localities included in this analysis. *U. edulis* from Yamaguchi and Shanghai, and *U. chinensis* from Xiamen, Hong Kong and Gulf of Thailand, are hence shown to be two distinct species but not the same species with clinal variation in this study. This study on the mitochondrial genes differentiation also supports the use of the arm sucker ring teeth shape (Natsukari and Okutani 1975) to distinguish between the two species.

From the COI and 16S rRNA gene trees, the three congeneric species, *U. chinensis, U. edulis* and *U. duvauceli* show similar divergence from one and another. While both the 16S rRNA and combined gene tree showed that the *U. chinensis* and *U. edulis* more closely related, the COI gene tree showed *U. chinensis* and *U. duvauceli* more closely related. A phylogenetic analysis of gonatid squids suggests that 16S rRNA provides some support for intergeneric relationships, in

addition to infrageneric relationships (Lindgren et al. 2005). On the other hand, COI, which was not able to resolve genus-level relationship, provides support at the individual and species levels. Thus it could be said that *U. chinensis* and *U. edulis*, were more closely related, which were in agreement with their similar morphological appearance. However, the bootstrap support for the relationship between the *Uroteuthis* species is weak. More species in the genus *Uroteuthis* should be included for further revealing phylogenetic relationship of *Uroteuthis* species.

There have been no misidentifications in the samples used in this study as all of them were grouped into their own clade. In a study of population structure of two *Loligo* species, *Loligo pealei* and *Loligo plei*, the intraspecific COI divergence (0.15-1.4%) indicated that the two species do not harbor cryptic species (Herke and Foltz 2002). All the samples of *U. chinensis* and *U. edulis* in this study showed a very low COI divergence with other conspecific individuals (range: 0-1.1%). And the sequence from a Thailand *U. chinensis* sample shows a low divergence (0.5-1.1%) to the sequences of Hong Kong and Xiamen *U. chinensis* samples. Thus it could be concluded that no cryptic species was found in this study according to the analysis of the mitochondrial COI gene. However, to answer this question completely, samples from more localities along their whole distribution range should be included, such as from Australia. Since it is believed that they are composed of several cryptic species, and some are probably endemic to Australia (Yeatman and Benzie 1994).

The GenBank DNA sequences included in this study are derived from some individuals that were correctly identified (COI: AF075394, AF075398, AF075400; 16S rRNA: AF110091, AJ000105, AF110092, AF110093), and also some individuals that were apparently misidentified (COI: AY185505; 16S rRNA: AJ000103, AF369955, AF369956). This shows that misidentifications of these squid species were common (assuming that there were no sequence contamination), as the sequences from misidentified individuals originate from two of the three research groups that reported the sequences included in the present study. The paralarva and immature individuals could not be identified based on morphology, and damaged specimens with missing body parts would lead to erroneous identification (Wakabayashi et al. 2006). This means that it is hard to identify them based on morphology only. Hence the mitochondrial gene segments sequenced in this study would be useful for species identification of juveniles.

3.4.2 Population structuring within *U. chinensis* and *U. edulis*

Analysis of the mitochondrial genes shows that the haplotype diversity was higher for COI than 16S rRNA gene. More haplotypes were also found in *U. chinensis* than in *U. edulis*. Although such a number of haplotypes are found in this study, there are no clustering of haplotypes with respect to localities and no significant genetic differentiation detected among populations in different localities revealed by analysis of F_{ST}. There are two shared COI haplotypes in *U. chinensis*, from Hong Kong and Xiamen and the others occur in single individuals. The haplotype of Gulf of Thailand individual is closest to the most common haplotype and is separated from it by three transversions. More samples from the Gulf of

Thailand are needed to determine if clustering occurs for Thailand individuals. Most *U. chinensis* individuals share the same 16S rRNA haplotype, with the Gulf of Thailand individuals separate from this haplotype by three mutations. This indicates the individuals from Gulf of Thailand may constitute a distinct population, but more samples from Gulf of Thailand are needed for further research. The case in the COI gene of *U. edulis* was similar, with most individuals from Yamaguchi (Japan) and Shanghai (China) sharing two haplotypes with others found in single individuals. Hong Kong (China) and Xiamen (China) are geographically close, which is similar for Shanghai (China) and Yamaguchi (Japan). In addition the squids are such mobile animals (Boyle 1990), it is believed that the squids in these places do not possess any population structure as they may be able to crossbreed, or they may be part of a large panmictic population. *U. duvauceli*, which belongs to the same genus, can be used for comparison as there are samples from Shanghai, Xiamen and Hong Kong, and also sequences of Gulf of Thailand and Andaman Sea individuals from GenBank. From the two mitochondrial genes, it shows that the individuals from Hong Kong, Xiamen and Shanghai group together with little divergence, and this grouping is divergent from the sequences of Gulf of Thailand and Andaman Sea individuals. So it seems the squids in Shanghai, Xiamen and Hong Kong are part of a panmictic population, and squids in Thailand may be partly reproductively isolated population. Also the two mitochondrial genes seem to be able to reveal population structuring. However, firm conclusions on their population structure cannot be made in this stage due to the limited number and proximity of the sampling sites in this study so that the applicability of the mitochondrial genes to act as the effective marker for revealing population structure of *U. chinensis* and *U. edulis* cannot be ascertained. Nevertheless, the use of COI gene is able to reveal population structure

in another two loliginid squids, *L. plei* and *L. pealei*, across the northern Gulf of Mexico and the northwestern Atlantic Ocean (Herke and Foltz 2002).

The COI and 16S rRNA gene sequences of *U. chinensis* from the Gulf of Thailand in GenBank are included in this analysis. The Gulf of Thailand haploptype is distinct from those in Hong Kong and Xiamen. It is possible that the Gulf of Thailand population is genetically different from the population in East Asia. It also shows that the two mitochondrial genes may be variable enough to resolve population structure. Although the high mobility of squid may lead to homogenization, three distinct lineages were detected in another mobile animal, the redlip mullet (*Chelon haematocheilus*) (Liu et al. 2007). The analysis of mitochondrial DNA control region revealed three *C. haematocheilus* populations in the Sea of Japan, the East China Sea and the South China Sea, caused possibly by lower sea levels during Pleistocene glacial period (Liu et al. 2007). As the distribution ranges of *C. haematocheilus*, *U. chinensis* and *U. edulis* are highly overlapped, and all of them are highly mobile animals with high fecundity and only spawn once a year, the geographical and tectonic history that caused genetic divergence in *C. haematocheilus* in North-western Pacific may also have effect on *U. chinensis* and *U. edulis*. To further investigate the population structure of the two species, and the possibility of using the mitochondrial genes as the molecular marker for population study, more samples from different localities along the distribution range are needed.

Chapter 4

Isolation of microsatellite loci for *Uroteuthis chinensis*

4.1 Introduction

Microsatellites are short tandem repeats of DNA, and the variation in repeat number of microsatellites gives rise to a large numbers of distinguishable alleles within a population. Microsatellite markers are commonly used in determining population structure, as they have higher resolution in population differentiation than allozyme and mtDNA markers (Brierley et al. 1995; O'Connell and Wright 1997; Shaw et al. 1999; Reichow and Smith 2001; Shaw et al. 2004).

The application of microsatellite markers in squid started about ten years ago. Markers were isolated in oceanic squid of genus *Illex* (Adcock et al. 1999), and in some loliginid squids, such as *Loligo forbesi* (Shaw 1997; Emery et al. 2000), *L. opalescens* (Reichow and Smith 1999), *L. vulgaris* (Guarniero et al. 2003), *L. gahi* (Shaw and Adcock 2002), and *L. bleekeri* (Iwata et al. 2003).

The objective of the present study was to isolate microsatellite loci from *Uroteuthis chinensis* and *U. edulis*. The possibility of cross-specific amplification of the isolated loci, which were from this study and from two *Loligo* species in other studies, in three *Uroteuthis* species was also tested. Since microsatellite isolated could be applied in future studies of the population structure of these species.

4.2 Materials and methods

Mantle tissues of *U. chinensis*, *U. edulis* and another species *U. duvauceli* samples were used for total genomic DNA extraction with QIAamp DNA Mini Kit (QIAGEN). After extraction, the DNA was eluted in 200 µl of double distilled H_2O. The quality of DNA extracts was evaluated by 1% agarose gel electrophoresis and ethidium bromide staining. DNA of the three species was used for microsatellite isolation. Cross-specific amplification of the isolated loci was tested in the three species.

The total genomic DNA was digested to fragments of approximately 500 bp in size by restriction enzyme *Rsa*I (Promega, Madison, WI, USA). A double-stranded linker was ligated onto both ends of each DNA fragment. DNA fragments with microsatellite sequences were enriched using Dynabeads (Dynal, Oslo, Norway) with the microsatellite oligos GATA and CTAT repeats. Microsatellite-containing fragments were captured by the Dynabeads, and all other DNA fragments were washed away. The microsatellite-containing fragments were recovered by polymerase chain reaction (PCR). The recovered DNA was incorporated into the cloning vector PCRscript (Stratagene, La Jolla, CA, USA) using the TA cloning kit (Invitrogen, Carlsbad, CA, USA). The cloning vector was incorporated into the bacterial host *E. coli* JM109. The inserts from positive colonies were amplified by PCR. The products were then subjected to sequencing reaction and loaded onto ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) for analysis. Primers for PCR were designed from the unique sequences flanking the

microsatellite isolated, using the programme OLIGO 4.1 (National Biosciences, Plymouth, MN, USA).

