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Genetic and morphological relationships of mud crabs, genus *Scylla*, from throughout the Indo-Pacific

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Genetic and morphological relationships of mud crabs, genus Scylla, from throughout the Indo-Pacific.

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1 Concerning this publication

This publication is the report of an Australian Centre for International Agricultural Research (ACIAR) funded "Small Project". The authors have prepared this Report for distribution to the many people who generously supplied material for the study. Much of the information contained in this Report is being prepared for publication. Therefore, this Report should not be the citation for the material that is contained within it. Please refer to the authors for the correct citation. Care should be exercised in the use of the taxonomic names recommended in this Report, the names are pending the finalisation of our study of the type material.

I trust that you find the Report interesting. Any questions about the content of the Report should be addressed to:

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2 Abstract

Conflicting scientific views and a high probability that mud crabs from different areas are different species suggests that there is an urgent need to clarify the taxonomic status of these animals prior to aquaculture studies. Two genetic techniques, allozyme electrophoresis and mitochondrial DNA sequence analysis, provided a simple and direct method of determining the genetic relationships of species and the extent of differentiation. These genetic data were then used to delineate species and the results applied to morphological data to determine each species' morphological characteristics. Three distinct species were found within the genus, with all three data types producing complementary results. Sexual dimorphism within and between species probably accounts for much of the confusion in identification and nomenclature. A new nomenclature of the three species is suggested.

3 General introduction

An understanding of the taxonomic details of the genus Scylla de Haan, throughout the Indo-Pacific is central to the development of improved wild-stock management, as well as a more successful aquaculture industry based on the mud crab (Brown 1994). The uncertainty of genetic relationships is recognised as one of the primary constraints to the management of the wild fishery and development of aquaculture (BOBP 1992).

While it is now widely recognised that the mud crabs of the Indo-Pacific region belong to more than one morph of the genus Scylla (BOBP 1992) there is considerable confusion of the taxonomic nomenclature (Joel and Raj 1980). Forsskål (1755) first named the species Cancer serratus from material collected from the Red Sea, near Jedah. The whereabouts of Forsskål's material is not known and the type specimen is presumed to be lost. Since this time another seven species or varieties have been proposed. Estampador (1949a,b) performed an extensive study of mud crabs in Philippine waters and recognised three species: Scylla serrata (Forsskål 1775), S. oceanica (Dana 1852), S. tranquebarica (Fabricus 1798); and one variety S. serrata var. paramamosain Estampador 1949. These morphs were distinguishable by colour patterns, relative size, cheliped spination, chromosome 'form' and process of gamete development.

Differences in morphology have long been recognised for almost all regions where the mud crab is found. Estampador's (1949a,b) conclusions about the morphological features of different species have been widely accepted by some authors. Serene (1952) found four distinct morphs in waters of Vietnam and basically agreed with Estampador's taxonomy although he considered S. tranquebarica to be a variety of S. oceanica. Ong (1964) distinguished four morphs of Scylla in Malaysia. Chayarat and Kaewridh (1978) determined that three morphs occurred in waters of Chantaburi in Thailand. Kathirvel (1981) found two morphs in the Cochin area of India and Joel and Raj (1980) reported S. tranquebarica and S. serrata from Pulicat Lake, Tamil Nadu as well as several areas on the east coast of India. In Australia Taylor (1984) reported two different morphs in Western Australia. To date no studies to determine genetic relationships between these different forms have been completed.

Justification for the classification of mud crabs into different species and varieties is controversial and all morphs were placed in synonomy by Stephenson and Campbell (1960), a move supported by Ong (1964). However, from the great variation in colouration, maximum size and preferred habitat of the different morphs it is clear that more than one species of Scylla exists, often sympatric, throughout the crabs' distribution (BOBP 1992). What is interesting from an aquaculture viewpoint is that certain aspects of these species' biology appear to be different. Kathirvel (1981) reported a maximum carapace size of approximately 200 mm and size of first maturity for females of 120 mm for S. oceanica. Comparable sizes (maximum, first maturity) for the species named S. serrata (by some authors) were 120 mm and 85 mm, and for the common Australian mangrove crabs 240mm and 150mm respectively (Brown 1994). [Care needs to be exercised in the use of this and other information as the reported species' names differ from region to region.] Chen (1990) reported that of the three morphs recognised by crab farmers in Taiwan, the 'white' crab or S. oceanica is considered the most suitable for culture as it grows larger, is less aggressive and more tolerant of a wide range of salinities. While comparative growth rates of the different morphs have not been determined, it seems that significant benefits could be gained through the selection of faster growing, larger morphs for aquaculture.

A knowledge of the morphology and distribution of any genetically distinct species and their population structure are important prerequisites for the development of aquacultural practices and the successful implementation of fisheries management regulations. Electrophoretic techniques provide a simple and direct method of determining the genetic relationships of mud crabs and the extent of species and population differentiation. The advantage of electrophoretic genetic methods over traditional morphological taxonomy is that breeding relationships and the absence of gene flow can be quantified. Therefore conclusions as to breeding structure of each species and the ability of isolated populations to interbreed in nature are more specific than those based on morphology. In addition such conclusions can be used to provide morphological information, based on the known “biological” species, to identify clearly the different morphs.

4 Collection of samples

Specimens were obtained from locations throughout the Indo-Pacific; including Australia, the Philippines, Malaysia, Thailand, Vietnam, India, Pacific Island countries, west to the east African coast and north to Okinawa (Table 2.1 and Figure 2.1), with the assistance of many helpful people. At least six specimens were requested of each recognised morphological type from each locality because this is an ideal number to determine genetic relationships between closely related species (Keenan 1991). Although a single sample allows the determination of fixed genetic differences (Sarich 1977), a sample of between six to twelve animals allows the determination of moderate levels of polymorphism and indicates genetic loci where species share alleles. The sample of mud crabs from near Jedah in the Red Sea was obtained to provide a basis for taxonomic nomenclature, as the original type material collected by Forskal in 1755 came from this locality. In most countries, crabs were obtained from local fish markets and transported to Australia either alive or frozen. Care was taken to identify the source of the samples and wild caught crabs were requested, cultured crabs were avoided where possible. Some collections, particularly those from Malaysia and the Philippines were much more extensive and accurate site information was collected.

Upon arrival in the laboratory, the morphology of each crab was examined and it was classified as a "green" or "brown" morph using the diagnostic diagrams in Taylor (1984). Not all crabs could be classified by this method. The sex, maturation stage and carapace width of individual crabs were recorded and the carapace of the crab removed to provide access to the internal organs. Tissue samples routinely dissected from each crab were leg muscle tissue [two samples; one for allozyme electrophoresis, the second for mtDNA research] and hepatopancreas tissue. Other tissues that were tested included ovary, gill, heart and sub-cuticle. The tissues were placed into cold 1.5 ml microcentrifuge tubes with a small amount (3-5 drops) of invertebrate homogenising buffer (Siciliano and Shaw 1976). Tissues for enzyme analysis were not homogenised but were centrifuged in a microfuge for 15 minutes prior to electrophoresis and after thawing, to extract concentrated cytoplasm from the cells (Keenan 1994). Samples were stored in an ultrafreezer at -75°C until required. The dissected crabs were labelled and stored at -30°C prior to the collection of morphometric data.

Table 2.1 Collection summary of Scylla samples

Site	No.	Location	Collector(s)
1	3	Aust.-Gulf of Carpentaria	Mike Potter
2	25	Aust.-Moreton Bay	Luciano Serifini
3	23	Aust.-Northern Territory	Ian Knuckey
4	3	Aust.-Western Australia	Peter Davie
5	7	Fiji	Krishna Swamy
6	4	Pakistan-Karachi	Ted Burton
7	7	Japan-Okinawa	N. Shikatani
8	7	Malaysia-Bako	Josephine Pang
9	6	Malaysia-Belawai	Josephine Pang
10	8	Malaysia-Buntal	Josephine Pang
11	8	Malaysia-Sabah	Josephine Pang
12	9	Malaysia-Santubong	Josephine Pang
13	10	Malaysia-Semara	Josephine Pang
14	8	Malaysia-Sibu Laut	Josephine Pang
15	12	Malaysia-sample	Josephine Pang
16	5	Mauritius	M. Munbodh
17	6	New Caledonia	Tony Lewis
18	3	Philippines-Mindanao	Arnulfo Marasigan
19	4	Philippines-Negros	Arnulfo Marasigan
20	41	Philippines-Panay	Arnulfo Marasigan
21	8	Singapore	Peter Ng/Peter Davie/Ted Burton
22	9	Solomon Islands	Johann Bell
23	8	Taiwan	Tin-Yam Chan
24	4	Thailand-Bangkok	Ted Burton
25	6	Thailand-Phuket	Ted Burton
26	6	Vietnam	Le thanh Hung/Nguyen Tac An
27	7	Yemen-Red Sea	John Thoroughgood
28	6	Indonesia-Kupang	Chan Lee
29	12	South Africa	Sean Fennessy
Total	**265**		

Figure 2.1 map of collection area. Numbers refer to sites listed in Table 2.1

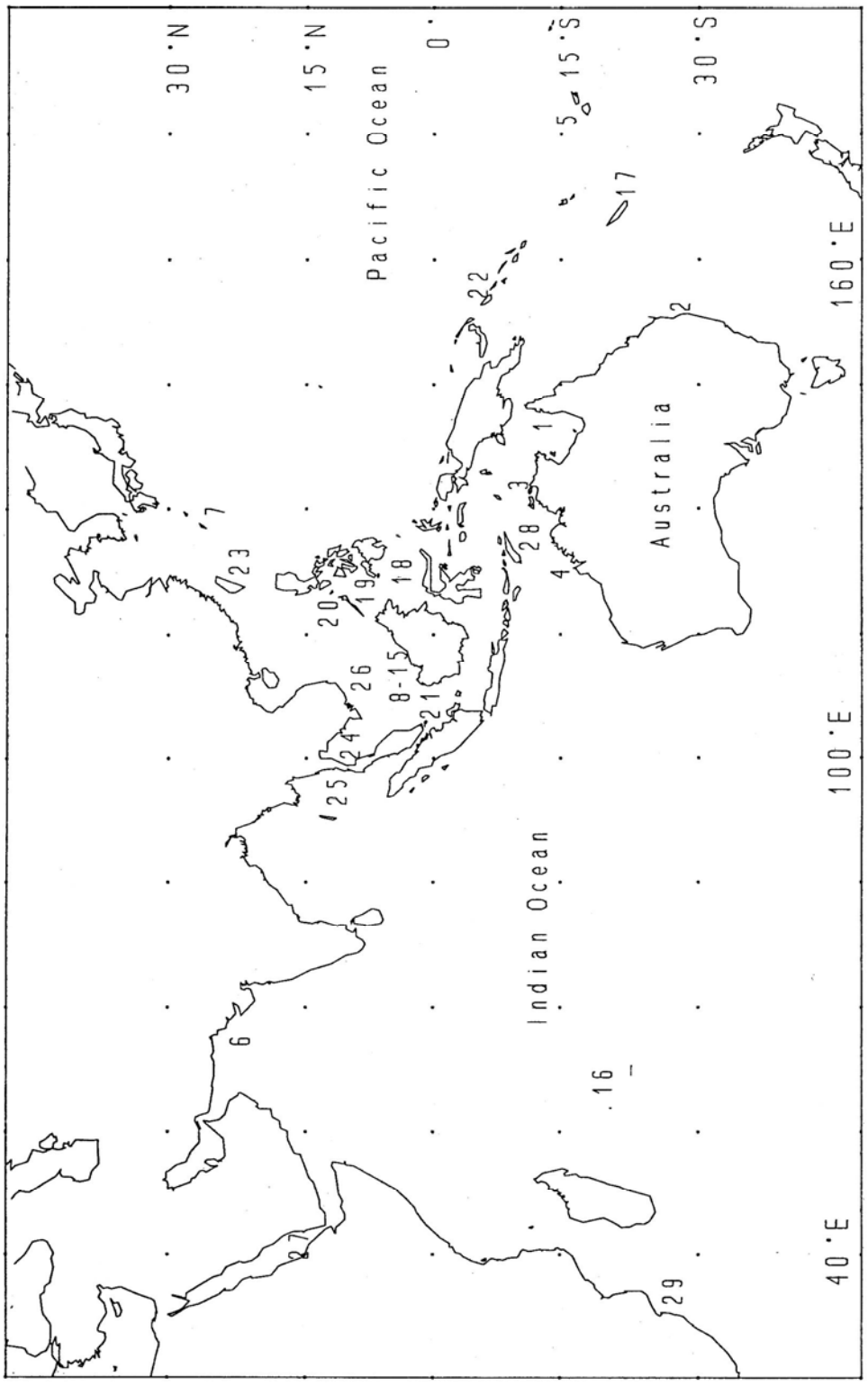


Figure 2.1 Map of collection area. Numbers refer to sites listed in Table 2.1

5 Allozyme electrophoresis

5.1 Introduction

Allozyme electrophoresis is a very powerful method for the determination of species differences (Keenan and Shaklee, 1985; Richardson et al, 1986). The common definition of a species as "a group of interbreeding individuals not interbreeding with another such group, being a taxonomic unit including geographical races and varieties and having 2 names in binomial nomenclature, the generic and specific epithet, similar and related species being grouped into a genus" taken from Holmes (1979), is based on the collection of breeding (genetic) information similar to that which can be obtained using allozyme electrophoresis. Using this definition, the identification of a species can be based upon the presence of fixed genetic differences between two different groups, which indicate that there is no genetic exchange. These characters can then be used as diagnostic characters (e.g. Musyl and Keenan, 1992) for the species.

