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MORPHOLOGICAL AND MITOCHONDRIAL DNA CHARACTERISTICS OF TWO CULTURED SPECIES OF *CUPPED-OYSTERS* (BIVALVIA: OSTREIDAE) IN HONG KONG: TOWARDS A SIGNIFICANT TAXONOMIC NAME CHANGE

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ABSTRACT

Cultured cupped-oysters species in Hong Kong have rarely been differentiated properly due to similarities in shell characters. This study attempted to characterize putative *Crassostrea gigas* and *Crassostrea ariakensis* by morphological and genetic means. Allometric ratios of shell height/length and depth/length were significantly different between the two species. Restriction fragment length polymorphism (RFLP) of a PCR-amplified mitochondrial COI (cytochrome oxidase I) fragment was used to examine genetic differentiation between putative *C. gigas* from Hong Kong and previously studied populations of *Crassostrea* from Europe, Japan and Taiwan. The two Hong Kong species are not only morphologically, but also genetically different from each other in terms of all the four restriction sites tested (*Taq*I, *Sau*3A, *Hha*I and *Mse*I) showing that RFLP in this COI fragment is a useful tool to distinguish them.

Putative *Crassostrea gigas* oysters in Hong Kong present a new and unique monomorphic COI haplotype when compared with *C.gigas* and *C. angulata* from Europe and Asia. The numbers of nucleotide differences between this Hong Kong *Crassostrea* species and *C. gigas*/*C. angulata* populations were high (~13–15) and significantly different (all p-values of pairwise test < 0.01). These results, therefore, suggest that the Hong Kong individuals represent a new species of cupped-oyster and this is currently being described.

INTRODUCTION

Oysters of the genus *Crassostrea* (Sacco, 1897) are important commercially worldwide. In Hong Kong, the cultivation of species of *Crassostrea* is reputed to have a ~700 year history and the industry is today located around Deep Bay, in northwestern Hong Kong (Morton and Wong 1975). *Crassostrea* taxonomy in the East and South China Seas has, however, been problematic. At least nine Asian species have been described based on morphological criteria. These are *C. ariakensis* (Wakiya, 1929), *C. belcheri* (Sowerby, 1871), *C. gigas* (Thünberg, 1793), *C. inequivalvis* (Sowerby, 1871), *C. lugubris* (Sowerby, 1871), *C. nippona* (Seki, 1934), *C. sikamea* (Amemiya, 1928), *C. paulucciae* (Crosse, 1869), *C. rivularis* (Gould, 1861) and *C. vitrefacta* (Sowerby, 1871) (Bernard *et al.* 1993).

The identities of the cultured species of cupped-oysters in Hong Kong have been discussed for many years. Bromhall (1958) examined a series of specimens of Deep Bay oysters but was unable to arrive at a conclusion with regard to an identity. This author suggested that on morphological and ecological grounds it belongs to the *Crassostrea angulata* (Lamarck, 1819), *C. virginica* (Gmelin, 1791), *C. gigas*, *C. commercialis* (=*Saccostrea commercialis*) (Iredale and Roughley, 1933) and *Ostrea rivularis* group of species. Morris (1985) identified the Deep Bay oyster, also on the basis of shell characters, as *Crassostrea gigas*. The oyster has also been given this name by Mok (1973, 1974a, b), Morton (1975), Morton and Wong (1975), Wong *et al.* (1981), Phillips *et al.* (1982), Morton and Morton (1983), Cheung and Wong (1992) and Chan *et al.* (1999). *C. rivularis* (Gould, 1861), however, is also reported to occur in local oyster beds (Morton and Morton 1983; Young and Melville 1993). Due to morphological similarities, however, the two species have rarely been differentiated properly. The aim of the present study was to compare the genetic diversity of Hong Kong's cultured species of *Crassostrea* in Deep Bay at both inter- and intra-specific levels with that of previously studied oyster samples. Information and results from the present work provide baseline information necessary for oyster farming studies. They are also expected to help strategic policy decisions in relation to the local oyster fishing industry and the conservation and management of marine invertebrates. The objectives of this study were, therefore: (1), to morphologically and molecularly distinguish putative *Crassostrea gigas* from *C. rivularis*; (2), to determine the specific identity of Hong Kong *Crassostrea* species by comparing mitochondrial DNA restriction fragment length polymorphism (RFLP) patterns with European and Asian taxa (this new species is currently being described by Lam and Morton [2003]) and (3), to monitor genetic variability in populations of Hong Kong *Crassostrea* using the COI (cytochrome oxidase I) gene.

MATERIALS AND METHODS

Sample collection

Individuals of cultivated, putative *Crassostrea gigas* (n = 50), and C. *rivularis* (n = 35) were obtained from an oyster farm in Lau Fau Shan, Deep Bay, Hong Kong, in February 2001 (Fig. 1). Forty-eight wild *Crassostrea* individuals were also collected from a rocky shore at Pak Nai, ~ 2 km southeast of this oyster farm (Fig. 1). The seed of all the studied populations was, thus, from the same wild set in the Pearl River Estuary.