In addition to developing species-specific loci, a number of microsatellite locus primer pairs previously developed from other loliginid species were also screened. They were 15 loci isolated from *Loligo forbesi* by Shaw (1997) and Shaw et al. (2000), and five loci isolated from *Loligo vulgaris* by Guarniero et al. (2003).

Preliminary tests of the possibility of PCR amplification of the isolated loci were performed on one individual each of *U. edulis*, *U. chinensis* and *U. duvauceli*. The PCR condition was 3 minutes at 94°C, then 32 cycles of 30 seconds at 94°C, 30 seconds at the specific annealing temperature (see Table 4.1), and 30 seconds at 72°C, with a final extension at 72°C for 3 minutes. The PCR reaction mixes contained 1.5 µl of DNA extract, 0.4 µM of each primer, 0.2 µM of dNTPs (Qiagen, Hilden, Germany), 1 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), 1.5 mM of magnesium chloride (Invitrogen, Carlsbad, CA, USA), and 1X Mg^{2+} free PCR buffer, in a final reaction volume of 30 µl. The loci that gave PCR products of appropriate size were then subjected to PCR reactions performed on 27 individuals of *U. chinensis* (23 from Hong Kong (China), 4 from Xiamen (China)), 3 individuals of *U. edulis* (from Yamaguchi (Japan)) and 2 individuals of *U. duvauceli* (from Hong Kong). Amplified products were resolved on 6% denaturing polyacrylamide gels and visualized by the silver staining method (Tegelström 1986). Product sizes were determined by comparison to 30-330 bp DNA sequence standard (Invitrogen, Carlsbad, CA, USA).

4.3 Results

A total of nine DNA sequences were obtained from the positive colonies. Of these nine sequences that contain repeats, four were isolated from *U. chinensis*, four from *U. edulis*, and one from *U. duvauceli*. Primers were designed from these sequences. Two primer sets (Uchi1 & Uchi3), which were developed based on two sequences of *U. chinensis*, gave consistent amplification of polymorphic products in *U. chinensis* (Table 4.1, Figs. 4.1 and 4.2), but not *U. edulis* and *U. duvauceli*.

The cross-specific amplification of 5 primer sets from *L. vulgaris* gave no PCR product. For the cross-specific amplification of the 15 primer sets from *L. forbesi*, only one (Lfor3) gave consistent amplification of polymorphic products in *U. chinensis* and *U. edulis* (Table 4.1, Fig. 4.3), but not *U. duvauceli*.

Optimization and screening of variability were performed for the two *U. chinensis*-specific loci (Uchi1 & Uchi3) using 27 individuals of *U. chinensis* (23 from Hong Kong and 4 from Xiamen), and the one cross-specific locus (Lfor3) using 27 individuals of *U. chinensis* (23 from Hong Kong and 4 from Xiamen) and 3 individuals of *U. edulis* (from Yamaguchi). The results are presented in Table 4.1. The levels of variability detected were high. The number of alleles ranged from 15 to 26 per locus, and observed heterozygosities (H_O) from 77.8 to 90.0%.

Table 4.1 Core repeat, primer sequences (5' to 3'), and optimal annealing temperature (T_a) for two microsatellite loci isolated from *Uroteuthis chinensis*. n1 and n2 are numbers of individuals successfully genotyped in *U. chinensis* and *U. edulis* respectively, and H_O and H_E are observed and expected heterozygosity. Data are also given at the locus isolated from *Loligo forbesi* (Shaw 1997).

Locus	Repeat	Primers 5'-3'	T_a (°C)	Allele size range (bp)	n1 / n2	No. alleles	H_E/H_O (%)
Uchi1	$(TATG)_9TATC(TTTC)_2(TATC)_3TTTC(TATC)_3...(TATC)_{10}$	F: TAAAATGTAACTTCCGAAATACC R: ACACAAAAGCAAACAGACACAGG	47.5	187-317	27 / 0	25	93.92/ 77.78
Uchi3	$(GA)_3(TAGA)_2CA(GATA)_6(GA)_{11}GTGAAC(GA)_2GG(GA)_9$	F: GATGACTAATAAGGATGGTTGGA R: TGAGATCAAAACAGGTTGAACTT	50.2	225-311	27 / 0	26	96.58/ 88.89
Primers published for *Loligo forbesi*							
Lfor3	$(AAT)_{22}$	F: GGTCATGTCATTCTCTGCAC R: ACATTTATCCATTAACAGAGTAGCA	56	119-164	27 / 3	15	92.26/ 90

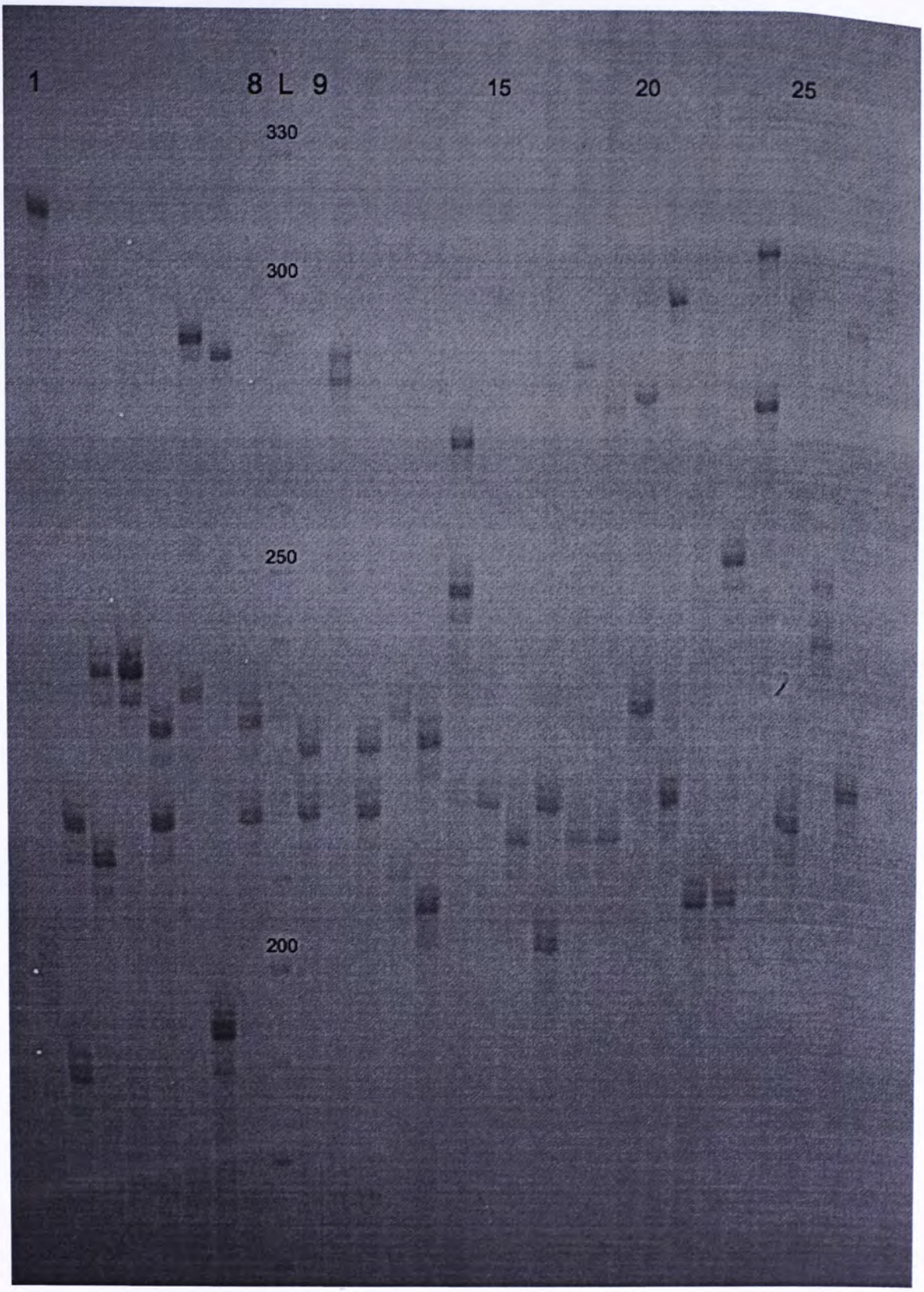

Fig. 4.1 Microsatellite alleles at locus Uchi1. L: ladder, 1-23: *U. chinensis* from Hong Kong (China), 24-27: *U. chinensis* from Xiamen (China). (As there were shadow bands generated by PCR, the alleles were scored as those with the highest intensity.)