Before proceeding further, some of the genetic terms that are going to be used will be defined.

Term	Definition
locus	(plural: loci) the basic unit of inheritance, equivalent to a gene, referring to a particular location on a chromosome. In this study, each locus codes for a a specific enzyme.
allele	a gene can occur in one or more forms, each alternative form is called an allele.
monomorphic	a locus (gene) with only one allele.
polymorphic	a locus (gene) with two or more (common, f ≥ 0.01) alleles.
homozygous	a locus with two copies of the same allele (for a diploid chromosome).
heterozygous	a locus with two different alleles (for a diploid chromos ome).
fixed difference	absence of heterozygotes at a polymorphic locus

The null hypothesis that we examined for mud crabs (genus Scylla) was; whether morphologically identifiable groups of crabs (morphs) possess no fixed genetic differences because there is interbreeding between the morphs and therefore they are all members of a single species. This hypothesis would be shown to be incorrect if congruent fixed genetic differences were observed at two or more loci (Richardson et al 1986), which would provide strong evidence that there is more than a single species within the genus. Furthermore, if sufficient animals were examined, the presence or absence of rare heterozygotes (i.e. hybrids) between the morphs could be determined for the loci where fixed differences were observed. If no heterozygotes were found for these loci in sympatric populations, then there is strong evidence that speciation has developed to a stage where hybridisation can no longer occur and that the morphs constitute "species" as defined above.

5.2 Materials and methods

Specimens were initially screened for general proteins using polyacrylamide gel electrophoresis (PAGE) to identify closely related specimens (Keenan and Shaklee 1985, Shaklee and Keenan 1986). Using this technique the crabs were divided into two classes based on the position of a single obvious protein band (see Figure 3.1). [However, later research suggested that this band represented the muscle protein, arginine kinase (ARGK) which was polymorphic at a low frequency in the "brown" crabs, and therefore would not always discriminate between the "green" and "brown" morphs. Furthermore, the "spined" morph could not be readily distinguished because it appeared to have the same general protein pattern as the common "brown" morph.]

Buffers and tissues suitable for starch gel electrophoretic screening were determined for 61 enzyme systems (Table 3.1) using a small number of samples from each of the two classes that could be readily recognised using the general protein method described above. Results of this initial screening are summarised in Tables 3.1, 3.2 and 3.3. The best loci-buffer combinations

(Table 3.2) were then used to gather genetic data for each locus from each sample. This allozyme genetic data was examined for the presence of fixed genetic differences to determine major taxonomic groupings.

Table 3.1 Details of enzyme systems examined

No.	Enzyme/Protein Name	E.C. Number	Abbrev.
1	aspartate aminotransferase	2.6.1.1	MT
2	acid phosphatase	3.1.3.2	ACP
3	4-methylumbelliferyl phophatase	3.1.1.55?	ACP-D
4	adenosine deaminase	3.5.4.4	ADA
5	alcohol dehydrogenase (DH)	1.1.1.1	ADH
6	aconitate hydratase	4.2.1.3	AH
7	adenylate kinase	2.7.4.3	AK
8	alkaline phosphatase	3.1.3.1	AKP
9	alanine aminotransferase	2.6.1.2	ALAT
10	a-amylase	3.2.1.1	AMY
11	arginine kinase	2.7.3.3	ARGK
12	cytidine deaminase	3.5.4.5	CDA
13	creatine kinase	2.7.3.2	CK
14	dihydrolipoamide dehydrogenase	1.8.1.4	DDH
15	enolase	4.2.1.11	ENOL
16	esterase	3.1.-.-	EST
17	esterase-D (umbelliferase)	3.1.-.-	ESTD
18	fructose-bisphosphate aldolase	4.1.2.13	FBALD
19	formaldehyde dehydrogenase	1.2.1.1	FDH
20	fumarate hydratase	4.2.1.2	FH
21	β-galactosidase	3.2.1.23	bGAL
22	glyceraldehyde-3-phosphate DH	1.2.1.12	GAPDH
23	guanine deaminase	3.5.4.3	GDA
24	glutamate dehydrogenase	1.4.1.2	GDH
25	a-glucoside	3.2.1.20	aGLU
26	glycerol-3-phosphate DH	1.1.1.8	G3PDH
27	glucose-6-phosphate DH	1.1.1.49	G6PDH
28	general proteins	-.-.-.-	GenProt
29	glucose-6-phosphate isomerase	5.3.1.9	GPI
30	glutathione reductase	1.6.4.2	GR
31	guanylate kinase	2.7.4.8	GUK
32	hexose di-phosphate	3.1.3.11	HDP
33	hexokinase	2.7.1.1	HK
34	L-iditol dehydrogenase	1.1.1.14	IDDH
35	isocitrate dehydrogenase (NADP+)	1.1.1.42	IDHP
36	lipoamide dehydrogenase	1.6.4.3?	LADH
37	leucine aminopeptidase	3.4.11.-	LAP
38	L-lactate dehydrogenase	1.1.1.27	LDH
39	a-mannosidase	3.2.1.24	aMAN
40	malate dehydrogenase	1.1.1.37	MDH
41	malic enzyme (NADP+)	1.1.1.40	MDHp
42	mannose-6-phosphate isomerase	5.3.1.8	MPI
43	β-napthyl amidase	3.4.11.1	NA
44	octanol dehydrogenase	1.1.1.73	ODH

45	octopine dehydrogenase	1.5.1.11	OPDH
46	peptidase (gly-leu substrate)	3.4.-.-	PEP-GL
47	peptidase (leu-gly substrate)	3.4.-.-	PEP-LG
48	peptidase (leu-gly-gly substrate)	3.4.-.-	PEP-LGG
49	peroxidase	1.11.1.7	PER
50	phosphogluconate dehydrogenase	1.1.1.44	PGDH
51	phosphoglycerate kinase	2.7.2.3	PGK
52	phosphoglucomutase	5.4.2.2	PGM
53	pyruvate kinase	2.7.1.40	PK
54	purine nucleoside phophorylase	2.4.2.1	PNP
55	strombine dehydrogenase	1.5.1.22	SDH
56	superoxide dismutase	1.15.1.1	SOD
57	succinate dehydrogenase	1.3.99.1	SUDH
58	tyrosine aminotransferase	2.6.1.5	TAT
59	uridine kinase	2.7.4.-	UMPK
60	xanthine dehydrogenase	1.1.1.204	XDH
61	xanthine oxidase	1.1.3.22	X0

Table 3.2 Best buffer and enzyme combinations found for the examination of genetic variation in mud crabs using starch gel electrophoresis. Polyacrylamide electrophoresis (PAGE) at different concentrations was used where indicated. H = hepatopancreas, M = muscle, 0 =ovary.

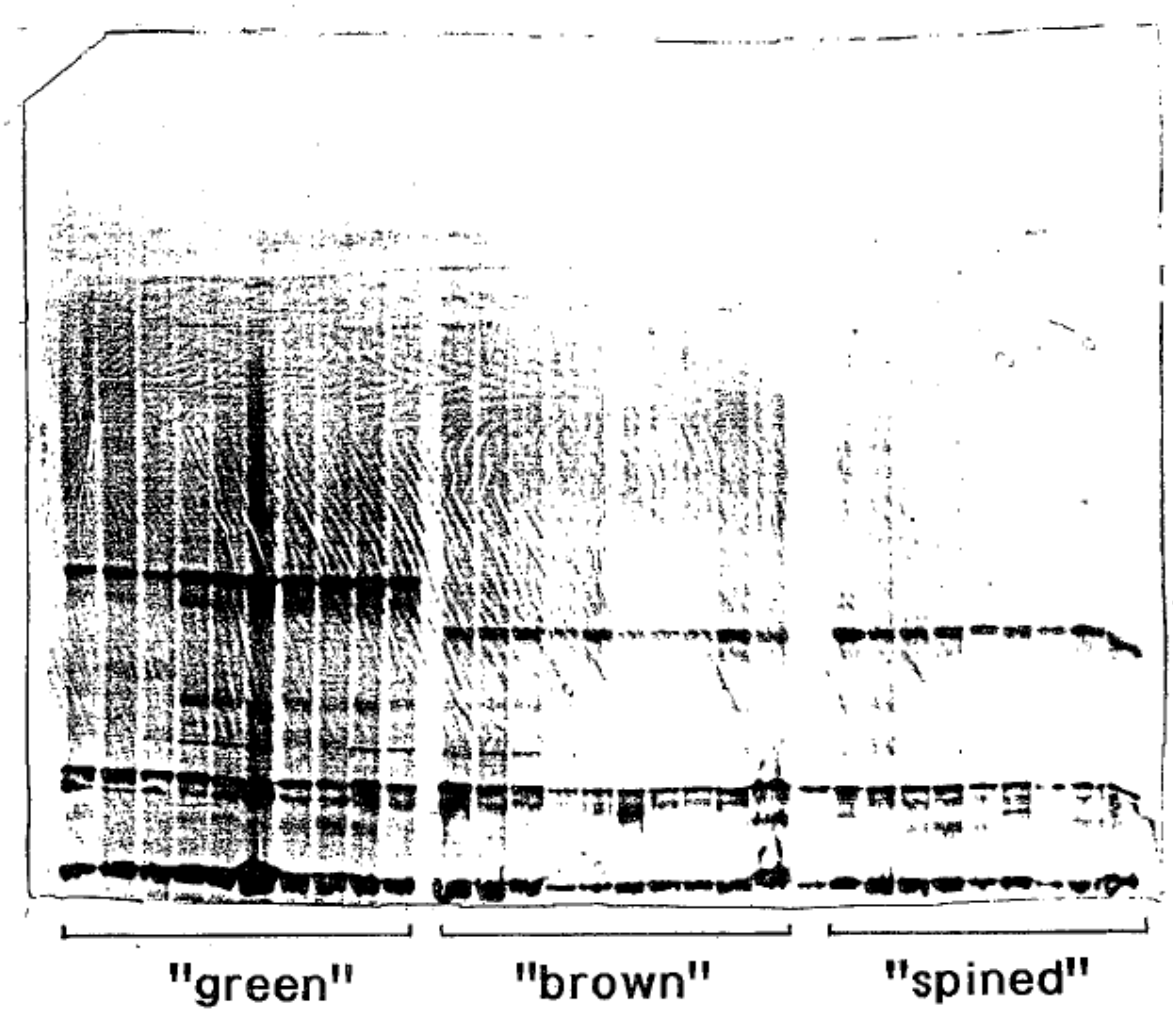
No.	Locus	Buffer1	Buffer2	*Tissue*	Resolution
1	AAT-H	Poulik	TM	*H*	
2	AAT-M	LiOH	Poulik or TM	*M*	good, use bottom locus
3	ADA-H	LiOH	EBT	*H*	good, variable
4	ADAM	LiOH	TM	*M*	good, variable
5	ADH	EBT	TM	*M*	stains up weakly on LDH
6	AK	TM		*M*	moderate
7	ALAT	TM		*M,O*	good, deteriorates in older samples
8	AMY	LiOH(PAGE 8%)		*H*	difficult to interpret, many loci
9	ARGK	TM		*M*	excellent
10	ENOL	TRIC		*M*	good
11	FBALD	TM		*M*	good
12	bGAL	TM		*H*	difficult to repeat separation
13	GAPDH	TRIC		*M*	good
14	GDH	Poulik		*H*	polymorphic, weak staining
15	G3PDH	CAME	TRIC	*M*	subbands, not clear
16	G6PDH	TRIC	TM	*M*	streaky, add NADP to gel
17	GenProt	LiOH(PAGE 10%)		*M*	same as ARGK
18	GPI	TRIC		*M*	excellent
19	HK	TRIC	TM	*H*	poor, variable staining, use high concentration of substrate for this locus
20	IDH	TM		*M*	2 invariant loci
21	LDH	EBT		*M*	also stains up ADH
22	MDH-1	TRIC		*M*	good
23	MDH-2	TRIC		*M*	good
24	MDHp	TRIC		*M*	moderate
25	MPI	EBT	TM LiOH(PAGE 8%)	*M,H*	excellent
26	PEP-GL	EBT		M	*clear*
27	PEP-LG1	LiOH		*M*	good, some heterozygotes odd
28	PEP-LG2	LiOH		*M*	good
29	PEP-LGG	TRIC		*H*	may be slight variation, not crisp
30	PGDH	CAME		*M*	excellent
31	PGK	LiOH		*M*	poor
32a	PGM	TRIC		*M*	subbands, medium
32b	PGM	CAME		*M*	good, strong
33	PK	CAME		*M*	no variation, good, AK stains
34	PNP	TRIC		*H*	poor, variable staining
35	SOD-1	LiOH(PAGE 10%)		*HAT*	clear (also GDH Poulik)
36	SOD-2	LiOH(PAGE 10%)		*H*	also on ADH gel

Source of electrophoresis buffer recipes: CAME (Clayton and Tretiak,1972); EBT, LiOH, LiOH(PAGE), Poulik, TRIC (Shaklee and Keenan 1986); TM, (Shaw and Prasad 1970).