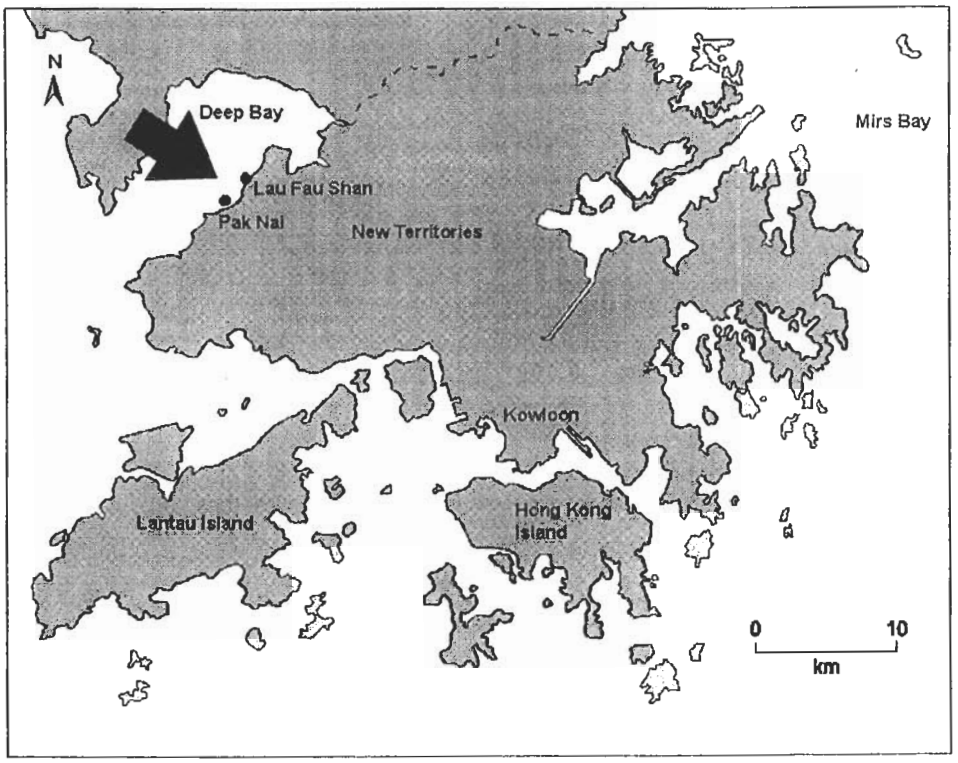

Fig. 1. A map of Hong Kong showing the sample collection sites at Lau Fau Shan and Pak Nai.

Comparative shell morphology and shell allometry

Comparisons were made between the shell characters of the putative *Crassostrea gigas* and *C. rivularis* individuals to clarify conchological distinctions. Shell characters examined were: (1), shape and surface sculpture; (2), external and internal shell colour; (3), hinge line and ligament position and extent; (4), attachment area of the left valve and (5), shape, position, colour and relative size of the adductor muscle scar. Shells were measured in terms of height (the dorsal:ventral axis), length (the anterior:posterior axis) and depth (the lateral axis). Each dimension was measured to the nearest 0.1 mm with Vernier calipers.

Mitochondrial DNA RFLP analysis

Genomic DNA was isolated from gill tissues of individual oysters using the phenol/chloroform method as described by Moore (1993). The partial COI segment of the mtDNA was amplified by PCR using LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG–3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA–3') as forward and backward primers, respectively (Folmer *et al.* 1994). PCR reactions were set up in a 50 ml volume solution composed of 0.4 μM of each primer, 2.5 mM $MgCl_2$, 2mM dNTPs, 5

µl of 10 x reaction buffer and 1.5 units of Taq polymerase (SilverStar): 0.2 µg of total genomic DNA was added as a template. The amplification cycles produced double-stranded PCR products as follows: denaturation at 94°C for two minutes, 35 cycles including denaturation at 94°C for one minute, annealing at 50°C for two minutes and extension at 72°C for one minute, followed by a final extension for seven minutes at 72°C.

The amplified segments of DNA were screened for restriction site polymorphism using four endonucleases: *Taq*I, *Sau*3A, *Hha*I and *Mse*I. These enzymes were chosen as they show restriction polymorphism of the COI segment among *Crassostrea gigas* and *C. angulata* populations (Boudry *et al.* 1998). Restriction reactions were performed for two hours in a 20 µl volume solution composed of 10 µl PCR product, 2 µl 10 x reaction buffer and two units of restriction endonucleases. The digested samples were electrophorized on 2% agarose gels in 1 x TAE buffer and stained using ethidium bromide. Restricted fragments were visualised under UV trans-illuminator. Composite genotypes (haplotypes) were designated four lower case letters, representing the different profiles for each of the four restriction endonucleases, as in Boudry *et al.* (1998). The sizes of the restriction fragments were estimated by comparison with a one hundred base pair (bp) ladder. Only fragments > 100 bp were recorded.

Data analysis

Three allometric shell ratios, height/depth, height/length and depth/length were subjected to *t*-tests to detect differences between the two species by using SPSS version 10.0.0 (SPSS Inc. 1989–1999).

To separate putative *Crassostrea gigas* and *C. rivularis* from Hong Kong, the RFLP patterns obtained above were compared with those of *C. angulata*, *C. gigas* and *C. ariakensis* (Boudry *et al.* 1998; Huvet 2000). Two *C. ariakensis* (provided in 1995 by the Shellfish Research Laboratory, Rutgers State University, New Jersey, U.S.A.) showed haplotype L (Table 3), which is the major haplotype presented by Hong Kong *C. rivularis*. These two samples were thus assumed to be genetically identical with respect to the COI gene and data for *C. ariakensis* was not included, therefore, in subsequent analyses.