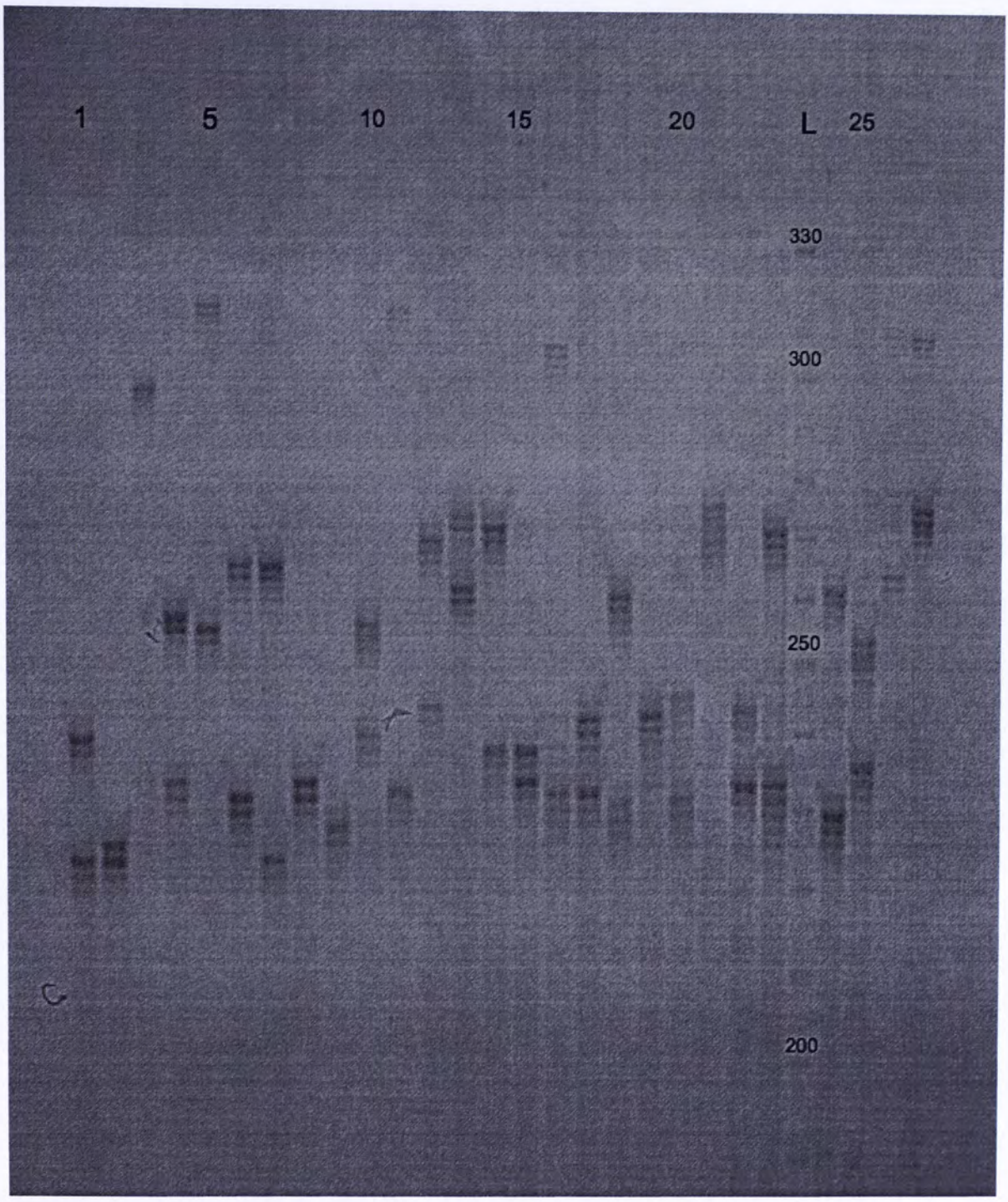

Fig. 4.2 Microsatellite alleles at locus Uchi3. L: ladder, 1-23: *U. chinensis* from Hong Kong (China), 24-27: *U. chinensis* from Xiamen (China). (As there were shadow bands generated by PCR, the alleles were scored as those with the highest intensity.)

Fig. 4.3 Microsatellite alleles at locus Lfor3. L: ladder, 1-23: *U. chinensis* from Hong Kong (China), 24-27: *U. chinensis* from Xiamen (China), 28-30: *U. edulis* from Yamaguchi (Japan). (As there were shadow bands generated by PCR, the alleles were scored as those with the highest intensity.)

4.4 Discussion

Two microsatellite loci isolated from *U. chinensis* in the present study were highly polymorphic in *U. chinensis*. For the 15 and 5 loci isolated from *L. forbesi* (Shaw 1997; Emery et al. 2000) and *L. vulgaris* (Guarniero et al. 2003) respectively, only one locus from *L. forbesi* is applicable for the cross-specific amplification in the two target species. This primer set, Lfor3, also gave consistent polymorphic products in *Loligo gahi* (Shaw and Adcock 2002) and *L. vulgaris* (Emery et al. 2000). The level of genetic variability uncovered in the three loci is high, as reflected in the degree of heterozygosity. The heterozygosity in the present study ranged from 77.8 to 90.0%, compared to the average heterozygosity in allozyme studies of *L. gahi* (0.07) (Carvalho and Loney 1989) and *L. opalescens* (0.037) (Augustyn and Grant 1988). This study shows that the squid microsatellite loci are highly variable and ideal for population genetic studies. The observed heterozygosities are consistently lower than the expected heterozygosities for the three loci in this study. This departure from Hardy-Weinberg equilibrium can be ascribed to non-random mating (inbreeding), selection and/or the presence of subdivisions within the population. The microsatellite loci will be useful for future study of population structure in these two species.

Chapter 5

Conclusions

The present study aimed to elucidate morphological and genetic relationships of *Uroteuthis chinensis* and *Uroteuthis edulis*. Polymorphisms in the mitochondrial COI and 16S rRNA genes, as well as three microsatellite loci, were also investigated. The main conclusions from this study are summarized as follows.

1. The percentage of total arm length of left arm IV that is modified by hectocotylization, and the teeth shape and number of arm sucker rings, are useful for distinguishing *U. chinensis* and *U. edulis*. This result is concordant with previous observations by other authors. Other morphometric characters included in the present study were not informative for differentiating *U. chinensis* and *U. edulis*, such that no new morphological characters informative for differentiating the two species were found.

2. The genetic study on the mitochondrial DNA of *U. chinensis* and *U. edulis* revealed clearly that these are two distinct species. The populations of *U. chinensis* and *U. edulis* do not represent a clinal variation of the same species along the coast of East Asia, based on the samples in this study. No cryptic species were found in this study. However, more samples from different sites are needed for resolving this issue, since *U. chinensis* and *U. edulis* are widely distributed.

3. The applicability of the mitochondrial COI and 16S rRNA genes as effective markers for population structure of *U. chinensis* and *U. edulis* cannot be ascertained from results of the present study. Two microsatellite loci isolated from *U. chinensis* gave consistent polymorphic products for *U. chinensis*. A microsatellite locus isolated from *Loligo forbesi* (Lfor3) gave consistent polymorphic products in both *U. chinensis* and *U. edulis*. These microsatellite loci will be useful for future study of population structure in these two *Uroteuthis* species.

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Appendix 1: Aligned COI sequences used in this study

Dots indicate nucleotide identical to 1 (top row); missing data are coded with the letter N; gaps at the end of sequences are coded with '-'.
1-10: *U. chinensis* (Hong Kong), 11-18: *U. chinensis* (Xiamen), 19: *U. chinensis* (Gulf of Thailand, AF075394), 20: *L. chinensis* (AY185505), 21-30: *U. edulis* (Yamaguchi), 31-36: *U. edulis* (Shanghai), 37-43: *U. duvauceli* (Hong Kong), 44-45: *U. duvauceli* (Xiamen), 46-48: *U. duvauceli* (Shanghai), 49-50: *U. duvauceli* (Andaman Sea, AF075398, AF075400), 51-54: *Sepioteuthis lessoniana* (Hong Kong)