Table 3.3 Other buffer and enzyme combinations that were examined for genetic variation in mud crabs using starch and polyacrylamide electrophoresis.

Locus	Buffer1	Buffer2	Tissue	Resolution
ACP	LiOH	EBT	H	smeary
ACP-D	TM	TC-1	H	try it again
AH				didn't work
AKP	EBT		H	variable staining - try it again
AMY	PAGE 8%		M	difficult to interpret
CDA				didn't work
CK				no locus in crustaceans
DDH	LiOH	EBT	H	try it again
EST	PAGE		H	smeary
EST-D	LiOH	TRIC	M	smeary
FDH	TC-1	LiOH	H,M	try it again
FH	TM	EBT		try it again
GDA	TRIC			poor
aGLU	LiOH	TC-1	H	good, 2 loci - try it again
GR	Poulik	LiOH	O	OK try again
GUK	EBT	LiOH	H	same as UMPK?
HDP	TRIC	TM	M	try it again
IDDH			M,O	nothing
LADH	Poulik		H,O	crisp, 2 variable loci - try it again
LAP				didn't work
aMAN	LiOH	TC-1	H	weak staining
NA	LiOH	TRIC	H	OK try again
ODH	EBT		M	weak
OPDH	TM	EBT	M	not variable
PCDH				nothing
PDH				nothing
PER	TC-1 TRIC	LiOH	H	cathodal, smeary TRIC
PER	TM	Poulik	H	sharp, weak - try it again
SDH	Poulik		M	clear - do it again
SUDH				nothing
TAT				nothing
UMPK	EBT	LiOH	H	variable, clear - try it again
XD H				didn't work
XO	EBT		H	try it again

Figure 3A General protein staining of a polyacrylamide gel of various crabs specimens showing the differences in banding patterns between "green", "brown" and "spined" morphs.



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5.3 Results

Variation was observed in the mobility of alleles at 22 of the 36 enzymatic loci screened. Table 3.4 summarises the enzymatic mobility of the allelic variation observed in samples from each species. Of the 36 loci examined, only 14 loci showed no apparent genetic variation in the amino acid structure of their enzymes (proteins). These loci were ENOL,FBALD, GAPDH, GDH, G3PDH, IDH, LDH, MDHp, GPK, PNP, SOD-1, and SOD-2. At 22 of the loci, variation either within or between the species was observed. At 16 loci, polymorphism was observed within one or more species (AAT-H, AAT-M, ADAH, ADA-M, AK, AMY, ARGK, bGAL, GenProt, GPI, MPI, PEP-GL, PEPL-LG1, PEP-LG2, PGDH and PK) and at 11 loci, fixed genetic differences between species were observed. The loci showing fixed genetic differences between pairs of species are summarised in Table 3.5. Note that some of these loci are also polymorphic within a species. For simplicity and consistency, the three identified species are referred to as "green", "brown" and "spined" throughout this report.

For the AMY (amylase) enyzme stain, the pattern of allelic variation observed was difficult to interpret but the general pattern was different for all species, and this has been scored as a single fixed difference in Table 3.4. However, these results for the amylase loci would underestimate the variation present. At least three amylase loci were observed by this study, two in liver and one in muscle tissue and all were variable. Six amylase loci have been reported in the literature for some crustacean species (Van Wormhoudt et al, 1995). The high concentration of the enzymes at this locus was interesting. The amylase from hepatopancreas was observed to digest the starch gels for some buffer systems. When applying the samples to polyacrylamide gels, the sample wicks were "run in" for only 3 minutes to decrease overstaining of the gel.

To summarise these results, fixed differences between species were observed at the loci tabulated below. Amylase is not included in these results because of the difficulty in interpretation.

green vs brown	*ADH, ALAT, G6PDH, HK, MPI, PEP-LG1, PEP-LGG, PGM*
brown vs spined	*MPI, PEP-LG1, PGM*
green vs spined	*ADH, ALAT, ARGK GenProt, (PGM,)*

At the PGM locus, when using the TRIC buffer system, it was sometimes possible to separate the "green" and "spined" alleles. However, this was not always repeatable because the enzyme mobility was not great and longer separation times than those used were required.

Table 3.4 Summary of observed alleles for each crab type, expressed as relative mobilities using the "green" crab as the reference point. Polymorphic loci are identified by the presence of more than one allele which are listed in the "additional" alleles column. ? = data missing, usually a result poor staining intensity.

No.	*Locus*	"green" morph alleles		"brown" morph alleles		"spinal" morph alleles	
		common	additional	common	additional	common	additional
1	*AAT-H*	100	77	100		?	
2	*AAT-M*	100		100	130, 60	100	
3	*ADA-H*	100	106	100		100	
4	*ADA-M*	100		100	125	100	
5	*ADH*	100		75		75	
6	*AK*	100		100	140	100	
7	*ALAT*	100		95		95	
8	*AMY*	100		95		90	
9	*ARGK*	100		75	100	75	
10	*ENOL*	100		100		100	
11	*FBALD*	100		100		100	
12	*bGAL*	100	120	120	115	120	
13	*GAPDH*	100		100		100	
14	*GDH*	100		100		?	
15	*G3PDH*	100		100		100	
16	*G6PDH*	100		74		?	
17	*GenProt*	100		85	100, 92	85	
18	*GPI*	100	158, 66	100	133, 58	42	100
19	*HK*	100		95		?	
20	*IDH*	100		100		100	
21	*LDH*	100		100		100	
22	*MDH-1*	100		100		100	
23	*MDI-1-2*	100		100		100	
24	*MDHp*	100		100		100	
25	*MP1*	100	103	95	90	100	
26	*PEP-GL*	100		100	78	100	
27	*PEP-LG1*	100		150	200	100	
28	*PEP-LG2*	100		100	120, 75	100	
29	*PEP-LGG*	100		98		?	
30	*PGDH*	100		100	105, 95	100	
31	*PGK*	100		100	?		
32a	*PGM (TRIC)*	100		85		107	
32b	*PGM(CAME)*	100		85		100	
33	*PK*	100		100	118	100	
34	*PNP*	100		100		100	
35	*SOD-1*	100		100		100	
36	*SOD-2*	100		100		100	

Table 3.5 Summary of the mobilities of alleles at loci showing fixed genetic differences between the species

No.	Locus	Relative Mobilites			
		green	brown		spined
5	*ADH*	100	75		75
7	*ALAT*	100	95		95
8	*AMY*	100	95		90
9	*ARGK*	100	75	100	75
16	*G6PDH*	100	74		?
17	*GenProt*	100	85	100, 92	85
19	*HK*	100	95		?
24	*MPI*	100 103	95	90	100
26	*PEP-LG1*	100	150 200		100
27	*PEP-LGG*	100	98		?
	| 31 *PGM* (TRIC)	100	85		107
	| 31a PGM(CAME)	100	85		100

Another two loci, *bGAL* and *GPI*, while showing significant differences in allele frequency between species, did not demonstrate fixed differences. For *bGAL* the 120 allele was shared by all species, and for GPI the 100 allele was observed in all species as shown below.

No.	Locus	Relative Mobilites		
		green	brown	spined
12	*bGAL*	100 120	120 115	120
18	*GPI*	100 158, 66	100 133, 58	42100

Polymorphism within species was detected for the "brown" species at 13 loci, for the "green" species at 5 loci and for the "spined" species at a single locus. These polymorphic loci could be used to conduct a population level analysis, which has not been attempted as part of this study. Such a study would be possible if sample sizes were to be increased to at least 50 crabs from each major location per species.

5.4 Discussion

The absence of heterozygotes (i.e. hybrids) between the different species "morphs" (Table 3.4, 3.5), for the loci where fixed differences were observed, provides evidence that there is no genetic exchange between them. As no heterozygotes were found between these "morphs" in sympatric samples, as defined on the basis of fixed genetic differences, then there is strong evidence that speciation has developed to a stage where hybridisation can no longer occur and that the "morphs" constitute "species" as defined above.

However, the pattern of fixed differences is very unusual, in that there are no obvious unique enzymes that indicate a "species". Rather each species has a unique combination of alleles that are also shared with other species. This unusual distribution of alleles in each species suggests that the ancestral species must have been, prior to the speciation events, polymorphic for the loci where the alleles are now distributed between the species. Two loci, bGAL and GPI, still do not demonstrate fixed differences between species. These shared polymorphic alleles have been shown to be important in understanding the speciation process (Keenan 1991). From these results it is reasonable to conclude that speciation in Scylla has been a relatively recent event and genetic divergence, both in terms of the fixation of alternate alleles at polymorphic loci and the evolution of new alleles, has not had sufficient time to cause fixation at all loci.

From the loci examined there is one locus, PGM, and an one enzyme system, AMY, where there are indications of unique alleles for each species. These loci require further refinement in electrophoretic techniques. When the enzyme PGM was separated on the TRIC buffer system, all three species could be distinguished in some experiments. Under the TRIC electrophoretic

conditions, the enzyme migrated approximately 10 mm from the origin or sample insertion point. If electrophoresis was allowed to run for a longer period the separation between the "spined" 107 allele and the "green" 100 allele became larger and was easier to observe. It is possible that a modification of this buffer system may allow the separation of all three species which would then prove diagnostic for the species. Such changes could include adjusting the buffer chemicals to increase the pH and/or increase or decrease the ionic concentration. The development of such a diagnostic locus would prove useful in the field for species identification purposes. The situation with the amylase enzyme system is much more complex. There could be as many as six loci, some of which show genetic variation, that need to be characterised and quantified. Breeding experiments may be necessary to separate out the many different genotypes.

Using the techniques applied by this study, species discrimination can be accomplished by the electrophoresis of muscle samples on an EBT or TM (tris maleate) gel. If the gel was sliced in half and stained for the enzymes MPI (which distinguishes "browns" from the other two species), and ADH (which distinguishes "greens" from the other two species), all three species can be separated. The "spined" species has a different pattern of enzymes for these loci; with one "green" allele and one "brown" allele, as tabulated below (Table 3.6). ALAT and ARGK could also be used on the TM buffer to distinguish "greens" from the other two species.

Table 3.6 Species-discriminating loci for the TM or EBT gel buffer systems

species	allele mobility at each locus			
	ADH	*MPI*	*ALAT*	*ARGK*
green	100	100, 103	100	100
brown	75	95, 90	95	75, 100
spined	75	100	95	75

Table 3.7 summarises the species identity of each crab in each of the individual collections made for this study. This sumary provides a quick concept of the distribution of each species defined by this study. The distribution of mud crab species, using the nomenclature found in the scientific literature, is not reliable because of the confusion of names in the literature. Further work is required to carefully determine each species' distribution. However, from the data available in Table 3.7 some broad conclusions can be drawn. The "green" species is the most widely distributed species, ranging from the east African coast (South Africa, Mauritius and Yemen), through Australia (Northern Territory and Moreton Bay) and north Asia (Japan, Philippines and Taiwan) to the eastern Pacific Ocean (Fiji, Solomon Islands and New Caledonia). The "green" and "brown" species are sympatric from five areas; Gulf of Carpentaria, Western Australia (Taylor 1984), Panay, Taiwan and Kupang. All three species are only seen in one collection, from Panay Island, Philippines.