The genetic relationship among the haplotypes shown by the two Hong Kong cupped-oysters species, and samples of European and Asian *C. angulata* and *C. gigas* was analysed by calculating the mean number of substitutions per restriction site between all haplotype pairs. Inter-haplotypic distance matrix was generated by ARLEQUIN version 2.000 (Schneider *et al.* 2000). A minimum spanning tree of RFLP haplotypes was constructed using the connection length calculated using a method recommended by Rohlf (1973). To analyse genetic distance among populations, a pairwise matrix of nucleotide substitution differences (Nei and Li 1979) was computed using ARLEQUIN.

Results

Comparative shell morphology and allometry

Plate 1 shows the external shell morphologies of the two Hong Kong species of cyooed-oysters. Table 1 identifies the characteristic shell morphologies of the two species. In general, the two species have similar external and internal shell characters. Among the samples examined, the adductor muscle scar in putative *C. gigas* was always D-shaped

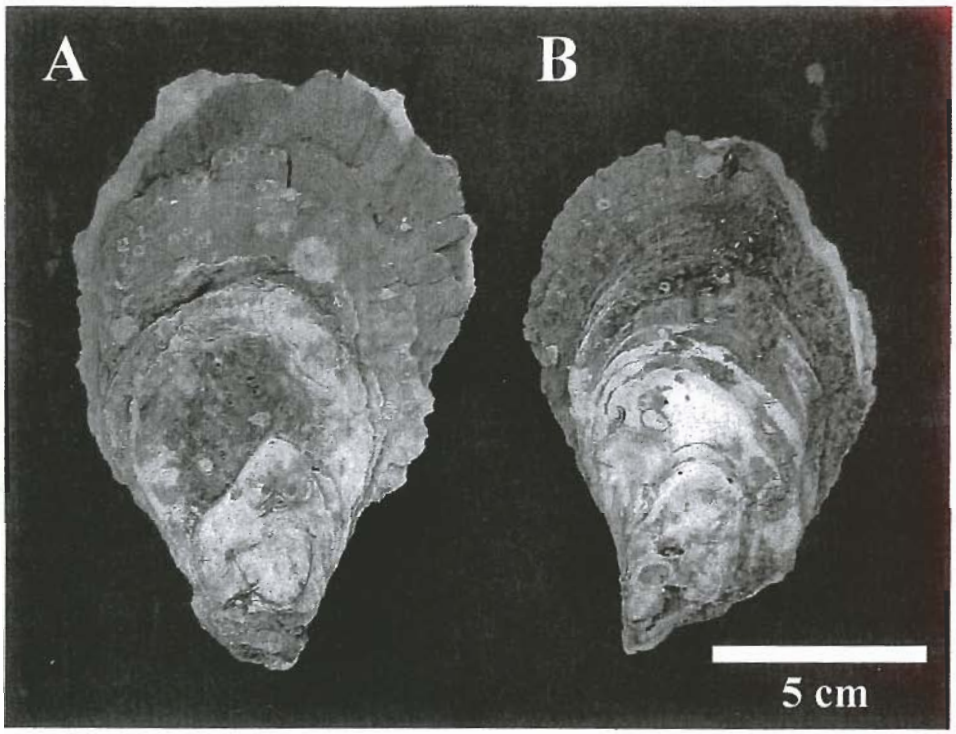

Plate 1. Right side view of both valves of A, *Crassostrea rivularis* (=*C. ariakensis*), and B, putative *C. gigas* from Deep Bay, Hong Kong.

and elongate dorso-ventrally whereas that in *C. rivularis* was always crescentic. The shape of the adductor muscle scar is suggested to be a reliable shell feature distinguishing these two species. The more erect scales in *C. rivularis* may aid identification in non-eroded, intact individuals. Figure 2 presents the mean and standard deviation values obtained for the three allometric ratios. Height/length and depth/length were significantly different between the two species (both $p < 0.05$, t-test) although height/depth was not ($p = 0.179$).

MtDNA RFLP analysis

Four different haplotypes were obtained from the RFLP analysis among the 133 oyster individuals studied. Approximate sizes of the restriction fragments (Plate 2), in bp, are given in Table 2. In general, both putative *Crassostrea gigas* and *C. rivularis* have different profiles with respect to all four restriction enzymes tested, i.e., *Taq*I, *Sau*3A, *Mse*I and *Hha*I. The identity of the two Hong Kong species was tested by comparing their restriction profiles with those of (1), American *C. rivularis* (=*ariakensis*); (2), Japanese and French *C. gigas* (Boudry *et al.* 1998) and (3), Portuguese and Taiwanese *C. angulata.*

Table 1

Shell morphologies of putative *Crassostrea gigas* (determined to be a new species of cupped-oyster by mt DNA RFLP analysis in the present study) and *C. rivularis* (=*C. ariakensis*) both collected from Lau Fau Shan, Hong Kong.