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          10         20         30         40         50         60         70
1  GTACTTCCTT GAGATTAATA ATTCGAACAG AGTTAGGAAA ACCAGGGTCG CTACTAAATG ATGATCAACT
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ..........
4  .......... .......... .......... .......... .......... .......... ....C.....
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
7  .......... .......... .......... .......... .......... .......... ..........
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14 .......... .......... .......... .......... .......... .......... ..........
15 .......... .......... .......... .......... .......... .......... ..........
16 .......... .......... .......... .......... .......... .......... ..........
17 .......... .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... .......... ..........
19 ---------- ---....... ......NNN. .......... C.AT..N... .......... ..........
20 ....A..A.. A.....G... ..C..T.... .......G.. ...T..C..C T..T...... ........T.
21 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
22 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
23 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
24 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
25 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
26 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
27 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
28 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
29 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
30 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
31 ---------- ---------- ---------- ------.G.. ...T..A..A ...T...... .C..C.....
32 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
33 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
34 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
35 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
36 .......A.. A.....G... .......... .AC....G.. ...T..A..A ...T...... .C..C.....
37 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
38 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
39 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
40 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
41 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
42 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
43 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
44 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
45 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
46 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
47 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
48 .......AC. A..GC..... ..C....... .......... ...T..T..A .....T.... ........T.
49 ---------- ---------- ---------- ----...... ...T..T..A .......... ........T.
50 .......AC. T.N.C..... ..C....... .A........ ...T..T..A .....T.... .......GT.
51 ....C..AC. A..........  ........C. .A.....T.. ...C..C..A T......... ....C...T.
52 ....C..AC. A..........  ........C. .A.....T.. ...C..C..A T......... ....C...T.
53 ....C..AC. A..G...... ........C. .A.....T.. ...C..C..A T......... ....C...T.
54 ....C..AC. A..........  ........C. .A.....T.. ...C..C..A T......... ....C...T.
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          80         90         100        110        120        130        140
1  ATATAACGTA GTAGTTACTG CTCACGGTTT TATTATAATT TTCTTCATAG TTATACCTAT TATAATTGGG
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......C.. ..........
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
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16 .......... .......... .......... .......... .......... .......... ..........
17 .......... .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... .......... ..........
19 .......... .......... .......... .......... .......... .......... ..........
20 ...C..T... .......... .A..T..... C......... ..T..T.... .......... .........A
21 .......... .......... ....T..... .......... ..T..T.... .......... ..........
22 .......... .......... ....T..... .......... ..T..T.... .......... ..........
23 .......... .......... ....T..... .......... ..T..T.... .......... ..........
24 .......... .......... ....T..... .......... ..T..T.... .......... ..........
25 .......... .......... ....T..... .......... ..T..T.... .......... ..........
26 .......... .......... ....T..... .......... ..T..T.... .......... ..........
27 .......... .......... ....T..... .......... ..T..T.... .......... ..........
28 .......... .......... ....T..... .......... ..T..T.... .......... ..........
29 .......... .......... ....T..... .......... ..T..T.... .......... ..........
30 .......... .......... ....T..... .......... ..T..T.... .......... ..........
31 .......... .......... ....T..... .......... ..T..T.... .....A.... ..........
32 .......... .......... ....T..... .......... ..T..T.... .......... ..........
33 .......... .......... ....T..... .......... ..T..T.... .......... ..........
34 .......... .......... ....T..... .......... ..T..T.... .......... ..........
35 .......... .......... ....T..... .......... ..T..T.... .......... ..........
36 .......... .......... ....T..... .......... ..T..T.... .......... ..........
37 .......... .....A.... .......... .......... ..T..T.... .......... ..........
38 .......... .....A.... .......... .......... ..T..T.... .......... ..........
39 .......... .....A.... .......... .......... ..T..T.... .......... ..........
40 .......... .....A.... .......... .......... ..T..T.... .......... ..........
41 .......... .....A.... .......... .......... ..T..T.... .......... ..........
42 .......... .....A.... .......... .......... ..T..T.... .......... ..........
43 .......... .....A.... .......... .......... ..T..T.... .......... ..........
44 .......... .....A.... .......... .......... ..T..T.... .......... ..........
45 .......... .....A.... .......... .......... ..T..T.... .......... ..........
46 .......... .....A.... .......... .......... ..T..T.... .......... ..........
47 .......... .....A.... .......... .......... ..T..T.... .......... ..........
48 .......... .....A.... .......... .......... ..T..T.... .......... ..........
49 .......... .....A.... ..........C......... ..T..T.... .......... ..........
50 ...C...... .....A.... ..........C......... ..T..T.... .......... ..........
51 ......T..T .......... .A........ .......... .....T.... .......... ..........
52 ......T..T .......... .A........ .......... .....T.... .......... ..........
53 ......T..T .......... .A........ .......... .....T.... .......... .........A
54 ......T..T .......... .A........ .......... .....T.... .......... ..........
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          150        160        170        180        190        200        210
1  GGTTTCGGAA ACTGATTAGT CCCATTAATA TTAGGAGCTC CAGATATAGC CTTCCCCCGT ATAAATAATA
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ..........
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
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11 .......... .......... .......... .......... .......... .......... ..........
12 .......... .......... .......... .......... .......... .......... ..........
13 .......... .......... .......... .......... .......... .......... ........C.
14 .......... .......... .......... .......... .......... .......... ..........
15 .......... .......... .......... .......... .......... .......... ..........
16 .......... .......... .......... .......... .......... .......... ..........
17 .......... .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... .......... ..........
19 .......... .......... .......... .......... .......... .......... ..........
20 ..A..T..T. .T...C.... A..T...... .....T.... ....C..... ...T..T..A .....C..C.
21 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
22 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
23 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
24 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
25 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
26 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
27 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
28 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
29 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
30 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
31 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
32 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
33 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
34 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
35 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
36 .....T.... .T........ T..C...... C....T..C. .......... T.....A... ..........
37 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
38 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
39 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
40 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
41 .....T..T. .T........ A..T...... C.......C. ....C..... T.....A... ..........
42 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
43 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
44 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
45 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
46 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
47 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
48 .....T..T. .T........ A..T...... C.......C. .......... T.....A... ..........
49 .....T..T. .T........ A..T..G... ........C. ....C..... T.....G... ..........
50 .....T..T. .T........ A..T.....G C.......C. .......... ......G... ..........
51 ..C..T..T. .....C.T.. ...TC.C... C.......A. .T........ A.....A..A ..........
52 ..C..T..T. .....C.T.. ...TC.C... C.......A. .T........ A.....A..A ..........
53 ..C.....T. .....C.T.. ...TC.C... C.......A. .T........ A.....A..A ..........
54 ..C..T..T. .....C.T.. ...TC.C... C.......A. .T........ A.....A..A ..........
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          220        230        240        250        260        270        280
1  TAAGATTCTG ATTACTTCCA CCTTCATTAA CACTACTATT AGCCTCATCC GCAGTTGAAA GAGGAGCCGG
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ..........
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
7  .......... ...G...... .......... .......... .......... .......... ..........
8  .......... .......... .......... .......... .......... .......... ..........
9  .......... .......... .......... .......... .......... .......... ..........
10 .......... .C........ .......... .......... .......... .......... ..........
11 .......... ...G...... .......... .......... .......... .......... ..........
12 .......... .......... .......... .......... .......... .......... ..........
13 .......... .......... .......... .......... .......... .......... ..........
14 .......... .......... .......... .......... .......... .......... ..........
15 .......... .......... .......... .......... .......... .......... ..........
16 .......... ...G...... .......... .......... .......... .......... ..........