The "brown" species is the most numerous in the collection, with large numbers from the Philippines and Malaysia. It is sympatric with the "spined" species in five locations; Karachi, Bako, Semara and Panay, as well as Singapore (personal observation). Both the "brown" and "spined" species would appear to have a distribution that is centralised in the South China Sea, where the "green" species is almost completely absent. However, as both the "brown" and "spined" are observed in the Karachi collection, all three species may be found around the Indian subcontinent and all three species are also reported from Japan (Fuseya and Watanabe 1995). The "spined" species has not been reported from Australia, but because of its similar morphology to the "green" species it may just be unrecognised.

Table 3.7 Summary of the number of Scylla per species by location, based on allozyme patterns

Collection Summary					
Site #	**# crabs**	**green**	**brown**	**spined**	**Location**
1	3	2	1		Aust.-Gulf of Carpentaria
2	25	25			Aust.-Moreton Bay
3	23	23			Aust.-Northern Territory
4	3		3		Aust.-Western Australia
5	7	7			Fiji
6	4		1	3	Pakistan-Karachi
7	7	7			Japan-Okinawa
8	7		5	2	Malaysia-Bako
9	6		6		Malaysia-Belawai
10	8		8		Malaysia-Buntal
11	8			8	Malaysia-Sabah
12	9		9		Malaysia-Santubong
13	10		9	1	Malaysia-Semara
14	8		8		Malaysia-Sibu Laut
15	12		11	1	Malaysia-sample
16	5	5			Mauritius
17	6	6			New Caledonia
18	3		3		Philippines-Mindanao
19	4		4		Philippines-Negros
20	41	2	27	12	Philippines-Panay
21	8		8		Singapore
22	9	9			Solomon Islands
23	8	1	7		Taiwan
24	4		4		Thailand-Bangkok
25	6		6		Thailand-Phuket
26	6		6		Vietnam
27	7	7			Yemen-Red Sea
28	6	1	5		Indonesia-Kupang
29	12	12			South Africa
Totals	**265**	**107**	**131**	**27**	

6 Mitochondrial DNA variation

6.1 Introduction

The above results from the allozyme electrophoresis, while clearly showing that there were three distinct groups of mud crabs with fixed differences between them, were unusual. From experience (Keenan and Shaklee, 1985; Keenan 1991), it is usual for different species to exhibit several unique alleles that can be used to distinguish a particular species from other closely related species. For the mud crabs, there was no problem distinguishing the "green" type from the "brown" type, but the third "spined" species appeared to have a unique genetic makeup which was an original combination of alleles present in either of these other two species. Examination of the mitochondrial DNA, in comparison to the allozyme results based on genomic DNA, would confirm or refute this interpretation.

6.2 Materials and methods

Sub-samples of crabs used for mitochondrial DNA (mtDNA) analysis were selected by using the allozyme database, based on the species group to which they belonged. Approximately 10 samples per species group were selected so that both within and between species levels of genetic variation could be determined. The samples which were successfully used for mtDNA sequence analysis are detailed in Table 4.1.

6.2.1 DNA extraction

DNA was extracted from frozen leg, muscle which was originally removed from the crab while still frozen, placed into the microcentrifuge tube without thawing and subsequently kept frozen (-75°C) until DNA extraction. This procedure was used to maximise the yield of high molecular weight DNA through minimising the fracture of DNA by repeated freezing and thawing.

Ten or 16 samples of crabs were extracted at a time. About 200 mg of leg muscle tissue from each crab was placed in a microcentrifuge tube. 500□l lysis buffer (100 mM EDTA, 10 mM Tris, 1% SDS, pH7) and 10□l Proteinase K (10 mg/ml) were added and the tissue was homogenised immediately with a small pestle. The samples were digested in a water bath (60°C) for 2 hours, with regular inversion for mixing. To remove RNA, 5□l of DNA free RNASE was mixed into the solution and the tubes were placed in a 37°C water bath for 1 hour.

Table 4.1 Samples examined for mtDNA genetic variation. Two mtDNA gene fragments were amplified by PCR and each sequenced in both forward and reverse directions. Sequences were combined to create a complete sequence (both) where possible. Successful derivation of a sequence is recorded in the table with a √

# No.	Locality	COIa	COIf	COI(both)	16sAR	16sBR	16s(both)
green							
Ss 5	Aust.-Gulf of Carpentaria	√	√	√			
Ss 12	Aust.-Moreton Bay	√	√	√	√		
Ss 34	Aust.-Northern Territory	√	√	√			
Ss 35	Aust.-Northern Territory	√	√	√	√	√	√
Ss 36	Aust.-Northern Territory	√	√	√	√	√	√
Ss 61	Yemen-Red Sea	√			√	√	√
Ss 62	Yemen-Red Sea		√				
brown							
Ss 4	Thailand-Bangkok	√	√	√			
Ss 6	Aust.-Gulf of Carpentaria	√	√	√	√	√	√
Ss 44	Thailand-Phuket	√	√	√			
Ss 114	Taiwan	√	√	√	√	√	√
Ss 115	Taiwan	√	√	√			
spined							
Ss 50	Pakistan-Karachi				√	√	√
Ss 163	Malaysia-Sabah	√	√	√	√	√	√
Ss 164	Malaysia-Sabah				√	√	√
Ss 206	Philippines-Panay	√	√	√	√	√	√
Ss 207	Philippines-Panay	√	√	√	√	√	√
Ss 211	Philippines-Panay	√	√	√	√	√	√

The following steps were carried out at room temperature. The tubes were spun for 10 minutes at 13,000 rpm to pellet cell debris. The supernatant was transferred to clean tubes and extracted three times, carefully pipetting the upper fraction each time into clean tubes, being careful not to take any of the interface. The first extraction was with an equal volume of buffered phenol (500□l) which was mixed gently for one minute and centrifuged at 13,000 rpm for 10 mins. The second extraction was done with phenol/chloroform/isoamyl alcohol (49:49:2 - 500□l), which was mixed gently for one minute and centrifuged at 13,000 rpm for 5 mins. The final extraction used chloroform/isoamyl alcohol (24:1 - 500□l) which was mixed gently for one minute and centrifuged at 13,000 rpm for 3 mins. This final supernatant (approximately 250□l) was transferred to clean tubes and precipitated with 1/2 volume (125□l) of ammonium acetate and 3 volumes (750□l) of ethanol. This mixture was inverted slowly to precipitate the DNA (which could often be seen) and allowed to sit for at least 10 mins before centrifuging at 13,000 rpm for 10 mins. The supernatant was carefully discarded and the pellet (loosened by flicking) was washed with 1 ml of 70% ethanol, with gentle inversion. The tube was again centrifuged at 13,000 rpm for 10 mins, the supernatant very carefully discarded and the pellet dried under vacuum in a SpeedVac for 5 mins or air dried for 1 hr.

Finally, the pellet of DNA was resuspended in 50□l of TE in a water bath at 37°C overnight. The presence of RNA and the approximate concentration of large fragment DNA was checked the next day by electrophoresis of 3□l of sample on a 0.6% TBE agarose gel run at 80 volts (~ 50 mA) for 1-2 hours with a λ sample control. Also, a 1:50 dilution was checked on a spectrophotometer to ascertain the concentration of nucleic acid and protein.

6.2.2 PCR amplification

The PCR amplification used 1□l of 1/10 dilution of template in a 50□l reaction. This was added to 49□l of reaction mixture which differed for each set of primers, as set out in Table 4.2, below. The primers used for both cytochrome oxidase I (COI) and 16s RNA (16s) genes were from Simon et al. (1991). They were:

COIa (21mer)	5 '	-	AGTATAAGCGTCTGGGTAGTC	- 3 '
COIf (20mer)	5 '	-	CCTGCAGGAGGAGGAGAYCC	- 3 ' (Y - C or T)
16sar (20mer)	5 '	-	CGCCTGTTTAACAAAAACAT	- 3 '
16sbr (22mer)	5'	-	CCGGTCTGAACTCAGATCACGT	- 3 '

Table 4.2 Concentration and volumes of solutions in reaction mixtures for PCR amplification or COI and 16s mtDNA.

Solution	initial concentration	COI	16s
		volume(□l)	volume(□l)
dd H2O		31.3	40.8
10x PCR buffer	*see below	5	5
dNTP	5 mM	2.5	1
Primer 1	10□M	2.5	1
Primer 2	10□M	2.5	1
MgCl	25 mM	5	-
Taq	5 u/□l	0.2	0.2
Total volume		**49.00**	**49.00**

**PCR buffer: 100 mM Tris-HCl (pH8.3), 15 mM MgCl2, 500 mM KCl.*

The reaction mixtures and template were added to Perkin-Elmer thin walled 200□l microtubes and placed into a Perkin-Elmer 9600 Thermocycler. Initial denaturation was at 94°C for 90 s. A reaction cycle (94°C for 5 s, 45°C for 20 s, 72°C for 20 s) was then repeated 35 times with a final extension step of 72°C for 5 minutes. To examine the PCR products, 5□l of each sample was run on a 1.4% TBE agarose gel for 1 hr at 80 volts, stained with 0.5□g ml-1 of ethidium bromide.

6.2.3 Sequencing protocol

On examination of the above PCR reaction, if there were other background amplified fragments in addition to the expected one, the extraneous products were removed by gel separation. To accomplish this, firstly the DNA was precipitated with propanol and concentrated to 10 □l in TE. The entire sample was then run on a 3 mm thick 1% TAE gel at 100 V. After ethidium bromide staining and photography each target band was excised with a clean scalpel blade and placed into a microcentrifuge tube with clean forceps. This was spun down to estimate the volume (~ 150 □l of gel in each tube).

Both the PCR product (if a single band) or the gel-excised band were purified from primers,dNTP's and buffer (and agarose) using either ©Gene-Clean (BIO-101) or QIAGEN quick-spin PCR purification columns, using their elution protocols. The purified DNA was resuspended in 20 □l of 0.1 x TE. 2 □l of sample was run on a 1.4% TBE agarose gel and the concentration of double-stranded PCR product was quantified by eye with reference to a known concentration sample.

Approximately 200 ng of PCR product was used as the template in a cycle-sequencing reaction with fluorescently labelled di-deoxy nucleotides (using the ABI PRISM kit and protocols). Each cycle-sequencing reaction used one of the same primers as those in the initial amplification. After phenol/chloroform extraction to remove excess fluorescent nucleotides and ethanol precipitation, the single-stranded extension products were electrophoresed and analysed on an ABI 373A automated sequencer. Approximately 400-500 bases were routinely sequenced in each direction for both mtDNA gene fragments in each individual (see Table 4.1).

6.2.4 Analysis

The sequences could be easily aligned manually using the ABI sequence alignment editor SeqEd. The sequences were manipulated and analysed using MEGA (Kumar et al, 1993) to provide sequence divergences and diversities, the resulting amino acid sequences for COI, and neighbour-joining and maximum parsimony phylogenetic trees. Sequences were exported from MEGA in NEXUS format for further maximum parsimony analysis performed by PAUP (Swofford 1985) and Hennig86 (Farris 1988).

6.3 Results

The sequences for the COI and 16s mtDNA segments, and the derived amino acid sequence for the COI gene, which was obtained using the yeast coding table in MEGA, are tabulated in Appendix I. These three sequences were used for the analysis procedures below.

Summaries within and between species of the numbers of transitions and transversions and the ns/nv ratio (no. of transitions / no. of transversions) are tabulated in Tables 4.3 and 4.4 for 16s and COI respectively. Information on alignment gaps in the 16s gene are provided in Table 4.5. There were no alignment gaps in the COI gene sequence. Site variability for each sequence and also the derived amino acid sequence are shown in Figure 4.1(a-c).

From each of these three datasets the data were summarised by the computation of phylogenetically informative dendrograms. Firstly, distance values were generated using the number of nucleotide differences between each pair of samples. The distance values calculated were then clustered using the UPGMA method to generate the dendrograms seen in Figures 4.2, 4.3 and 4.4. Similarly, maximum parsimony phylogenies were generated for each sequence. The phylograms found using this method describe the minimum number of base changes required to generate the branching structure. Often many trees of equal length are found and bootstrap analysis is then used to examine the robustness of the branching structure. The results of maximum parsimony analsysis for each of the two DNA sequence datasets can be seen in Figures 4.5 and 4.6.