Shell characteristics	New cupped-oyster	*Crassostrea rivularis* (=*C. ariakensis*)
1. Shape and surface sculpture	Long spatulate form; outline tongue-shaped. Left valve deeply cupped. Right valve usually flat.	Orbicular to long spatulate form; outline tongue-shaped. Left valve deeply cupped. Right valve is usually either flat or slightly convex near the dorsal side and slightly concave near the ventral margin.
2. External and internal	External colour whitish shell colour with broken rays of purplish brown. The scales of the outer shell layer radiating from the dorsal side are brown to light brown. Scales in *C. rivularis* are generally more erect. Internal colour whitish with hollow chambers of irregular size and position.	
3. Hinge line and ligament position and extent	Hinge line short; ligament occupies its full length.	
4. Attachment area of the left valve	Attachment area is variably small, usually not more than half shell height.	
5. Adductor muscle scar		
(a). Shape	D-shaped, outline of the adductor muscle on the dorsal side is straight to slightly convex.	D-shaped, outline of the adductor muscle on the dorsal side is concave.
(b). Position	In middle to ventral third of the pallial area.	
(c). Colour	White to purplish growth lines which vary among individuals.	Colour may be different on right and left valves in the same individual.
(d). Relative size	Large	

Table 2

Approximate sizes of the restriction fragments in base pairs (bp) for *Crassostrea angulata* and *Crassostrea gigas* digested with four endonucleases, i.e., *Taq*I, *Sau*3A, *Hha*I and *Mse*I. Combined results are from this study and Boudry *et al.* (1998).

	Restriction endonuclease																	
	*Taq*I					*Sau*3A						*Hha*I				*Mse*I			
Restriction profile	a	b	c	d	e	a	b	c	d	e	f	a	b	c	d	a	b	c	d
Fragment sizes (bp)	660	380	430	370	770 (not cut)	520	400	350	450	400	550	710	525	590	580	285	285	250	250
	280	280	270	130				110	110	110		125	200	200	160	220	160	200	
																115	115		

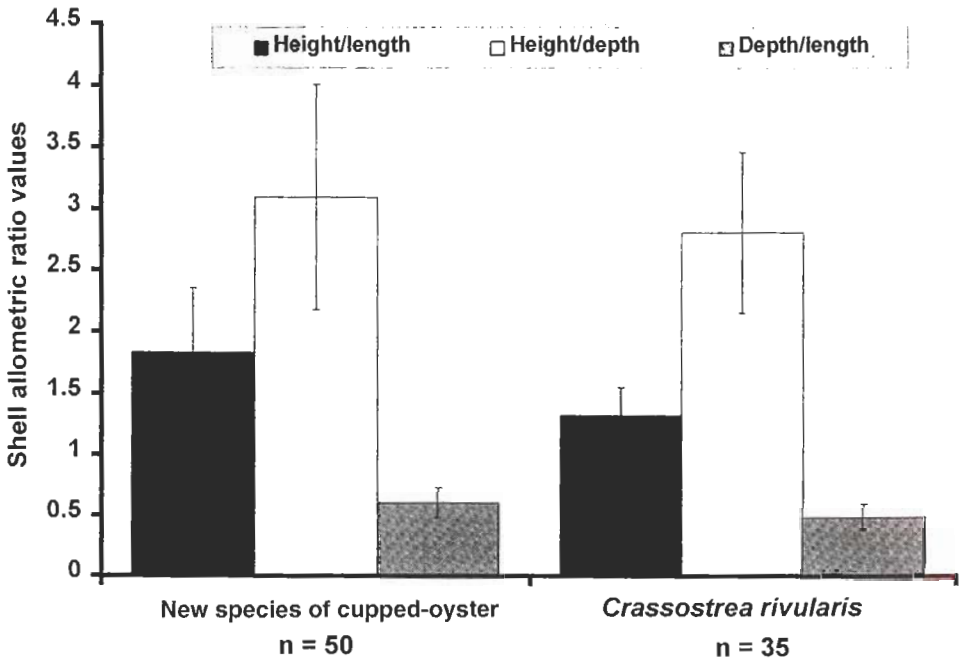

Fig. 2. Means and standard deviations of the shell allometric ratios, height/length, height/depth and depth/length for the new species of *Crassostrea* and *C. rivularis* (=*C. ariakensis*) from Deep Bay, Hong Kong.

RFLP patterns of *Crassostrea rivularis* from Hong Kong and *C. ariakensis* from the United States of America were extremely similar. *C. ariakensis*, therefore, is suggested to be the correct name for this Hong Kong species. Hong Kong *C. ariakensis* is polymorphic with respect to the *Sau* 3A restriction site only. It shows a major haplotype with a frequency of 0.94 (n = 33/35) and two minor ones, both of 0.04 (n = 1/35). Putative Hong Kong *C. gigas*, however, showed a monomorphic, new haplotype, named 'K', with respect to its restriction profile when compared with the results published on this (putative) species from elsewhere (Table 3 and Plate 3). This 'K' haplotype is different from the haplotypes shown by *C. gigas* and *C. angulata*. A minimum spanning tree linking the haplotypes presented by putative *C. gigas* from Hong Kong (Fig. 3) shows that the former is genetically closer to *C. ariakensis* than to *C. angulata* and even *C. gigas* with respect to the COI gene.

The numbers of nucleotide differences between what is being described as a new Hong Kong species of cupped-oyster and *C. gigas*/*C. angulata* from elsewhere were high (~13–15) and significantly different (all *p*-values of pairwise test < 0.01, Table 4). The Hong Kong species of cupped-oyster, therefore, is not *C. gigas* but a new species currently being described by Lam and Morton (2003).