17 .......... ...G...... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... .......... ..........
19 .......... .......... .......... .......... .......... .......... ..........
20 .......T.. .C..T.A... ......C.C. .T..T..T.. ...A..C..G ..T....... .C........
21 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
22 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
23 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
24 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
25 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
26 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
27 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
28 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
29 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
30 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
31 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
32 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
33 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
34 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
35 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
36 .......T.. .C....C... ..A...C... ..T..T.... ......C... .......... ....G..A..
37 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
38 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
39 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
40 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
41 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
42 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
43 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
44 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
45 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
46 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
47 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
48 .......T.. .......... ..A..T.... ..T..T..C. .......... .......... .......T..
49 .......... .C........ ..A..T.... ..T..T..C. .......... .......... .......T..
50 .......... ...G..C... ..A..T.... ..T..T..C. .........T .......... .......T..
51 .......... ...G..A..T ..A...C... ....C..T.. ...G..C..A .......... ..........
52 .......... ...G..A..T ..A...C... ....C..T.. ...G..C..A .......... ..........
53 .......... ...G..A..T ..A...C... ....C..T.. ...A..C..A .......... ..........
54 .......... ...G..A..T ..A...C... ....C..T.. ...G..C..A .......... ..........
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          290        300        310        320        330        340        350
1  AACAGGGTGA ACAGTATACC CACCCTTATC CAGTAACCTT TCTCATGCAG GTCCTTCAGT TGACTTGGCT
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ..........
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .....C.... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
7  .......... .......... .......... .......... .......... .......... ..........
8  .......... .......... .......... .......... .......... .......... ..........
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13 .......... .......... .......... .......... .......... .......... ..........
14 .......... .......... .......... .......... .......... .......... ..........
15 .......... .......... .......... .......... .......... .......... ..........
16 .......... .......... .......... .......... .......... .......... ..........
17 .......... .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... .......... ..........
19 .......... .......... .......... .......... .......... .......... ..........
20 ......T... .....C.... .....C.... T..A..T..C ........T. .......... A...C.C..C
21 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
22 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
23 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
24 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
25 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
26 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
27 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
28 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
29 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
30 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
31 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
32 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
33 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
34 ......C... .......... ....T..... T..A..T... .......... .C........ ....C.A..C
35 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
36 ......C... .......... ....T..... T..A..T... .......... .C.....G.. ....C.A..C
37 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
38 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
39 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
40 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
41 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
42 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
43 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
44 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
45 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
46 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
47 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
48 T..C...... .....T..T. .C..T..... ...A...T.A .....C..T. .A..C..... A..TC.A..C
49 T..T..A... .....T..T. .T..T..... ...A...T.A .....C..C. .......... A..T..A...
50 T..T..A... .....T..T. .T..T..... ...A.....A .....C..T. .A........ A...C.T..C
51 T.....A... ..C..C..T. .....C.C.. A.....T..A ........T. .A........ ...TC.T...
52 T.....A... ..C..C..T. .....C.C.. A.....T..A ........T. .A........ ...TC.T...
53 T.....A... ..C..C..T. .G...C.C.. A........A ........T. .A........ ...TC.T...
54 T.....A... ..C..C..T. .....C.C.. A.....T..A ........T. .A........ ...TC.T...
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          360        370        380        390        400        410        420
1  ATTTTCTCTC TACACTTAGC TGGAATCTCA TCCATCTTAG GTGCCATTAA CTTTATCACA ACTATTATAA
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ..........
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
7  .......... .......... .......... .......... .......... .......... ..........
8  .......... .......... .......... .......... .......... .......... ..........
9  .......... .......... .......... .......... .......... .......... ..........
10 .......... .......... .......... .......... .......... .......... ..........
11 .......... .......... .......... .......... .......... .......... ..........
12 .......... .......... .......... .......... .......... .......... ..........
13 .......... .......... .......... .......... .......... .......... ..........
14 .......... .......... .......... .......... .......... .......... ..........
15 .......... .......... .......... .......... .......... .......... ..........
16 .......... .......... .......... .......... .......... .......... ..........
17 .......... .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... .......... ..........
19 .......... .......... .......... .......... .......... .......... ..........
20 ........A. .T........ A..T.....C ..T..T.... .G..T..... .......... ..........
21 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
22 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
23 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
24 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
25 ........A. .T........ G..T..T..T ..T..TC... ....T..... T......... ..C.......
26 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
27 ........A. .T........ G..T..T..T ..T..TC... ....T..... T......... ..C.......
28 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
29 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
30 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
31 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
32 ..C.....A. .T........ G..T..T..T ..T..TC... ....T..... T......... ..C.......
33 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
34 ........A. .T........ G..T..T..T ..T..TC... ....T..... T......... ..C.......
35 ........A. .T........ G..T..T..T ..T..TC.G. ....T..... T......... ..C.......
36 ........A. .T........ G..T..T..T ..T..TC... ....T..... T......... ..C.......
37 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
38 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
39 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
40 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
41 .......... .C..T..... ...T..T..T ..T..T.... ....A..... T..C..T... ..C.....G.
42 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
43 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
44 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
45 .......... .C........ ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
46 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
47 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
48 .......... .C..T..... ...T..T..T ..T..TC... ....A..... T..C..T... ..C.....G.
49 .....T.... .......... ...T..T..T ..T..T.... ....A..... T..C..T... ..........
50 .....T..A. .T..T..... A..T..T..T ..T..TC... ....A..... T..C..T... ..........
51 ..C.....A. .G..T..... ...T.....T ..T..TC... .A..A..... T.....T... ..C.....T.
52 ..C.....A. .G..T..... ...T.....T ..T..TC... .A..A..... T.....T... ..C.....T.
53 ..C.....A. ....T..... ...T.....T ..T...C... .A..A..... ......T... ..C.....T.
54 ..C.....A. .G..T..... ...T.....T ..T..TC... .A..A..... T.....T... ..C.....T.
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```
          430        440        450        460        470        480        490
1  ATATACGCTG AGAAGGCTTA TTAATAGAAC GAATATCATT ATTTGTATGA TCTGTTTTCA TTACAGCAAT
2  .......T.. .......... .......... .......... .......... .......... ..........
3  .......T.. .......... .......... .......... .......... .......... ..........
4  .......T.. .......... .......... .......... .......... .......... ..........
5  .......T.. .......... .......... .......... .......... .......... ..........
6  .......T.. .......... .......... .......... .......... .......... ..........
7  .......T.. .......... .......... .......... .......... .......... ..........
8  .......T.. .......... .......... .......... .......... .......... ..........
9  .......T.. .......... .......... .......... .......... .......... ..........
10 .......T.. .......... .......... .......... .......... .......... ..........
11 .......T.. .......... .......... .......... .......... .......... ..........
12 .......T.. .......... .......... .......... .......... .......... ..........
13 .......T.. .......... .......... .......... .......... .......... .......G..
14 .......T.. .......... .......... .......... .......... .......... ..........
15 .......T.. .......... .......... .......... .......... .......... ..........
16 .......T.. .......... .......... .......... .......... .......... ..........
17 .......T.. .......... .......... .......... .......... .......... ..........
18 .......T.. .......... .......... .......... .......... .......... ..........
19 .......T.. .......... .......... .......... .......... .......... ..........