Table 4.3 Observed frequencies of variable and similar sites from pairwise comparisons, within species, for the 16s gene sequence. ns: number of transitional differences; nv: number of transversional differences.

	Transitions				Transversions			Identical pairs					
	ns/nv	AG	TC	AT	AC	TG	CG	AA	TT	CC	GG	ns+nv	Total
within "green"													
Ss12 & Ss35	*	0	2	0	0	0	0	95	135	75	42	2	349
Ss12 & Ss36	*	0	2	0	0	0	0	101	135	75	42	2	355
Ss12 & Ss61	*	0	0	0	0	0	0	100	134	77	42	0	353
Ss35 & Ss36	*	0	0	0	0	0	0	147	169	92	53	0	461
Ss35 & Ss61	*	0	2	0	0	0	0	146	166	92	53	2	459
Ss36 & Ss61	*	0	2	0	0	0	0	156	166	92	53	2	469
within "brown"													
Ss6 & Ss114	0.000	0	0	0	1	1	0	142	156	81	53	2	434
within "spined"													
Ss50 & Ss163	*	1	0	0	0	0	0	155	175	83	50	1	464
Ss50 & Ss164	*	1	0	0	0	0	0	148	173	82	50	1	454
Ss50 & Ss206	0.667	1	1	0	0	3	0	155	161	81	50	5	452
Ss50 & Ss207	0.000	0	0	0	0	2	0	155	168	83	51	2	459
Ss50 & Ss211	*	2	0	0	0	0	0	153	174	82	51	2	462
Ss163 & Ss164	*	0	0	0	0	0	0	147	173	82	50	0	452
Ss163 & Ss206	0.500	0	1	0	0	2	0	154	162	81	51	3	451
Ss163 & Ss207	1.000	1	0	0	0	1	0	153	169	83	50	2	457
Ss163 & Ss211	*	1	0	0	0	0	0	151	174	82	51	1	459
Ss164 & Ss206	0.333	0	1	0	0	3	0	147	159	81	50	4	441
Ss164 & Ss207	0.500	1	0	0	0	2	0	148	166	82	50	3	449
Ss164 & Ss211	*	1	0	0	0	0	0	144	172	81	51	1	449
Ss206 & Ss207	2.000	1	1	0	0	1	0	153	161	81	52	3	450
Ss206 & Ss211	0.667	1	1	0	0	3	0	151	162	80	51	5	449
Ss207 & Ss211	1.000	2	0	0	0	2	0	151	167	82	51	4	455
between "green" and "brown"													
Ss6 & Ss12	3.444	5	26	4	3	1	1	96	122	58	38	40	354
Ss6 & Ss35	2.667	6	26	6	3	2	1	139	154	73	49	44	459
Ss6 & Ss36	2.667	6	26	6	3	2	1	149	154	73	49	44	469
Ss6 & Ss61	2.833	6	28	6	3	2	1	148	151	73	49	46	467
Ss114 & Ss12	3.875	6	25	3	3	1	1	83	108	56	37	39	323
Ss114 & Ss35	3.200	7	25	4	3	2	1	126	139	70	48	42	425
Ss114 & Ss36	3.200	7	25	4	3	2	1	135	139	70	48	42	434
Ss114 & Ss61	3.400	7	27	4	3	2	1	134	136	70	48	44	432
between "brown" and "spined"													
Ss6 & Ss50	1.000	1	14	11	2	2	0	151	160	75	51	30	467
Ss6 & Ss163	1.067	2	14	11	2	2	0	147	160	75	50	31	463
Ss6 & Ss164	1.067	2	14	11	2	2	0	140	158	74	50	31	453
Ss6 & Ss206	0.944	2	15	11	2	5	0	147	146	73	50	35	451
Ss6 & Ss207	0.882	1	14	11	2	4	0	147	153	75	51	32	458
Ss6 & Ss211	1.133	3	14	11	2	2	0	145	159	74	51	32	461
Ss114 & Ss50	1.154	2	13	8	3	2	0	137	145	72	50	28	432
Ss114 & Ss163	1.333	3	13	8	2	2	0	135	146	72	50	28	431
Ss114 & Ss164	1.231	3	13	8	3	2	0	133	143	71	49	29	425

	Transitions				Transversions			Identical pairs					
	ns/nv	AG	TC	AT	AC	TG	CG	AA	TT	CC	GG	ns+nv	Total
Ss114 & Ss206	1.133	3	14	8	3	4	0	134	133	70	50	32	419
Ss114 & Ss207	1.071	2	13	8	3	3	0	135	140	72	50	29	426
Ss114 & Ss211	1.308	4	13	8	3	2	0	132	144	72	50	30	428
between "green" and "spined"													
Ss12 & Ss50	13.000	5	21	1	1	0	0	99	125	61	38	28	351
Ss12 & Ss163	12.500	4	21	1	1	0	0	98	125	61	38	27	349
Ss12 & Ss164	12.500	4	21	1	1	0	0	92	124	60	38	27	341
Ss12 & 5s206	4.000	4	16	1	1	3	0	97	117	60	38	25	337
Ss12 & Ss207	6.000	5	19	1	1	2	0	97	122	61	38	28	346
Ss12 & Ss211	12.500	5	20	1	1	0	0	96	125	61	39	27	348
Ss35 & Ss50	13.000	6	20	1	1	0	0	144	159	77	49	28	457
Ss35 & Ss163	12.500	5	20	1	1	0	0	144	159	77	49	27	456
Ss35 & Ss164	12.500	5	20	1	1	0	0	137	157	76	49	27	446
Ss35 & Ss206	4.400	5	17	1	1	3	0	144	149	75	49	27	444
Ss35 & Ss207	6.250	6	19	1	1	2	0	142	153	77	49	29	450
Ss35 & Ss211	12.500	6	19	1	1	0	0	140	159	76	50	27	452
Ss36 & Ss50	13.000	6	20	1	1	0	0	154	159	77	49	28	467
Ss36 & Ss163	12.500	5	20	1	1	0	0	151	159	77	49	27	463
Ss36 & Ss164	12.500	5	20	1	1	0	0	144	157	76	49	27	453
Ss36 & Ss206	4.400	5	17	1	1	3	0	151	149	75	49	27	451
Ss36 & Ss207	6.250	6	19	1	1	2	0	150	153	77	49	29	458
Ss36 & Ss211	12.500	6	19	1	1	0	0	149	159	76	50	27	461
Ss61 & Ss50	14.000	6	22	1	1	0	0	154	157	77	49	30	467
Ss61 & Ss163	13.500	5	22	1	1	0	0	150	156	77	49	29	461
Ss61 & Ss164	13.500	5	22	1	1	0	0	143	154	76	49	29	451
Ss61 & Ss206	4.600	5	18	1	1	3	0	150	147	75	49	28	449
Ss61 & Ss207	6.500	6	20	1	1	2	0	149	151	77	49	30	456
Ss61 & Ss211	13.500	6	21	1	1	0	0	148	156	76	50	29	459

Table 4.4 Observed frequencies of variable and similar sites from pairwise comparisons, within species, for the COI gene sequence. ns: number of transitional differences; nv: number of transversional differences.

	Transitions				Transversions			Identical pairs					
	ns/nv	AG	TC	AT	AC	TG	CG	AA	TT	CC	GG	ns+nv	Total
within "green"													
Ss5 & Ss12	7.500	8	7	0	0	2	0	155	229	97	92	17	590
Ss5 & Ss34	*	2	0	0	0	0	0	160	233	101	92	2	588
Ss5 & Ss35	3.000	2	1	0	0	1	0	159	217	99	93	4	572
Ss5 & Ss36	1.000	2	0	2	0	0	0	160	232	101	93	4	590
Ss5 & Ss61	4*	2	2	0	0	0	0	65	123	49	44	4	285
Ss5 & Ss62	*	6	3	0	0	0	0	89	102	52	51	9	303
Ss12 & Ss34	8.500	10	7	0	0	2	0	155	227	97	89	19	587
Ss12 & Ss35	15.000	10	5	0	0	1	0	154	215	96	90	16	571
Ss12 & Ss36	4.250	10	7	2	0	2	0	155	226	97	90	21	589
Ss12 & Ss61	*	0	0	0	0	0	0	65	125	49	46	0	285
Ss12 & Ss62	1.500	0	3	0	0	2	0	92	99	52	54	5	302
Ss34 & Ss35	1.000	0	1	0	0	1	0	161	216	99	92	2	570
Ss34 & Ss36	0.000	0	0	2	0	0	0	163	230	102	92	2	589
Ss34 & Ss61	*	4	2	0	0	0	0	66	123	49	42	6	286
Ss34 & Ss62	*	7	3	0	0	0	0	89	101	53	49	10	302
Ss35 & Ss36	0.333	0	1	2	0	1	0	161	214	99	93	4	571
Ss35 & Ss61	6.000	4	2	0	0	1	0	65	113	47	42	7	274
Ss35 & Ss62	*	7	4	0	0	0	0	89	96	52	50	11	298
Ss36 & Ss61	3.000	4	2	2	0	0	0	66	120	49	42	8	285
Ss36 & Ss62	*	7	3	0	0	0	0	89	102	53	50	10	304
Ss61 & Ss62	*	0	0	0	0	0	0	1	4	4	4	0	13
within "brown"													
Ss4 & Ss6	*	4	3	0	0	0	0	161	196	125	93	7	582
Ss4 & S544	*	4	3	0	0	0	0	161	200	126	93	7	587
Ss4 & Ss114	*	4	3	0	0	0	0	162	201	126	93	7	589
Ss4 & Ss115	1.000	0	3	0	0	3	0	164	200	125	95	6	590
Ss6 & Ss44	*	0	0	0	0	0	0	163	199	125	95	0	582
Ss6 & Ss114	*	0	2	0	0	0	0	164	199	124	95	2	584
Ss6 & Ss115	4.000	4	4	0	0	2	0	162	197	122	93	10	584
Ss44 & Ss114	*	0	2	0	0	0	0	164	204	125	95	2	590
Ss44 & Ss115	8.000	4	4	0	0	1	0	162	202	123	93	9	589
Ss114 & Ss115	6.000	4	2	0	0	1	0	163	204	124	93	7	591
within "spined"													
Ss163 & Ss206	3.000	1	2	0	0	1	0	168	206	92	89	4	559
Ss163 & Ss207	2.000	1	3	0	1	1	0	162	207	91	89	6	555
Ss163 & Ss211	3.000	1	2	0	1	0	0	161	204	90	89	4	548
Ss206 & Ss207	*	0	0	0	0	0	0	162	195	93	94	0	544
Ss206 & Ss211	*	0	0	0	0	0	0	162	191	92	93	0	538
Ss207 & Ss211	*	0	0	0	0	0	0	161	197	91	94	0	543
between "green" and "brown"													
Ss4 & Ss5	3.182	16	54	13	5	4	0	144	182	85	85	92	588
Ss4 & Ss12	2.760	15	54	15	4	6	0	143	179	85	87	94	588
Ss4 & Ss34	3.045	14	53	13	5	4	0	147	181	86	84	89	587