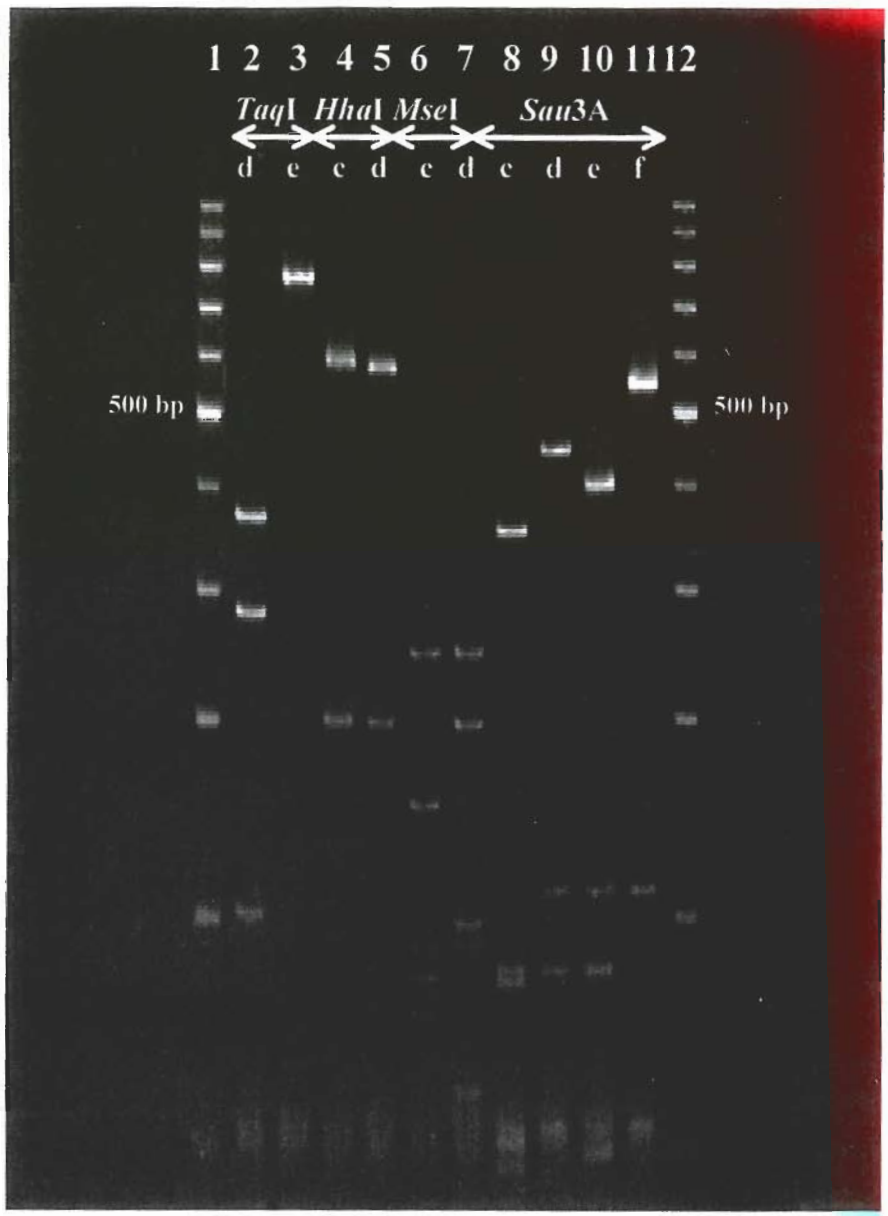

Plate 2. Electrophoretic separation on 8% acrylamide gel of amplified COI mitochondrial DNA gene fragments restriction products from the new species of cupped-oyster and *C. rivularis* (=*C. ariakensis*). Lanes 1 and 12 are the molecular mass standard (100bp ladder, Promega). Lanes 2 and 3, profiles for *Taq*I restriction of the new species of *Crassostrea* and *C. rivularis*, respectively. Lanes 4 to 5, profiles for *Hha*I restriction of the new species of cupped-oyster and *C. rivularis*, respectively. Lanes 6 and 7, profiles for *Mse*I restriction of the new species of cupped-oyster and *C. rivularis* (=*C. ariakensis*), respectively. Lanes 8 to 11, profiles for *Sau*3A restriction of the new species of cupped-oyster, *C. rivularis* major and two minor types, respectively.