20 .......A.. ......TC.. .......... ..C.C..T.. ....A.C... ..A..A..T. ....T..T..
21 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
22 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
23 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
24 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
25 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
26 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
27 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
28 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
29 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
30 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
31 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
32 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
33 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
34 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
35 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
36 .......T.. ......TC.T ..G....... .......... ......T... .......... ..........
37 .......T.. ......T... .......... .......T.. .......... ........T. ..........
38 .......T.. ......T... .......... .......T.. .......... ........T. ..........
39 .......T.. ......T... .......... .......T.. .......... ........T. ..........
40 .......T.. ......T... .......... .......T.. .......... ........T. ..........
41 .......T.. ......T... .......... .......T.. .......... ........T. ..........
42 .......T.. ......T... .......... .......T.. .......... ........T. ..........
43 .......T.. ......T... .......... .......T.. .......... ........T. ..........
44 .......T.. ......T... .......... .......T.. .......... ........T. ..........
45 .......T.. ......T... .......... .......T.. .......... ........T. ..........
46 .......T.. ......T... .......... .......T.. .......... ........T. ..........
47 .......T.. ......T... .......... .......T.. G......... ........T. ..........
48 .......T.. ......T... .......... .......T.. .......... ........T. ..........
49 .......T.. ......A... .......... .......T.. .......... ........T. ..........
50 .......T.. ...C..A... .....G.... .......T.. .......... .......... ..........
51 .......A.. ......T... C.T....... .CT..C.T.. .....CC... .....C..T. ....T..T..
52 .......A.. ......T... C.T....... .CT..C.T.. .....CC... .....C..T. ....T..T..
53 .......A.. ......T... C.T.....G. .CT..C.T.. .....CC... .....C..T. ....T..T..
54 .......A.. ......T... C.T....... .CT..C.T.. .....CC... .....C..T. ....T..T..
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          500        510        520        530        540        550        560
1  TCTATTGCTT CTTTCCCTCC CAGTATTAGC TGGAGCAATT ACTATACTCT TAACTGACCG AAATTTTAAC
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ...C......
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ...C......
7  .......... .......... .......... .......... .......... .......... ..........
8  .......... .......... .......... .......... .......... .......... ...C......
9  .......... .......... .......... .......... .......... .......... ..........
10 .......... .......... .......... .......... .......... .......... ..........
11 .......... .......... .......... ...G...... .......... .......... ..........
12 .......... .......... .......... .......... .......... .......... ..........
13 .......... .......... .......... .......... .......... .......... ...C......
14 .......... .......... .......... .......... .......... .......... ..........
15 ......A... .......... ....G..... .......... .......... .......... ..........
16 .......... .......... .......... .......... .......... .......... ..........
17 .......... .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......T. .T........ ..........
19 .......... .......... .......... .......... .......... .......... ..........
20 C.....A..A ..A.....T. ....TC.... G..T..T... ..A.....T. .......... .........T
21 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
22 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
23 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
24 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
25 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
26 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
27 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
28 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
29 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
30 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
31 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
32 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
33 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
34 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
35 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
36 .T....A..C .....T..A. .T..TC.... C......... .........A. .......... ...C..C...
37 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
38 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
39 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
40 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
41 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
42 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
43 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
44 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
45 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
46 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
47 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
48 .T....AT.A ..C..T..T. .......... A......... .........TC .T.....T.. ..........
49 .T..C.AT.A ..C..T..T. ....G..... A......... .TN......C .T........ .T.C...C..
50 .T....AT.A ..C..T..T. .......... A.....T... .........TC .T.....T.. ...C......
51 CT..C.T... ..A..AT.A. .T..T..... A.....C... ..A...T.AC .T..A..... ...C.....T
52 CT..C.T... ..A..AT.A. .T..T..... A.....C... ..A...T.AC .T..A..... ...C.....T
53 CT..C.C... ..A..AT.A. .T..T..... G..G..C... ..A...T.AC .T..A..... ...C.....T
54 CT..C.T... ..A..AT.A. .T..T..... A.....C... ..A...T.AC .T..A..... ...C.....T
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          570        580
1  ACTACCTTTT TTGATCCGAG AGGGGGA
2  .......... .......... .......
3  .......... .......... .......
4  .......... .......... .......
5  .......... .......... .......
6  .......... .......... .......
7  .......... .......... .......
8  .......... .......... .......
9  .......... .......... .......
10 .......... .......... .......
11 .......... .......... .......
12 .......... .......... .......
13 .......... .......... .......
14 .......... .......... .......
15 .......... .......... .......
16 .......... .......... .......
17 .......... .......... .......
18 .........A. .......... ..-----
19 .....N.... .......... ..-----
20 .....T..C. ....C..T.. T..A...
21 ..C..A..C. ....C..A.. ...T..T
22 ..C..A..C. ....C..A.. ...T..T
23 ..C..A..C. ....C..A.. ...T..T
24 ..C..A..C. ....C..A.. ...T..T
25 ..C..A..C. ....C..A.. ...T..T
26 ..C..A..C. ....C..A.. ...T..T
27 ..C..A..C. ....C..A.. ...T..T
28 ..C..A..C. ....C..A.. ...T..T
29 ..C..A..C. ....C..A.. ...T..T
30 ..C..A..C. ....C..A.. ...T..T
31 ..C..A..C. ....C..A.. ...T..T
32 ..C..A..C. ....C..A.. ...T..T
33 ..C..A..C. ....C..A.. ...T..T
34 ..C..A..C. ....C..A.. ...T..T
35 ..C..A..C. ....C..A.. ...T..T
36 ..C..A..C. ....CN.A.. ...T..T
37 .....A.... ....C..A.. ...A...
38 .....A.... ....C..A.. ...A..-
39 .....A.... ....C..A.. ...A...
40 .....A.... ....C..A.. ...A...
41 .....A.... ....C..A.. ...A...
42 .....A.... ....C..A.. ...A...
43 .....A.... ....C..A.. ...A...
44 .....A.... ....C..A.. ...A...
45 .....A.... ....C..A.. ...A...
46 .....A.... ....C..A.. ...A...
47 .....A.... ....C..A.. ...A...
48 .....A.... ....C..A.. ...A...
49 ....NNG... AN..C..A.- -------
50 .....A.... ....C..A.. ...C---
51 ..C..T..C. .C..C..A.. ...T...
52 ..C..T..C. .C..C..A.. ...T...
53 ..C..T..C. .C..C..A.. ...T..G
54 ..C..T..C. .C..C..A.. ...T...
```

Appendix 2: Aligned 16S rRNA sequences used in this study

Dots indicate nucleotide identical to 1 (top row); missing data are coded with the letter N; gaps at the end of sequences are coded with '-'
1-10: *U. chinensis* (Hong Kong), 11-14: *U. chinensis* (Xiamen), 15: *U. chinensis* (Gulf of Thailand, AF110091), 16: *L. chinensis* (AF369955), 17: *L. chinensis* (AJ000105), 18-22: *U. edulis* (Yamaguchi), 23-26: *U. edulis* (Shanghai), 27: *L. edulis* (AJ000103), 28: *L. edulis* (AF369956), 29-34: *U. duvauceli* (Hong Kong), 35: *L. duvauceli* (AJ000101), 36: *U. duvauceli* (Gulf of Thailand, AF110093), 37: *U. duvauceli* (Andaman Sea, AF110092), 38-41: *Sepioteuthis lessoniana* (Hong Kong)

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           10         20         30         40         50         60         70
1  GAGTTGGGCC TGCTCGGTGA AACGTTTT-A ACAGCTGCGG TATTTTAACT GTACTAAGGT AGCATAATAA
2  .......... .......... ........-. .......... .......... .......... ..........
3  .......... .......... ........-. .......... .......... .......... ..........
4  .......... .......... ........-. .......... .......... .......... ..........
5  .......... .......... ........-. .......... .......... .......... ..........
6  .......... .......... ........-. .......... .......... .......... ..........
7  .......... .......... ........-. .......... .......... .......... ..........
8  .......... .......... ........-. .......... .......... .......... ..........
9  .......... .......... ........-. .......... .......... .......... ..........
10 .......... .......... ........-. .......... .......... .......... ..........
11 .......... .......... ........-. .......... .......... .......... ..........
12 .......... .......... ........-. .......... .......... .......... ..........
13 .......... .......... ........-. .......... .......... .......... ..........
14 .......... .......... ........-. .......... .......... .......... ..........
15 --.A....... .......... .....C..-. .......... .......... .......... ..........
16 .......... .......... ..A.....-. .......... .......... .......... ..........
17 .......... .......... ........-. .......... .......... .......... ..........
18 .......... .......... ..T..-..-. .......... .......... .......... ..........
19 .......... .......... ..T..-..-. .......... .......... .......... ..........
20 .......... .......... ..T..-..-. .......... .......... .......... ..........
21 .......... .......... ..T..-..-. .......... .......... .......... ..........
22 .......... .......... ..T..-..-. .......... .......... .......... ..........
23 .......... .......... ..T..-..-. .......... .......... .......... ..........
24 .......... .......... ..T..-..-. .......... .......... .......... ..........
25 .......... .......... ..T..-..-. .......... .......... .......... ..........
26 .......... .......... ..T..-..-. .......... .......... .......... ..........
27 .......... .......... .TAT....T. .......... .......... .......... ..........
28 .......... .....A.... TGTAAA..T. .......... .......... .......... ..........
29 .......... .......... .TAT....-. .......... .......... .......... ..........