	Transitions				Transversions			Identical pairs					
	ns/nv	AG	TC	AT	AC	TG	CG	AA	TT	CC	GG	ns+nv	Total
Ss4 & Ss35	2.913	14	53	13	5	5	0	145	166	83	85	90	569
Ss4 & Ss36	2.792	14	53	15	5	4	0	147	180	86	85	91	589
Ss4 & Ss61	3.250	8	31	7	3	2	0	58	91	43	40	51	283
Ss4 & Ss62	3.200	8	24	9	1	0	0	84	83	46	49	42	304
Ss6 & Ss5	3.045	16	51	13	5	4	0	144	180	84	85	89	582
Ss6 & Ss12	2.600	15	50	15	4	6	0	143	178	84	87	90	582
Ss6 & Ss34	2.909	14	50	13	5	4	0	147	179	85	84	86	581
Ss6 & Ss35	2.783	14	50	13	5	5	0	145	167	83	85	87	567
Ss6 & Ss36	2.667	14	50	15	5	4	0	147	178	85	85	88	583
Ss6 & Ss61	3.167	9	29	8	3	1	0	58	94	42	40	50	284
Ss6 & Ss62	3.000	7	23	8	1	1	0	84	79	46	49	40	298
Ss44 & Ss5	3.045	16	51	13	5	4	0	144	184	85	85	89	587
Ss44 & Ss12	2.708	15	50	15	4	5	0	143	183	85	87	89	587
Ss44 & Ss34	2.909	14	50	13	5	4	0	147	184	86	84	86	587
Ss44 & Ss35	2.783	14	50	13	5	5	0	145	171	83	85	87	571
Ss44 & Ss36	2.667	14	50	15	5	4	0	147	182	86	85	88	588
Ss44 & Ss61	3.167	9	29	8	3	1	0	57	95	43	40	50	285
Ss44 & Ss62	3.000	7	23	8	1	1	0	85	82	46	49	40	302
Ss114 & Ss5	3.045	16	51	13	5	4	0	145	185	85	85	89	589
Ss114 & Ss12	2.708	15	50	15	4	5	0	144	184	85	87	89	589
Ss114 & Ss34	2.909	14	50	13	5	4	0	148	184	86	84	86	588
Ss114 & Ss35	2.783	14	50	13	5	5	0	146	171	83	85	87	572
Ss114 & Ss36	2.667	14	50	15	5	4	0	148	183	86	85	88	590
Ss114 & Ss61	3.167	9	29	8	3	1	0	58	95	43	40	50	286
Ss114 & Ss62	3.000	7	23	8	1	1	0	85	82	46	49	40	302
Ss115 & Ss5	2.760	16	53	13	5	7	0	145	183	83	85	94	590
Ss115 & Ss12	2.577	15	52	15	4	6	1	144	182	83	88	93	590
Ss115 & Ss34	2.640	14	52	13	5	7	0	148	182	84	84	91	589
Ss115 & Ss35	2.750	14	52	13	5	6	0	146	169	81	85	90	571
Ss115 & Ss36	2.444	14	52	15	5	7	0	148	181	84	85	93	591
Ss115 & Ss61	3.250	8	31	7	3	2	0	59	94	41	40	51	285
Ss115 & Ss62	2.583	8	23	9	1	2	0	84	82	46	49	43	304
Between "brown" and "spined"													
Ss4 & Ss163	3.000	8	52	10	6	3	1	154	180	81	86	80	581
Ss4 & Ss206	2.292	9	46	10	6	6	2	153	163	82	86	79	563
Ss4 & Ss207	1.821	9	42	12	7	7	2	147	167	82	86	79	561
Ss4 & Ss211	2.038	9	44	12	6	6	2	147	162	80	86	79	554
Ss6 & Ss163	2.900	9	49	11	6	3	0	153	178	81	86	78	576
Ss6 & Ss206	2.391	10	45	11	6	5	1	152	161	82	86	78	559
Ss6 & Ss207	1.889	10	41	13	7	6	1	146	164	81	86	78	555
Ss6 & Ss211	2.120	10	43	12	7	5	1	146	161	79	86	78	550
Ss44 & Ss163	3.105	9	50	11	6	2	0	153	184	81	86	78	582
Ss44 & Ss206	2.619	10	45	11	6	3	1	152	166	82	86	76	562
Ss44 & Ss207	2.040	10	41	13	7	4	1	146	170	82	86	76	560
Ss44 & Ss211	2.304	10	43	12	7	3	1	146	165	80	86	76	553
Ss114 & Ss163	3.105	9	50	11	6	2	0	154	184	81	86	78	583
Ss114 & Ss206	2.500	10	45	11	6	4	1	153	166	82	86	77	564

	Transitions				Transversions			Identical pairs					
	ns/nv	AG	TC	AT	AC	TG	CG	AA	TT	CC	GG	ns+nv	Total
Ss114 & Ss207	1.962	10	41	13	7	5	1	147	170	82	86	77	562
Ss114 & Ss211	2.208	10	43	12	7	4	1	147	165	80	86	77	555
Ss115 & Ss163	2.850	8	49	10	6	3	1	155	184	80	87	77	583
Ss115 & Ss206	2.304	9	44	10	6	5	2	154	166	81	88	76	565
Ss115 & Ss207	1.815	9	40	12	7	6	2	148	170	81	88	76	563
Ss115 & Ss211	2.125	9	42	12	6	4	2	148	166	79	88	75	556
between "green" and "spined"													
Ss5 & Ss163	3.231	12	30	7	4	2	0	153	210	78	85	55	581
Ss5 & Ss206	2.111	13	25	8	4	5	1	151	190	81	85	56	563
Ss5 & Ss207	1.810	13	25	10	4	6	1	146	191	80	85	59	561
Ss5 & Ss211	1.900	13	25	9	5	5	1	145	187	79	85	58	554
Ss12 & Ss163	3.071	14	29	8	3	3	0	151	209	78	86	57	581
Ss12 & Ss206	2.000	14	24	9	3	5	2	150	189	81	86	57	563
Ss12 & Ss207	1.727	15	23	10	4	6	2	144	191	80	86	60	561
Ss12 & Ss211	1.850	14	23	10	4	4	2	144	188	79	86	57	554
Ss34 & Ss163	3.077	10	30	7	4	2	0	156	209	79	84	53	581
Ss34 & Ss206	2.118	11	25	8	4	4	1	154	189	82	84	53	562
Ss34 & Ss207	1.800	11	25	10	4	5	1	149	190	81	84	56	560
Ss34 & Ss211	1.895	11	25	9	5	4	1	148	186	80	84	55	553
Ss35 & Ss163	3.000	10	29	7	4	2	0	154	196	77	85	52	564
Ss35 & Ss206	2.250	11	25	8	4	3	1	152	177	80	85	52	546
Ss35 & Ss207	1.842	11	24	10	4	4	1	147	179	78	85	54	543
Ss35 & Ss211	1.944	11	24	9	5	3	1	146	177	77	85	53	538
Ss36 & Ss163	2.667	10	30	9	4	2	0	156	207	79	85	55	582
Ss36 & Ss206	1.800	11	25	10	4	5	1	154	187	82	85	56	564
Ss36 & Ss207	1.565	11	25	12	4	6	1	149	188	81	85	59	562
Ss36 & Ss211	1.636	11	25	11	5	5	1	148	184	80	85	58	555
Ss61 & Ss163	2.625	7	14	6	1	1	0	62	115	41	39	29	286
Ss61 & Ss206	2.500	8	12	6	1	1	0	61	108	42	39	28	278
Ss61 & Ss207	2.714	8	11	5	1	1	0	59	110	43	39	26	277
Ss61 & Ss211	2.714	8	11	5	1	1	0	59	109	43	39	26	276
Ss62 & Ss163	4.600	8	15	2	2	1	0	89	89	42	48	28	296
Ss62 & Ss206	2.111	7	12	3	2	3	1	89	75	44	48	28	284
Ss62 & Ss207	1.750	8	13	5	2	4	1	85	75	42	48	33	283
Ss62 & Ss211	1.818	7	13	5	2	3	1	85	77	41	48	31	282

Table 4.5 Observed number of alignment gaps in each 16s DNA sequence with a gap length = 1

Ss 16s	3
Ss114 16s	3
Ss12 16sAR	3
Ss35 16s	3
Ss36 16s	3
Ss61 16s	3
Ss50 16s	2
Ss163 16s	2
Ss164 16s	2
Ss206 16s	2
Ss207 16s	2
Ss211 16s	2

Figure 4.1a Numbers of variable sites in non-overlapping windows of 50 bases for the 16s DNA sequence

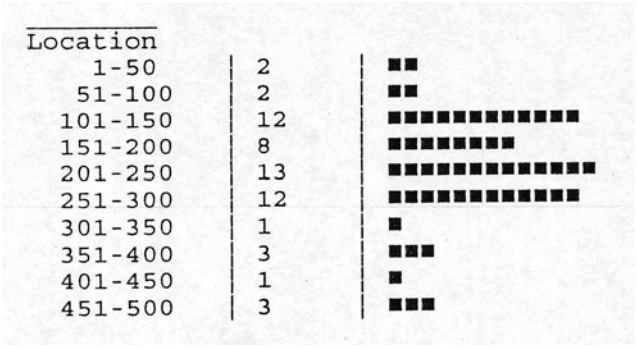


Figure 4.1b Numbers of variable sites in non-overlapping windows of 50 bases for the COI DNA sequence

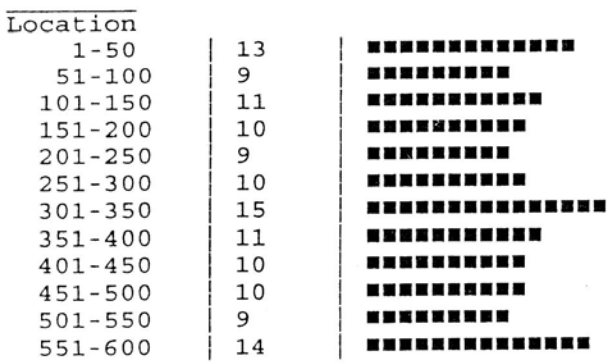


Figure 4.1c Numbers of variable sites in non-overlapping windows of 50 codons (3 bases) for the COI amino acid sequence

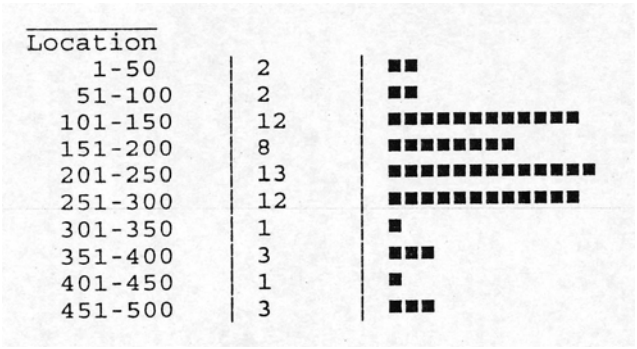


Figure 4.2 UPGMA dendrogram of base differences between mud crabs samples derived from the 16s DNA sequences

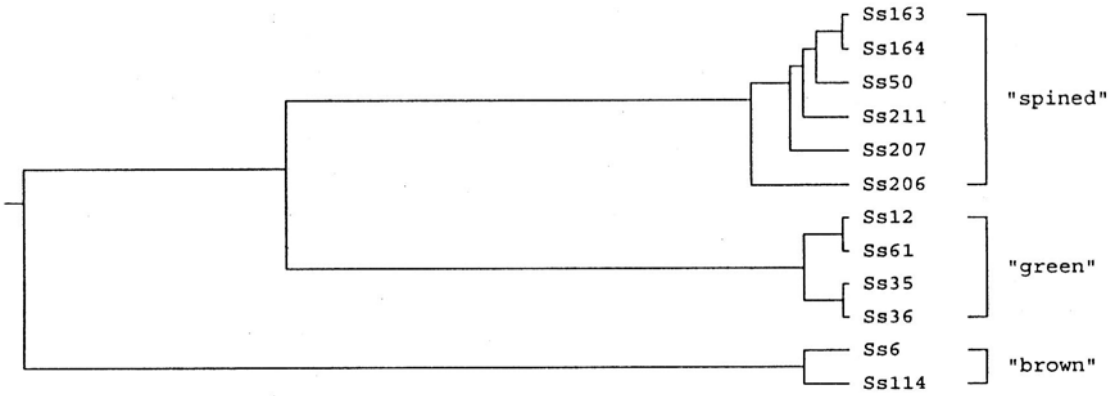


Figure 4.3 UPGMA dendrogram of base differences between mud crabs samples derived from the COI DNA sequences.