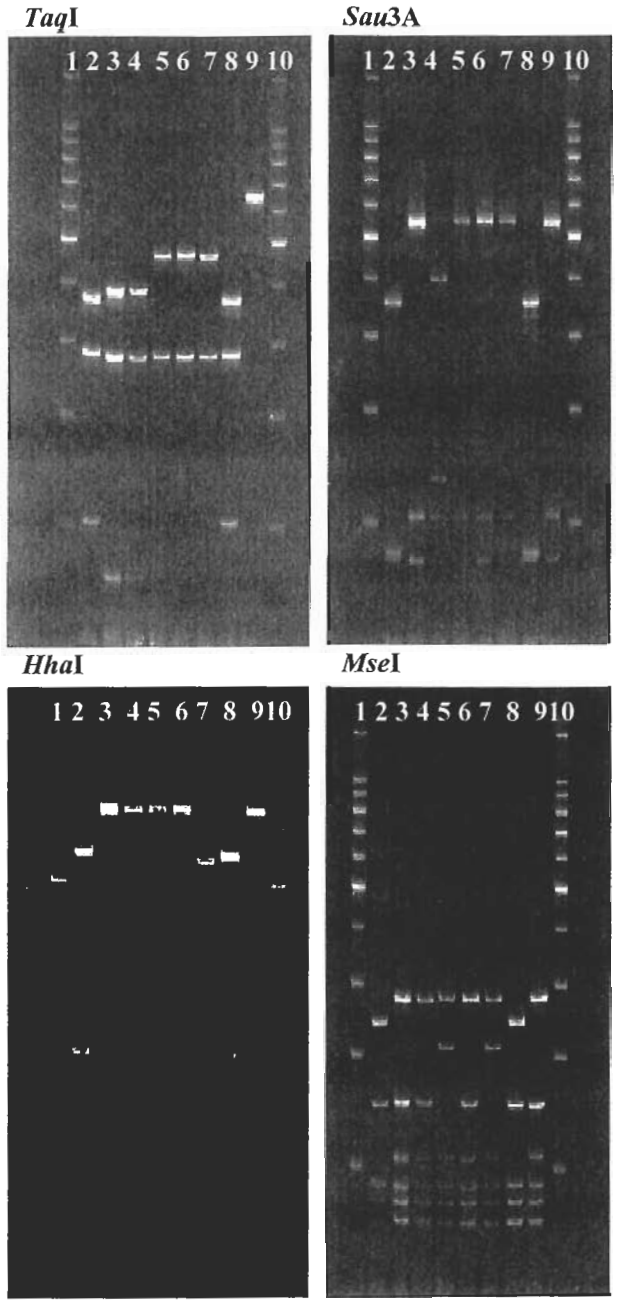

Plate 3. Electrophoretic separation on 8% acrylamide gel of amplified COI mitochondrial DNA gene fragments restriction products from the new species of cupped-oyster from Hong Kong, *C. gigas* and *C. angulata*. Lanes 1 and 12 are the molecular mass standard (100bp ladder, Promega). Lane 2, haplotype K (cultured new species of cupped-oyster from Hong Kong); lane 3, haplotype A; lane 4, haplotype B; lane 5, haplotype C; lane 6, haplotype D; lane 7, haplotype E; lane 8, haplotype K (wild new species of cupped-oyster from Hong Kong) and lane 9, haplotype J.

Table 3

Sample sizes (n) and haplotype frequencies for *Crassostrea gigas* and *C. angulata*. Data for Hong Kong samples were obtained from this study and others were re-organised from Boudry *et al.* (1998) and Huvet (2000). Haplotypes are denoted by capital latters, assigned to each combination of restriction profiles for the four restriction enzymes *Taq* I, *Sau* 3A, *Hha* I and *Mse* I.

					Haplotype									
					A	B	C	D	E	J	K	L	M	N
Country	Population	Abbreviation	Taxa	n	baaa	bbaa	caab	caaa	cabb	aaaa	dccc	eddd	eedd	efdd
Japan	Hiroshima	HIR	*C. gigas*	46			0.97		0.03					
South Korea	Chungmu	CHU	*C. gigas*	14			0.93	0.07						
Taiwan	Tungkang	TUN	*C. angulata*	40	0.9	0.07				0.03				
China	Hong Kong	HKC	New species of cupped-oyster (cultured)	50							1			
		HKW	New species of cupped-oyster (wild)	48								1		
		HKR	*C. rivularis*	35								0.94	0.3	0.3
Australia	Tasmania	TAS	*C. gigas*	11			1							
New Zealand		NEW	*C. gigas*	12			1							
United Kingdom	Guernsey	GUE	*C. gigas*	16										
	Bangor	BAN	*C. gigas*	10										
France	Seudre Estuary	SEU	*C. gigas*	20			0.95		0.05					
	Bonne Anse	BON	*C. gigas*	9			0.88			0.11				
	Charente	CHA	*C. gigas*	15	0.07		0.93							
	Arcachon	ARC	*C. gigas*	15			1							
	Rade de Brest	BRE	*C. gigas*	24			0.92		0.08					
	Gravelines	GRA	*C. gigas*	23			0.91	0.04	0.04					
Portugal	Ria Formosa	RFG	*C. gigas* (?)	11	0.82	0.09	0.09							
	Ria Formosa	RFA	*C. angulata*	11	0.82		0.18							
	Setutal	SEB	*C. angulata*	13	0.85	0.15								
Spain	Cadiz	CAD	*C. angulata*	26	0.92	0.08								