30 .......... .......... .TAT....-. .......... .......... .......... ..........
31 .......... .......... .TAT....-. .......... .......... .......... ..........
32 .......... .......... .TAT....-. .......... .......... .......... ..........
33 .......... .......... .TAT....-. .......... .......... .......... ..........
34 .......... .......... .TAT....-. .......... .......... .......... ..........
35 .......... .......... .TAT....T. .......... .......... .......... ..........
36 .......... .......... ..TT....T. .......... .......... .......... ..........
37 .......... .......... ..TT....-. .......... .......... .......... ..........
38 ....C..... .......... ..AT....-. .......... .......... .......... ..........
39 ....C..... .......... ..AT....-. .......... .......... .......... ..........
40 ....C..... .......... ..AT....-. .......... .......... .......... ..........
41 ....C..... .......... ..AT....-. .......... .......... .......... ..........
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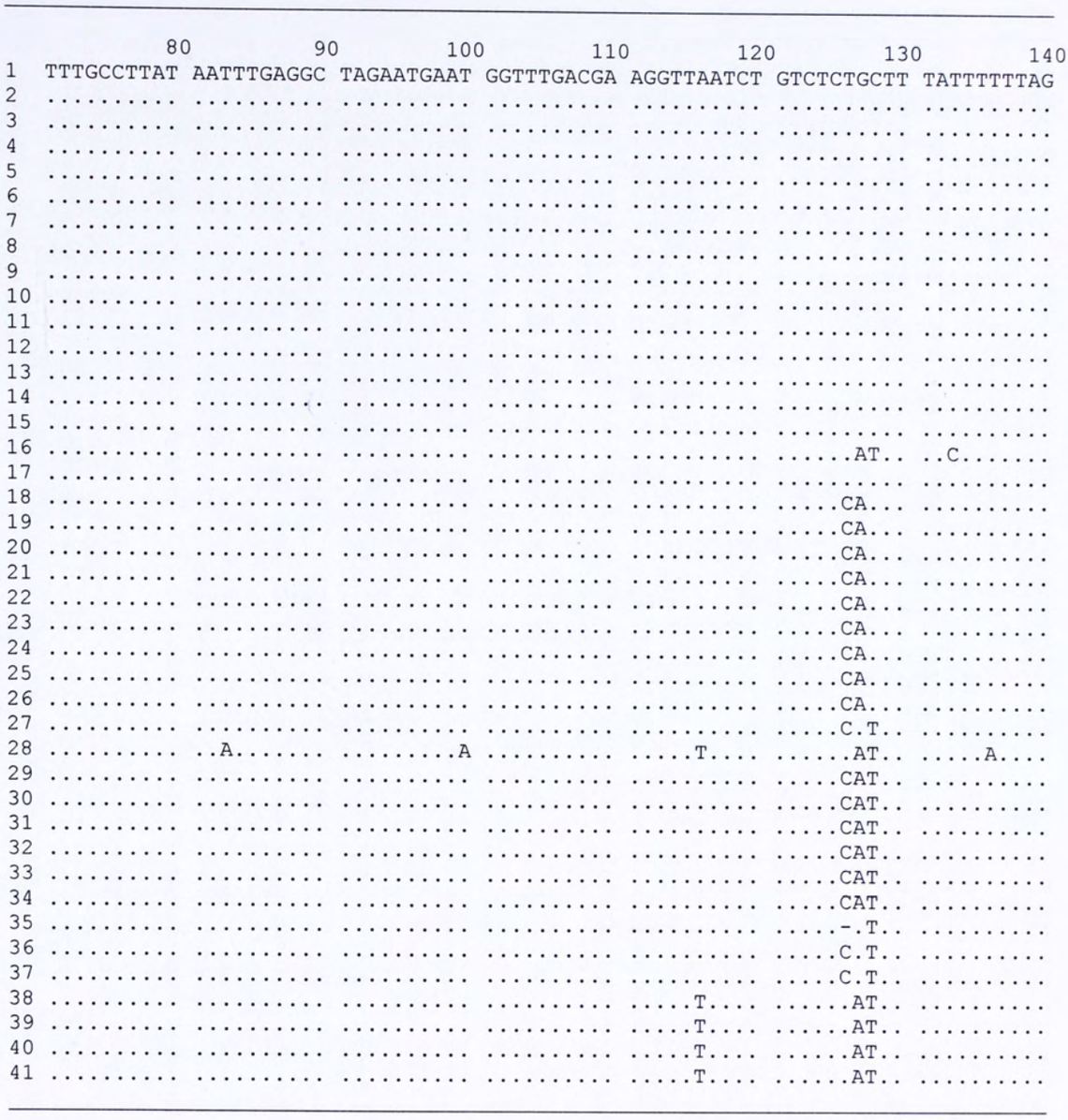

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          80         90        100        110        120        130        140
1  TTTGCCTTAT AATTTGAGGC TAGAATGAAT GGTTTGACGA AGGTTAATCT GTCTCTGCTT TATTTTTTAG
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ..........
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
7  .......... .......... .......... .......... .......... .......... ..........
8  .......... .......... .......... .......... .......... .......... ..........
9  .......... .......... .......... .......... .......... .......... ..........
10 .......... .......... .......... .......... .......... .......... ..........
11 .......... .......... .......... .......... .......... .......... ..........
12 .......... .......... .......... .......... .......... .......... ..........
13 .......... .......... .......... .......... .......... .......... ..........
14 .......... .......... .......... .......... .......... .......... ..........
15 .......... .......... .......... .......... .......... .......... ..........
16 .......... .......... .......... .......... .......... ......AT.. ..C.......
17 .......... .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... .....CA... ..........
19 .......... .......... .......... .......... .......... .....CA... ..........
20 .......... .......... .......... .......... .......... .....CA... ..........
21 .......... .......... .......... .......... .......... .....CA... ..........
22 .......... .......... .......... .......... .......... .....CA... ..........
23 .......... .......... .......... .......... .......... .....CA... ..........
24 .......... .......... .......... .......... .......... .....CA... ..........
25 .......... .......... .......... .......... .......... .....CA... ..........
26 .......... .......... .......... .......... .......... .....CA... ..........
27 .......... .......... .......... .......... .......... .....C.T.. ..........
28 .......... ..A....... .........A .......... .....T.... ......AT.. .....A....
29 .......... .......... .......... .......... .......... .....CAT.. ..........
30 .......... .......... .......... .......... .......... .....CAT.. ..........
31 .......... .......... .......... .......... .......... .....CAT.. ..........
32 .......... .......... .......... .......... .......... .....CAT.. ..........
33 .......... .......... .......... .......... .......... .....CAT.. ..........
34 .......... .......... .......... .......... .......... .....CAT.. ..........
35 .......... .......... .......... .......... .......... .....-.T.. ..........
36 .......... .......... .......... .......... .......... .....C.T.. ..........
37 .......... .......... .......... .......... .......... .....C.T.. ..........
38 .......... .......... .......... .......... .....T.... ......AT.. ..........
39 .......... .......... .......... .......... .....T.... ......AT.. ..........
40 .......... .......... .......... .......... .....T.... ......AT.. ..........
41 .......... .......... .......... .......... .....T.... ......AT.. ..........
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          150        160        170        180        190        200        210
1  AAATTAATTT TCATAGTGAA AAAGCTTGAA TTTCTTAAGG GGACGAGAAG ACCCTACTGA GCTTATAATT
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ..........
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
7  .......... .......... .......... .......... .......... .......... ..........
8  .......... .......... .......... .......... .......... .......... ..........
9  .......... .......... .......... .......... .......... .......... ..........
10 .......... .......... .......... .......... .......... .......... ..........
11 .......... .......... .......... .......... .......... .......... ..........
12 .......... .......... .......... ...T...... .......... .......... ..........
13 .......... .......... .......... .......... .......... .......... ..........
14 .......... .......... .......... .......... .......... .......... ..........
15 .......... .......... .......... .......... .......... .......... ..........
16 .......... .......... ..G....... ...T....A. .......... ......T... ....G.....
17 .......... .......... .......... ...TC.T... .-........ .......... ..........
18 .......... .......... .......... ...T...... .......... ......T... ..........
19 .......... .......... .......... ...T...... .......... ......T... ..........
20 .......... .......... .......... ...T...... .......... ......T... ..........
21 .......... .......... .......... ...T...... .......... ......T... ..........
22 .......... .......... .......... ...T...... .......... ......T... ..........
23 .......... .......... .......... ...T...... .......... ......T... ..........
24 .......... .......... .......... ...T...... .......... ......T... ..........
25 .......... .......... .......... ...T...... .......... ......T... ..........
26 .......... .......... .......... ...T...... .......... ......T... ..........
27 .......... .......... .......... ...T....A. .......... ......T... ..........
28 ..T....... .T-....... ......AT.. .A.T....A. .......... ......T... .....-....
29 .......... .......... .......... ...T....A. .......... ......T... ..........
30 .......... .......... .......... ...T....A. .......... ......T... ..........
31 .......... .......... .......... ...T....A. .......... ......T... ..........
32 .......... .......... .......... ...T....A. .......... ......T... ..........
33 .......... .......... .......... ...T....A. .......... ......T... ..........
34 .......... .......... .......... ...T....A. .......... ......T... ..........
35 .......... .......... .......... ...T....A. .......... ......T... ..........
36 .......... .......... .......... ...T....A. .......... ......T... ..........
37 .......... .......... .......... ...T....A. .......... ......T... ..........
38 .......... .......... .......... ...T....A. .......... ......T... ....G..G..
39 .......... .......... .......... ...T....A. .......... ......T... ....G..G..
40 .......... .......... .......... ...T....A. .......... ......T... ....G..G..
41 .......... .......... .......... ...T....A. .......... ......T... ....G..G..
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          220        230        240        250        260        270        280
1  TTATTA---T CACAT-ATAT TTATGTATAA T-GTGCTATA TAAATTTTAA TTGGGGTGAT TAAGGAATAA
2  .......---. .....-.... .......... .-........ .......... .......... ..........
3  .......---. .....-.... .......... .-........ .......... .......... ..........
4  .......---. .....-.... .......... .-........ .......... .......... ..........
5  .......---. .....-.... .......... .-........ .......... .......... ..........
6  .......---. .....-.... .......... .-........ .......... .......... ..........
7  .......---. .....-.... .......... .-........ .......... .......... ..........
8  .......---. .....-.... .......... .-........ .......... .......... ..........
9  .......---. .....-.... .......... .-........ .......... .......... ..........
10 .......---. .....-.... .......... .-........ .......... .......... ..........
11 .......---. .....-.... .......... .-........ .......... .......... ..........
12 .......---. .....-.... .......... .-........ .......... .......... ..........
13 .......---. .....-.... .......... .-........ .......... .......... ..........
14 .......---. .....-.... .......... .-........ .......... .......... ..........
15 .......---. .....-.... .......... .-........ .......... .......... ..........
16 ......TTA. T.G-.AT... ....TCT..T .G..AG.... .......... .......... ..........
17 .......---. .....-.... .......... .-........ ..C.A..... .....-.... ..........
18 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
19 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
20 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
21 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
22 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
23 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
24 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
25 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
26 .......---A T.TT.T...A A...T...GT .-...T.... .......... .......... ..........
27 ......CTA. TGTG.TT.G. .G.AT...C. .CA.AG.... .......... .......... ..........
28 .A....---- ..T.----.. .A.AT...GT .-----.TG. .......... .......... ..........
29 ......CTA. T.TGGTTAT. GA..T...C. .T..AG.... .......... .......... ..........
30 ......CTA. T.TGGTTAT. GA..T...C. .T..AG.... .......... .......... ..........
31 ......CTA. T.TGGTTAT. GA..T...C. .T..AG.... .......... .......... ..........
32 ......CTA. T.TGGTTAT. GA..T...C. .T..AG.... .......... .......... ..........
33 ......CTA. T.TGGTTAT. GA..T...C. .T..AG.... .......... .......... ..........
34 ......CTA. T.TGGTTAT. GA..T...C. .T..AG.... .......... .......... ..........
35 ......CTA. TGTG.TT.G. -G.AT...C. .CA.AG.... .......-.. .......... ..........
36 ......CTA. TGTG.TT.G. .G.AT...C. .CA.AG.... .......... .......... ..........
37 ......CTA. T.TG.TTGT. AA..TC..C. .T..AG.... .......... .......... ..........
38 ..TA..TAAC T.--.AT... .A..T....T AGT.AT.-.. ....C....G .........C ..........
39 ..TA..TAAC T.--.AT... .A..T....T AGT.AT.-.. ....C....G .........C ..........
40 ..-A..TAAC T.--.AT... .A..T....T AGT.AT.-.. ....C....G .........C ..........
41 ..TA..TAAC T.--.AT... .A..T....T AGT.AT.-.. ....C....G .........C ..........
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          290        300        310        320        330        340        350
1  TATAGCT-AT TTTATA---- -ACTTCCTTA GAT-ATTATA TTGTTGAATT AAGTAACCGA T-TAATTAAT
2  .......-.. ......---- -......... ...-...... .......... .......... .-.........
3  .......-.. ......---- -......... ...-...... .......... .......... .-.........
4  .......-.. ......---- -......... ...-...... .......... .......... .-..G.....
5  .......-.. ......---- -......... ...-...... .......... .......... .-.........