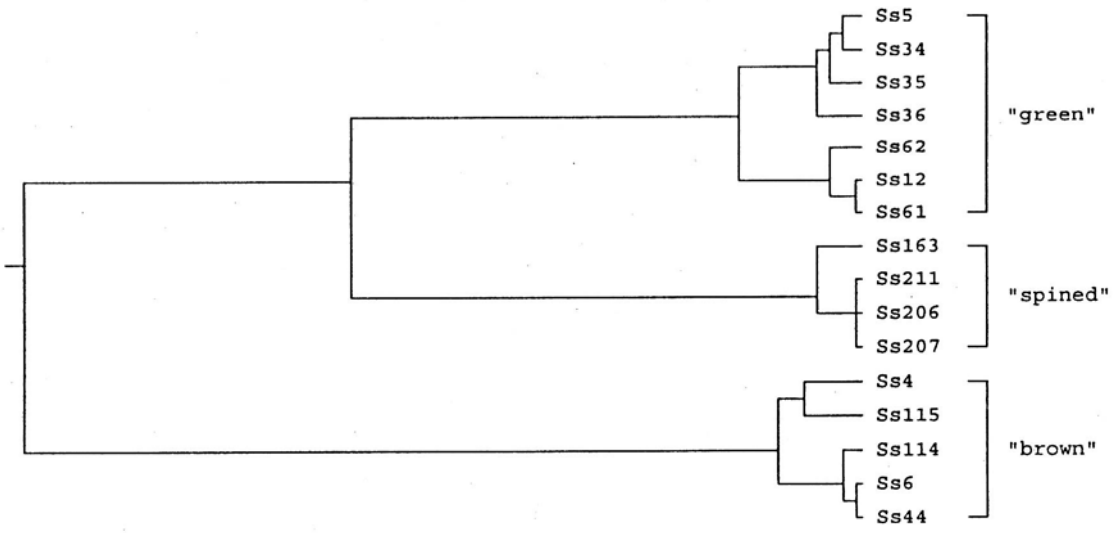


Figure 4.4 UPGMA dendrogram of amino acid differences between mud crabs samples derived by using yeast coding of the COI DNA sequences

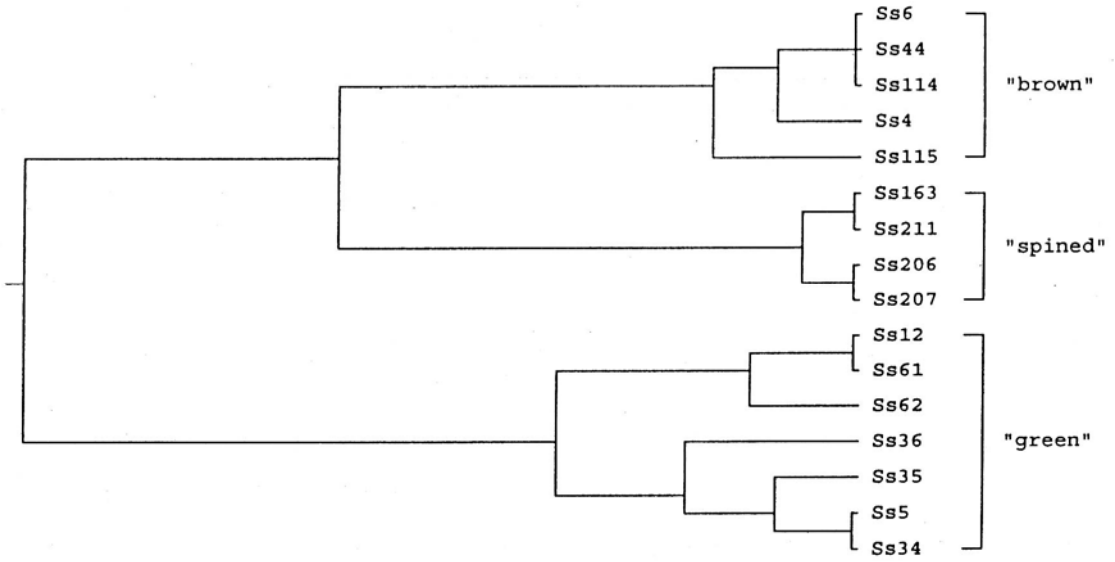


Figure 4.5 Maximum parsimony phylogram of 16s sequence data from mud crabs. Branch lengths not indicative of distance, just relationships, figures represent percentage of times trees structure was found in similar length trees

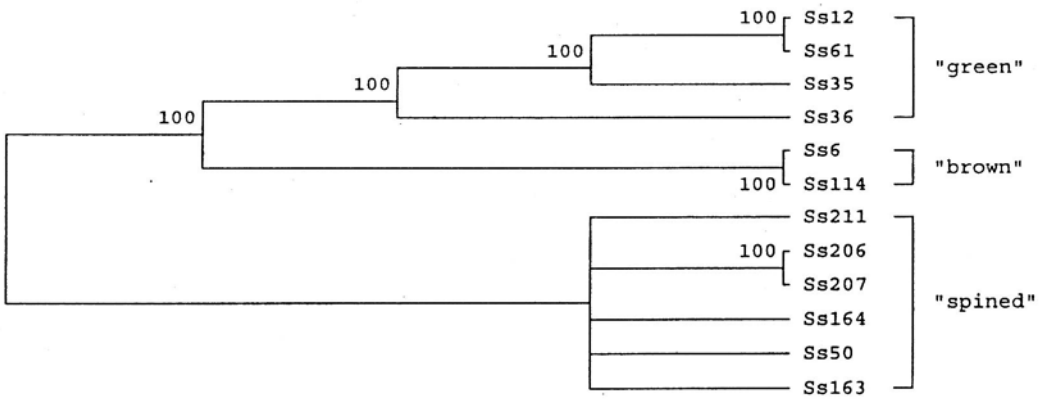
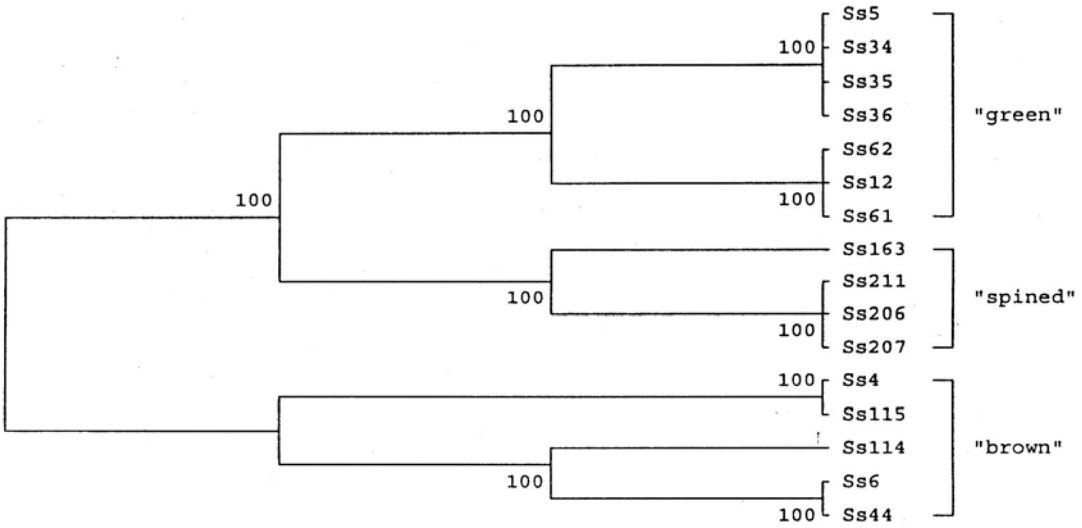


Figure 4.6 Maximum parsimony phylogram of COI sequence data from mud crabs. Branch lengths not indicative of distance, just relationships, figures represent percentage of times trees structure was found in similar length trees



6.4 Discussion

All the denrograms and phylograms presented above, for both mitochondrial gene sequences, produce the same grouping of specimens into three distinct clusters. The specimens within each cluster belong to the same species as defined by the allozyme research, and therefore each cluster represents a distinct species.

The COT gene sequence presented here is 594 bases long and its corresponding amino acid sequence is 198 codons. Similarly the 16s sequence presented here is 474 bases long. Table 4.6, below, summarises the within and between species variation for both sequences. Within species variation is clearly at least an order of magnitude less than the between species variation, which confirms the definition of the groups as species. While the samples examined are from geographically spaced locations, further work with samples from additional locations would most likely provide more information on population structure and relationships within each species. Within species variability may also increase from the results of such studies.

Table 4.6 Within and between species variation in mean number of variable codon sites, expressed as a percentage of total number of sites

	"green"		"brown"		"spined"	
	COI	16s	COI	16s	COI	16s
"green"	1.84	0.33				
"brown"	15.36	10.14	0.97	0.46		
"spined"	9.96	6.49	13.71	6.91	0.43	0.54

Both within and between species variation in the COI gene was greater than that found for the 16s RNA gene, with the exception of the "spined" within species comparison. This is expected because the COI gene, as a protein coding gene, has the potential to vary at silent sites in the third codon position. Mean within species gene variability for the COI gene was 1.29%, considerably higher than the 0.48 % found for the 16s gene sequence. Between species variability was approximately ten times greater than within species variability for COT at 13.20%. Between species variability for the 16s gene was about 15 times greater at 7.26% than that observed within species.

To define the generic and evolutionary relationships correctly the cladogram should be rooted with outgroup taxa, to the determine the most primitive and derived species. While the most useful outgroups would be other genera from the Portunidae, e.g. Thalamita and Portunus, data for these species is not currently available and we are in the process of obtaining sequence data from the family Penaeidae for this purpose.

7 Morphological variation

7.1 Introduction

Many authors have observed a high degree of morphological variation between different types of mud crabs, which they have used as the basis for their taxonomic classification. However, as pointed out recently by Fuseya and Watanabe (1995), it is still not clear whether the genus Scylla consists of 4 species (types), 3 species or 1 species, and because of the importance of this aspect to other mud crab research, further hereditary or genetic work is necessary. With our new knowledge of the genetic relationships of mud crabs it now appears that while Estampador (1949a,b) clarified much confusion about the species groupings of mud crabs [through very careful work on colour patterns, relative size, cheliped spination, chromosome 'form' and process of gamete development], without reference to the type material he was not able to identify correctly Scylla serrata (see below and Section 6). Therefore his other nomenclature decisions were based on an incorrect assumption. Many authors have used his descriptions since they were published, while many others have used their own interpretation of the taxonomy, and these have also added to the confusion of nomenclature for the group.

The genetic data, presented in Sections 3 & 4, show that there are three distinct species of mud crabs, showing no evidence of hybridization despite being sympatric in many areas. By employing the genetic data for grouping of specimens into species groups, the morphometric data was analysed by discriminant function analysis to produce a summary of identifiable characteristics for each species.

7.2 Materials and Methods

Morphological data were collected from 241 crabs, 237 of which were represented in the genetic samples. Therefore not all samples present in the collection (Table 2.1) were measured, however a sufficient representative sample of each species was obtained. Apart from recording the sex of the specimens, there were 24 measurements taken with digital calipers to the nearest 0.1 mm and 12 descriptive (coded) characters recorded. Diagrams of the positions of the measurement points are presented in Figures 5.1 a-e. Many of the crabs had broken or missing appendages and spines and were recorded as missing data. The measurement characters were size standardised through the creation of simple ratios. The details of the calculation of these ratios and details of the coded characters are provided in Table 5.1. A total of 39 variables were therefore available for analysis.