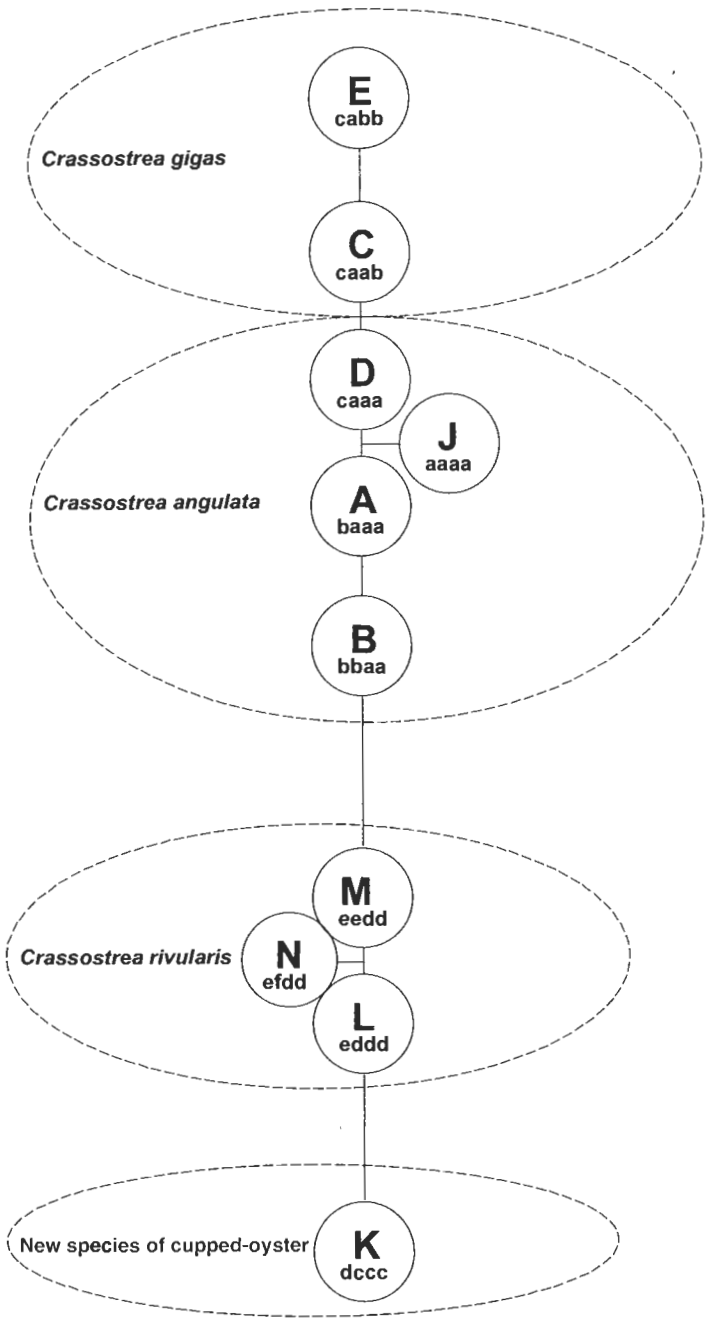

Fig. 3. Minimum spanning tree of RFLP haplotypes from populations of *Crasssotrea gigas, C. angulata, C. rivularis* (=*C. ariakensis*) and the new species of *Crassostrea* according to the results presented in Table 3. The sequence of the restriction enzyme is as follows: *Taq*I, *Sau*3A, *Hha*I and *Mse*I. Table 2 provides the restriction profiles for each enzyme. Connection length between each haplotype pairs are drawn to scale.

Table 4
Nucleotide substitution matrix for mtDNA RFLP analysis. Nei's raw number of nucleotide differences among *Crassostrea* populations was above diagonal. Significance values of pairwise test for genetic difference is below diagonal.

	HIR	CHU	TUN	HKC	HKW	HKR	TAS	NEW	GUE	BAN	SEU	BON	CHA	ARC	BRE	GRA	RFG	GFA	SEB	CAD
HIR	–	0.23	4.34	15	15	15	0.09	0.09	0.57	1.62	0.28	0.64	0.35	0.09	0.41	0.34	4	3.36	4.54	4.31
CHU	NS	–	4.12	14.86	14.86	15	0.14	0.14	0.64	1.74	0.34	0.67	0.39	0.14	0.48	0.39	3.79	3.18	4.31	4.08
TUN	**	**	–	13.05	13.05	14.9	4.25	4.25	4.75	5.85	4.45	4.13	3.99	4.25	4.58	4.34	0.89	1.02	0.69	0.5
HKC	**	**	**	–	0	10	15	15	15	15	15	14.67	14.87	15	15	14.91	13.27	13.36	13.15	13.08
HKW	**	**	**	NS	–	10	15	15	15	15	15	14.67	14.87	15	15	14.91	13.27	13.36	13.15	13.08
HKR	**	**	**	**	**	–	15	15	15	15	15	14.89	15	15	15	15	14.9	15	14.84	14.92
TAS	NS	NS	**	**	**	**	–	0	0.5	1.6	0.2	0.56	0.27	0	0.33	0.26	3.91	3.27	4.46	4.23
NEW	NS	NS	**	**	**	**	NS	–	0.5	1.6	0.2	0.56	0.27	0	0.33	0.26	3.91	3.27	4.46	4.23
GUE	**	NS	**	**	**	**	NS	NS	–	1.7	0.65	1.06	0.77	0.5	0.75	0.72	4.41	3.77	4.96	4.73
BAN	**	**	**	**	**	**	**	**	**	–	1.64	2.16	1.87	1.6	1.67	1.72	5.51	4.87	6.06	5.83
SEU	**	NS	**	**	**	**	NS	NS	**	**	–	0.76	0.47	0.2	0.5	0.44	4.11	3.47	4.66	4.43
BON	**	**	**	**	**	**	**	**	NS	**	**	–	0.78	0.56	0.89	0.8	3.86	3.28	4.35	4.12
CHA	**	NS	**	**	**	**	NS	NS	**	**	NS	*	–	0.27	0.6	0.52	3.69	3.1	4.19	3.96
ARC	NS	**	**	**	**	**	NS	NS	NS	**	NS	**	**	–	0.33	0.26	3.91	3.27	4.46	4.23
BRE	**	NS	**	**	**	**	NS	NS	*	**	NS	*	NS	NS	–	0.57	4.24	3.6	4.79	4.56
GRA	**	NS	**	**	**	**	NS	NS	**	**	NS	**	NS	NS	NS	–	4.01	3.39	4.55	4.31
RFG	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	–	1.23	1.01	0.83
GFA	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	–	1.19	0.96
SEB	**	**	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	–	0.62
CAD	**	**	NS	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	–

** = $p < 0.01$, * = $p < 0.05$, NS = not significant.

DISCUSSION

Using mtDNA RFLP analysis, Hong Kong putative *Crassostrea gigas* has been shown to be genetically different from *C. gigas, C. angulata* and *C. ariakensis*. As a consequence, we will call this the 'new cupped-oyster' herein and which is currently being described by Lam and Morton (2003). It is distinguishable, both morphologically and genetically from *C. ariakensis* obtained from the same area, i.e., Deep Bay, Hong Kong. Since shell characters of the two species are similar and differentiation by shell allometry requires a large sample size ($n > 30$ in this study), DNA markers are more reliable in identifying individuals. This study shows that COI RFLP analysis can distinguish between species of *Crassostrea* effectively and it should be extended to properly identify the other oyster species in the South China Sea. Sequencing of the COI gene will be the next step in a genetic analysis of these *Crassostrea* species.