6  .......-.. ......---- -......... ...-...... .......... .......... .-.........
7  .......-.. ......---- -......... ...-...... .......... .......... .-.........
8  .......-.. ......---- -......... ...-...... .......... .......... .-.........
9  .......-.. ......---- -......... ...-...... .......... .......... .-.........
10 .......-.. ......---- -......... ...-...... .......... .......... .-.........
11 .......-.. ......---- -......... ...-...... .......... .......... .-.........
12 .......-.. ......---- -......... ...-...... .......... .......... .-.........
13 .......-.. ......---- -......... ...-...... .......... .......... .-.........
14 .......-.. ......---- -......... ...-...... .......... .......... .-.........
15 .......-.. ......---- -......... ...-...... .......... .......... .-...C....
16 GG.TTG.AG. ....GT---- A......... TTAATAATG. .....A...A ........A. ----------
17 .......-.. ..-...---- -......... ...-...... .......... .......... ----------
18 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
19 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
20 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
21 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
22 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
23 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
24 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
25 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
26 .T.TAT.G.. ......GTGT A......... ..A-.AC... .....A.... .......... .-.T..AT..
27 AT.TT---.. GG....---- -......... ...T.A.... .....AG..- G....-.... .A..G....C
28 A.------.. A.....TAGT A......... ATAGT.A... C.....GG.. ..T.....A. .---------
29 AT.TTT--.. AA.G..---- -......... ...T...... .....A.... .......... .G.G....GC
30 AT.TTT--.. AA.G..---- -......... ...T...... .....A.... .......... .G.G....GC
31 AT.TTT--.. AA.G..---- -......... ...T...... .....A.... .......... .G.G....GC
32 AT.TTT--.. AA.G..---- -......... ...T...... .....A.... .......... .G.G....GC
33 AT.TTT--.. AA.G..---- -......... ...T...... .....A.... .......... .G.G....GC
34 AT.TTT--.. AA.G..---- -......... ...T...... .....A.... .G........ .G.G....GC
35 AT.TT---.. GG....---- -......... T..T.A.... .....AG... GT.A.-.... .A..G....C
36 AT.TTT--.. GG....---- -......... ...T.A.... .....AG... G......... .A..G....C
37 GT.TTT.-.. AA....---- -......... ...T.A.... .....AG... .......... .G.G.....C
38 ----------. A.A.GT---- A......... .-AGTAA.G. .....A...A ........A. A---------
39 ----------. A.A.GT---- A......... .-AGTAA.G. .....A...A ........A. A---------
40 ----------. A.A.GT---- A......... .-AGTAA.G. .....A...A ........A. A---------
41 ----------. A.A.GT---- A......... .-AGTAA.G. .....A...A ........A. A---------
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          360        370        380        390        400        410        420
1  AGAAT-TGTA TTATGTATTG --CTTATAAT ATAG--TTAC CATAGGGATA ACAGCGTAAT TTACTTAGAG
2  .....-.... .......... --......... ....--.... .......... .......... ..........
3  .....-.... .......... --......... ....--.... .......... .......... ..........
4  .....-.... .......... --......... ....--.... .......... .......... ..........
5  .....-.... .......... --......... ....--.... .......... .......... ..........
6  .....-.... .......... --......... ....--.... .......... .......... ..........
7  .....-.... .......... --......... ....--.... .......... .......... ..........
8  .....-.... .......... --......... ....--.... .......... .......... ..........
9  .....-.... .......... --......... ....--.... .......... .......... ..........
10 .....-.... .......... --......... ....--.... .......... .......... ..........
11 .....-.... .......... --......... ....--.... .......... .......... ..........
12 .....-.... .......... --......... ....--.... .......... .......... ..........
13 .....-.... .......... --......... ....--.... .......... .......... ..........
14 .....-.... .......... --......... ....--.... .......... .......... ..........
15 .....-.... .......... --......... ....--.... .......... .......... ..........
16 ---------- --TAT..... --......C. ....--.... .......... ..........T.......
17 -....-.... .........-. --......... ....--.... .......... .......... ..........
18 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
19 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
20 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
21 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
22 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
23 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
24 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
25 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
26 ..GT.-.A.. ..GG...... --......... ....--.... .......... .......... ..........
27 .T.G.G...T .AGA.....T GCT........ ..TAGT.... .......... ...-...... ..........
28 ---------- ---AA..... --......... .A..--.... .......... ..........T.......
29 .C...G...G .......... --......... ....--.... .......... .......... ..........
30 .C...G...G .......... --......... ....--.... .......... .......... ..........
31 .C...G...G .......... --......... ....--.... .......... .......... ..........
32 .C...G...G .......... --......... ....--.... .......... .......... ..........
33 .C...G...G .......... --......... ....--.... .......... .......... ..........
34 .C...G...G .......... --......... ....--.... .......... .......... ..........
35 .T.G.G...T -AGA...... --......... ....--.... .......... .......... ..........
36 .T.G.G...- .AGA...... --......... ....--.... .......... .......... ..........
37 .T...G.A.G .......... --......... ....--.... .......... .......... ..........
38 ---------- ..TAA..... --......... ....--.... .......... .......... ...T......
39 ---------- ..TAA..... --......... ....--.... .......... .......... ...T......
40 ---------- ..TAA..... --......... ....--.... .......... .......... ...T......
41 ---------- ..TAA..... --......... ....--.... .......... .......... ...T......
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          430        440        450        460        470        480        490
1  AGTTCTTATT GAAAAGTAAG ATTGCGACCT CGATGTTGGA TTAAAGTGAC CTTAAGGTGC AGAAGCTTTA
2  .......... .......... .......... .......... .......... .......... ..........
3  .......... .......... .......... .......... .......... .......... ..........
4  .......... .......... .......... .......... .......... .......... ..........
5  .......... .......... .......... .......... .......... .......... ..........
6  .......... .......... .......... .......... .......... .......... ..........
7  .......... .......... .......... .......... .......... .......... ..........
8  .......... .......... .......... .......... .......... .......... ..........
9  .......... .......... .......... .......... .......... .......... ..........
10 .......... .......... .......... .......... .......... .......... ..........
11 .......... .......... .......... .......... .......... .......... ..........
12 .......... .......... .......... .......... .......... .......... ..........
13 .......... .......... .......... .......... .......... .......... ..........
14 .......... .......... .......... .......... .......... .......... ..........
15 .......... .......... .......... .......... .......... ......---- ----------
16 .......... .....A.... .......... .......... .......A.. .......... ..........
17 .......... .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......A.. .......... ..........
19 .......... .......... .......... .......... .......A.. .......... ..........
20 .......... .......... .......... .......... .......A.. .......... ..........
21 .......... .......... .......... .......... .......A.. .......... ..........
22 .......... .......... .......... .......... .......A.. .......... ..........
23 .......... .......... .......... .......... .......A.. .......... ..........
24 .......... .......... .......... .......... .......A.. .......... ..........
25 .......... .......... .......... .......... .......A.. .......... ..........
26 .......... .......... .......... .......... .......A.. .......... ..........
27 .......... .......... .......... ....TG.... .......A.. .......... ..........
28 .......... .....A.... .......... .......... .......A.. .......... ..........
29 .......... .......... .......... .......... .......A.. .......... ..........
30 .......... .......... .......... .......... .......A.. .......... ..........
31 .......... .......... .......... .......... .......A.. .......... ..........
32 .......... .......... .......... .......... .......A.. .......... ..........
33 .......... .......... .......... .......... .......A.. .......... ..........
34 .......... .......... .......... .......... .......A.. .......... ..........
35 .......... .......... .......... .......... .......A.. .......... ..........
36 .......... .......... .......... .......... .......A.. .......... ..........
37 .......... .......... .......... .......... .......A.. .......... ..........
38 .....A.... .....A.... .......... .......... .......A.. ...G...... ........C.
39 .....A.... .....A.... .......... .......... .......A.. ...G...... ........C.
40 ..C..A.... .....A.... .......... .......... .......A.. ...G...... ........C.
41 .....A.... .....A.... .......... .......... .......A.. ...G...... ........C.
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            500         510         520
1  TGA-GGTAAA TCTGTTCGAT TTTTAAAACT TT
2  ...-...... .......... .......... ..
3  ...-...... .......... .......... ..
4  ...-...... .......... .......... ..
5  ...-...... .......... .......... ..
6  ...-...... .......... .......... ..
7  ...-...... .......... .......... ..
8  ...-...... .......... .......... ..
9  ...-...... .......... .......... ..
10 ...-...... .......... .......... ..
11 ...-...... .......... .......... ..
12 ...-...... .......... .......... ..
13 ...-...... .......... .......... ..
14 ...-...... .......... .......... ..
15 ---------- ---------- ---------- --
16 AT.A...... .......... .......... ..
17 ...-...... .......... .......... ..
18 .A.-...... .......... .......... ..
19 .A.-...... .......... .......... ..
20 .A.-...... .......... .......... ..
21 .A.-...... .......... .......... ..
22 .A.-...... .......... .......... ..
23 .A.-...... .......... .......... ..
24 .A.-...... .......... .......... ..
25 .A.-...... .......... .......... ..
26 .A.-...... .......... .......... ..
27 .A.-...... .......... .......... ..
28 AT.-...... .......... .......... ..
29 .A.-...... .......... .......... ..
30 .A.-...... .......... ...-...... ..
31 .A.-...... .......... .......... ..
32 .A.-...... .......... .......... ..
33 .A.-...... .......... .......... ..
34 .A.-...... .......... .......... ..
35 .A.-...... .......... .......... ..
36 .A.-...... .....----- ---------- --
37 .A.-...... ......---- ---------- --
38 GTG-...... .......... .......... ..
39 GTG-...... .......... .......... ..
40 GTG-...... .......... .......... ..
41 GTG-...... .......... .......... ..
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