Comparisons with European and other Indo-West Pacific *Crassostrea* conspecifics in terms of RFLP data suggest that the most commonly cultivated oyster in Hong Kong is a new species, admixed with a few individuals of *C. ariakensis*. Because of problematic identifications, the comparative biology of these two sympatric species is poorly known. This determining identification, however, opens up new perspectives for physiological studies on Hong Kong's cupped-oysters.

European *Crassostrea angulata* and *C. gigas*, however, have been studied extensively and can be differentiated both genetically and physiologically. Although these two species are morphologically very similar (Ranson 1967), they have been shown to be genetically different by molecular and karyological studies (Boudry *et al.* 1998; O'Foighil *et al.* 1998; Leitão *et al.* 1999). Studies have also shown that *C. angulata* and *C. gigas* are different in various physiological ways, including growth performance, food assimilation, filtration rate and activity at low temperatures (His 1972; Héral 1986).

The species of oyster cultured in Taiwan is *Crassostrea angulata* (Boudry *et al.* 1998). Despite the geographic proximity of the two localities, therefore, Hong Kong and Taiwan *Crassostrea* species are different. Since Hong Kong's new species of cupped-oyster shows a unique COI haplotype, the genetics of the species stock is thus (at present) unique to Deep Bay and the Pearl River Estuary. Any importation of seed to this area could change stock genetics.

In Asia, cultured species of *Crassostrea* show interesting latitudinal variations in biogeography, i.e., these change from pure *C. gigas* stocks in Japan, including Hokkaido, Hiroshima, Miyagi and Kyushu (O'Foighil *et al.* 1998) in the north, to *C. angulata* in Taiwan and the new species of cupped-oyster and *C. ariakensis* in Hong Kong, as we show in the present study. In a complementary study, Lam and Morton (2003) have examined the type material of all the Indo-West Pacific species of *Crassostrea* in order to provide the new Hong Kong one with a name. The structure of the minimum spanning tree showing genetic distances among the species of *Crassostrea* and the new cupped-oyster (Fig. 3) also coincides with their geographic distributions. That is, *C. gigas* is closer to *C. angulata* than to *C. ariakensis*, which is in turn more related to the co-occurring new *Crassostrea* species from Hong Kong. Cultured oysters in Vietnam, the Philippines and Thailand have been identified, based on shell characters, as *C. rivularis*, *C. iredalei* and *C. belcheri*, respectively (Talavera and Fautino 1933; Rosell 1991; Klinbunga *et al.* 2000; Tang 2001). Hong Kong's new species of cupped-oyster, however, is not synonymous with either *C. iredalei* or *C. belcheri* since it has a different

shape and the colour of the adductor muscle scar is different from that of the latter species (Talavera and Fautino 1933; Rosell 1991; personal observations). Preliminary results of both mtDNA partial 16S and COI sequence analyses show Hong Kong's new cupped-oyster is significantly genetically different from other Asian Pacific *Crassostrea* species such as C. *iredalei*, *C. belcheri, C. nippona, C. sikamea, C. angulata* and *C. gigas* (Lam and Morton 2003). It seems that these *Crassostrea* species are segregated along the Indo-West Pacific coastline with little geographic overlap. Cultured species of *Crassostrea,* such as *C. virginica*, are critically dependent upon estuarine conditions with high nutrient inputs and thus occur where salinities range optimally between 10 ‰ to 20 ‰ (Shumway 1996). *C. virginica* populations along the coastlines of the Eastern Atlantic and the Gulf of Mexico also comprise two distinct genetic assemblages, as revealed by mtDNA RFLP analysis (Reeb and Avise 1990). This genetic discontinuity was attributed to an influence on gene flow by historical vicarious events and contemporary hydrographies. This hypothesis can be extended to the Indo-West Pacific scenario, i.e., the speciation of *Crassostrea* here is most likely driven by geographical separation with restricted gene flow between individual estuaries.

A more complete picture of the inter-species and population genetics of Indo-West Pacific *Crassostrea* could be obtained if samples from other sites on the southern coast of China were to be studied. The islands of Okinawa seem to constitute a transition zone where *C. gigas* from Japan meets *C. angulata* from Taiwan. The population genetics of species of *Crassostrea* from these islands should, therefore, should show how *C. gigas* and *C. angulata* interact genetically. Nuclear markers of these populations should be studied to show whether hybridisation occurs among the two species. Despite a well-documented history of cultivation and described biology of cupped-oysters in Hong Kong (Morton 1975, 1977; Morton and Wong 1975; Morton and Morton 1983), questions concerning gene flow and hybridisation within and among Hong Kong and Asian populations will be resolved only by studying nuclear markers, such as ITS–1 (Hedgecock *et al.* 1999) and microsatellites (Huvet *et al.* 2000).

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