Figure 5.1 (a-c) Measurements taken from the carapace, frontal lobe and sternum

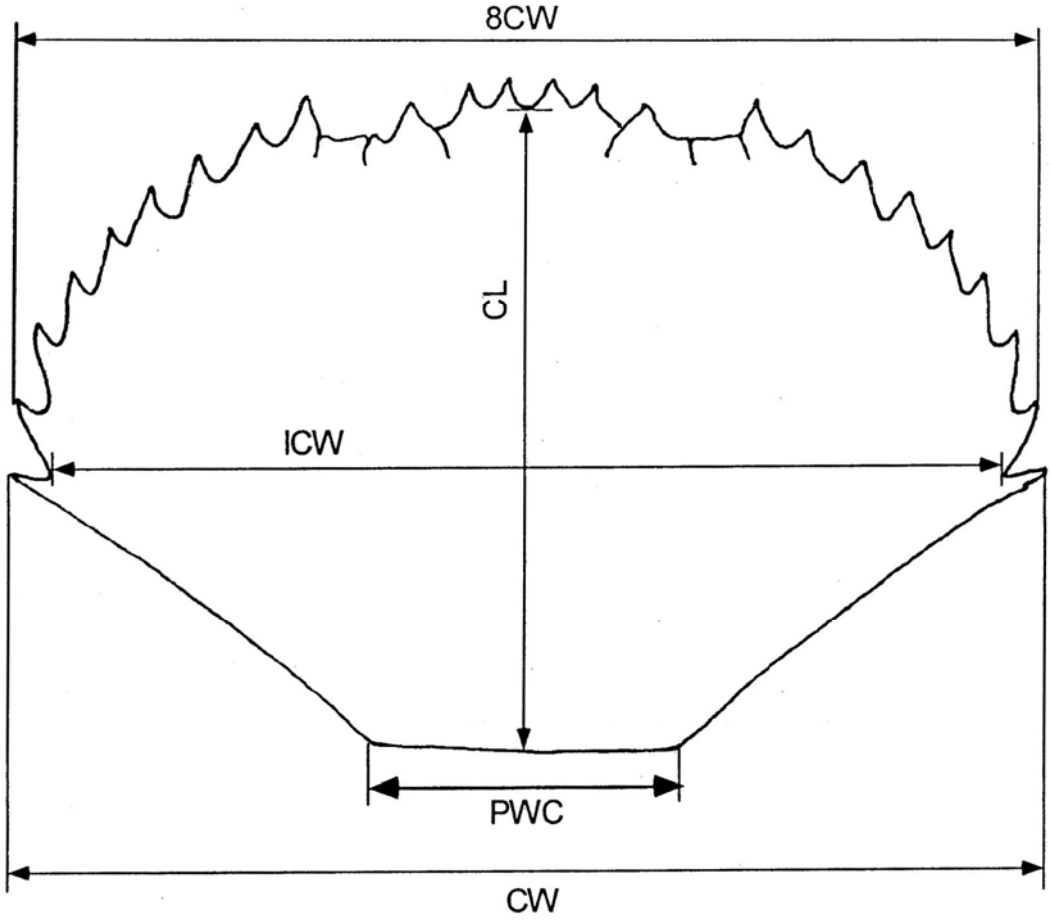


Figure 5.1a. Measurements taken from the carapace

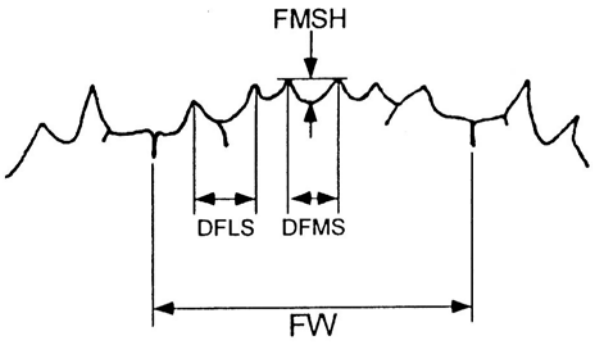


Figure 5.1b. Measurements taken from the frontal lobe

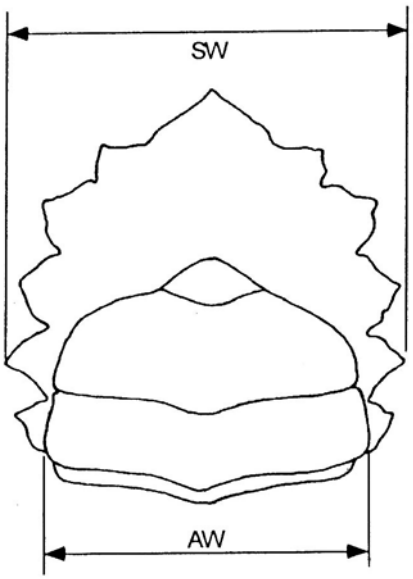


Figure 5.1c. Measurements taken from the sternum

Figure 5.1 (d-e)Measurements taken from the periopods and chelipeds

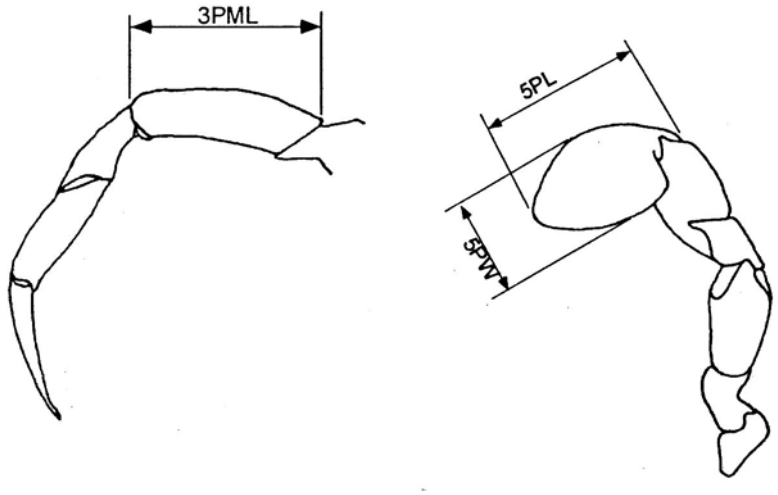


Figure 5.1d. Measurements taken from the periopods

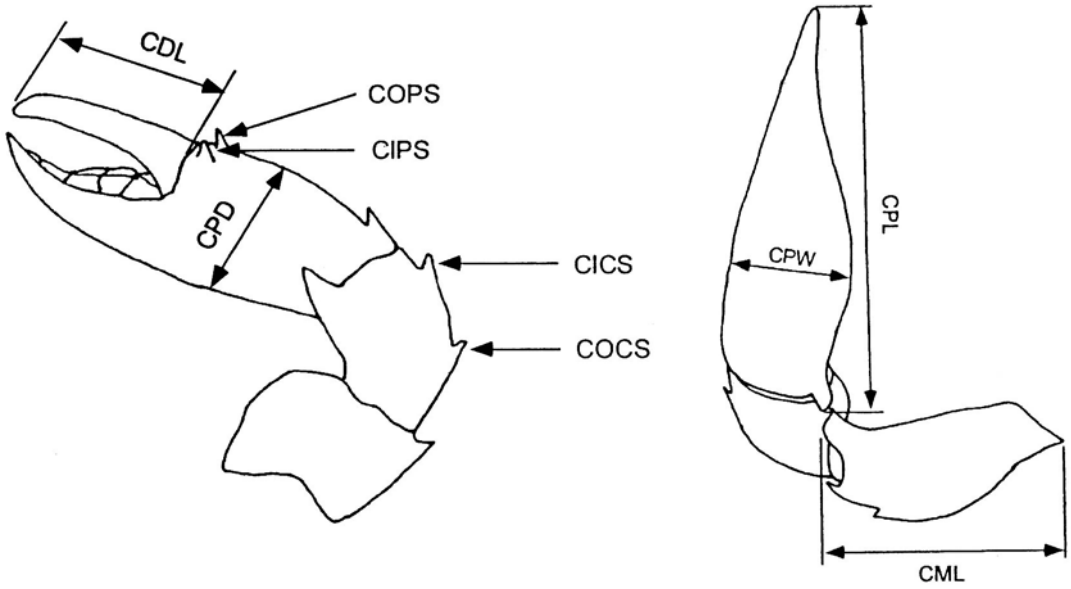


Figure 5.1e. Measurements taken from the chelipeds

Table 5.1 Mud crab morphometric data for statistical analysis

A. Carapace data

1. 9th Lateral spine height (LSH) / Internal carapace width (ICW) where LSH = (CW d ICW) / 2
2. Carapace width (CW) / Carapace width at spine 8 (8CW)
3. Carapace length (CL) I Internal carapace width (ICW)
4. Body depth (BD) I Internal carapace width (ICW)
5. Posterior width of carapace (PWC) / Internal carapace width (ICW)
6. Carapace frontal width (FW) / Internal carapace width (ICW)
7. Posterior width of carapace (PWC) / Frontal width (FW)
8. Frontal median spine height (FMSH) I Frontal width (FW)
9. Frontal median spine height (FMSH) I Distance between frontal median spines (DFMS)
10. Distance between frontal median spines (DFMS) I Frontal width (FW)
11. Distance between frontal lateral spines (DFLS) I Frontal width (FW)
12. Distance between frontal median spines (DFMS) I Distance between frontal lateral spines (DFLS)
13. Sternum width (SW) / Internal carapace width (ICW)
14. Abdomen width (AW) I Sternum width (SW)
15. Pattern on abdomen (AP)
    0 = none
    1 = fine
    2 = reticulated/polygonal

B. Cheliped data

16. Propodus length (CPL) / Internal carapace width (ICW)
17. Dactyl length (CDL) I Propodus length (CPL)
18. Propodus width (CPW) I Propodus length (CPL)
19. Propodus depth (CPD) / Propodus length (CPL)
20. Propodus width * Propodus depth (CPW*CPD*0.7854) / Propodus length (CPL)
21. Inner propodus spine (CIPS) I Propodus length (CPL)
    0 = no spine
22. Outer propodus spine (COPS) / Propodus length (CPL)
23. Inner carpus spine (CICS) I Propodus length (CPL)
24. Outer carpus spine (COCS) / Propodus length (CPL)
25. Merus length (CML) I Propodus length (CPL)
26. Cheliped propodus patterning (CPP)
    0 = none
    1 = fine
    2 = reticulated/polygonal

C. Periopod data

27. 5th periopod dactyl width (PW5) / 5th periopod dactyl length (5PL)
28. 3rd periopod merus length (P3ML) I Internal carapace width (ICW)
29. Pattern on periopod 1 (PIP)
30. Pattern on periopod 2 (P2P)
31. Pattern on periopod 3 (P3P)
32. Pattern on periopod 4 (P4P)
33. Pattern on periopod 5 (P5P)

    For items 28 to 32: 0 = none
    1 = fine
    2 = reticulated/polygonal

D. Descriptive data

34. Crab sex (sex)

1 = male
2 = female

35. Frontal median spine shape (FMSS)
0 = blunt/rounded and shallow
1 = triangular to slightly rounded and shallow
2 = steep, rounded and deep

36. Antero-lateral spine shape (ALSS)
0 = normal (anterior truncated)
1 = flatter, broader (anterior more concave)
2 = more conical and pointier

37. Carapace colour (CaraC)
0 = green
1 = browny green
2 = greeny brown
3 = brown

38. Cheliped colour (ChelC) = background colour (other colour present)
0 = green (brown)
1 = brown (green)
2 = orange (green)
3 = green (orange)
4 = brown (orange)
5 = purple/blue

39. Cheliped tip colour (CTC)
0 = not different from rest of propodus
1 = orange to rust
2 = purple

A forward stepwise discriminant function analysis, with missing values substituted by means, was conducted to determine the characters that best discriminated between the three species as determined by the allozyme pattern of each specimen. An F-value of 3.7, which approximately represents the 0.05 significance level for the number of samples examined, was used as the minimum F value for variables to enter the model and variables were removed from the model if their respective F to remove value was smaller than 3.6.

7.3 Results

The results of the forward stepwise discriminant function analysis are presented in Table 5.2 and illustrated graphically in Figure 5.2. The model provided almost 100% discrimination between species for the 15 variables included in the model. However, there was no single character that provided clearly discriminating information between all three species although FMSH/FW showed by far the highest discrimination. Many variables showed a degree of overlap between the species and also a degree of sexual dimorphism. Appendix II summarises the data providing mean values, sample sizes, standard deviations and variances for species, and species by sex, breakdowns.

Table 5.2 Results of forward Stepwise Discriminant Analysis by species

Last variable removed: PW5 5PL	F (2, 222) = 3.753461	p < .02494
Wilks' Lambda: .0378341 approx.	F (26, 444) = 70.71766	p <0.00000

Summary of Stepwise Analysis								
Variable Enter/Remove	Step	F to entr/rem	df1	df2	p-level	No. of vars. in	Lambda	F-value
FMSH FW-(E)	1	352.3926	2	234	0.000000	1.00000	.249258	352.3926
FMSS-(E)	2	70.8977	2	233	.000000	2.00000	.154957	179.4514
CICS_CPL-(E)	3	39.2857	2	232	.000000	3.00000	.115754	149.9659
P3P-(E)	4	22.5413	2	231	.000000	4.00000	.096852	127.8153
CPL ICW-(E)	5	16.2027	2	230	.000000	5.00000	.084892	111.8792
CIPS-CPL-(E)	6	18.7088	2	229	.000000	6.00000	.072969	103.1245
CARAC-(E)	7	13.3831	2	228	.000003	7.00000	.065303	94.8877
FW ICW-(E)	8	10.6045	2	227	.000040	8.00000	.059723	87.7340
SW:ICW-(E)	9	17.6501	2	226	.000000	9.00000	.051655	85.3761
AP-(E)	10	13.4827	2	225	.000003	10.00000	.046126	82.2629
CHELC-(E)	11	9.9077	2	224	.000075	11.00000	.042378	78.5570
CML CPL-(E)	12	8.6449	2	223	.000242	12.00000	.039328	75.1233
PW5:5PL-(E)	13	4.3840	2	222	.013573	13.00000	.037834	70.7177

Squared Mahalanobis Distances

Species	green	brown	spined
green	0.00000	49.35399	23.07419
brown	49.35399	0.00000	32.67575
spined	23.07419	32.67575	0.00000

F-values; df = 15,220

SPECIES	green	Brown, spined	
green	--	151.1140	29.10229
brown	151.1140	--	45.66498
spined	29.1023	45.6650	--

p-levels

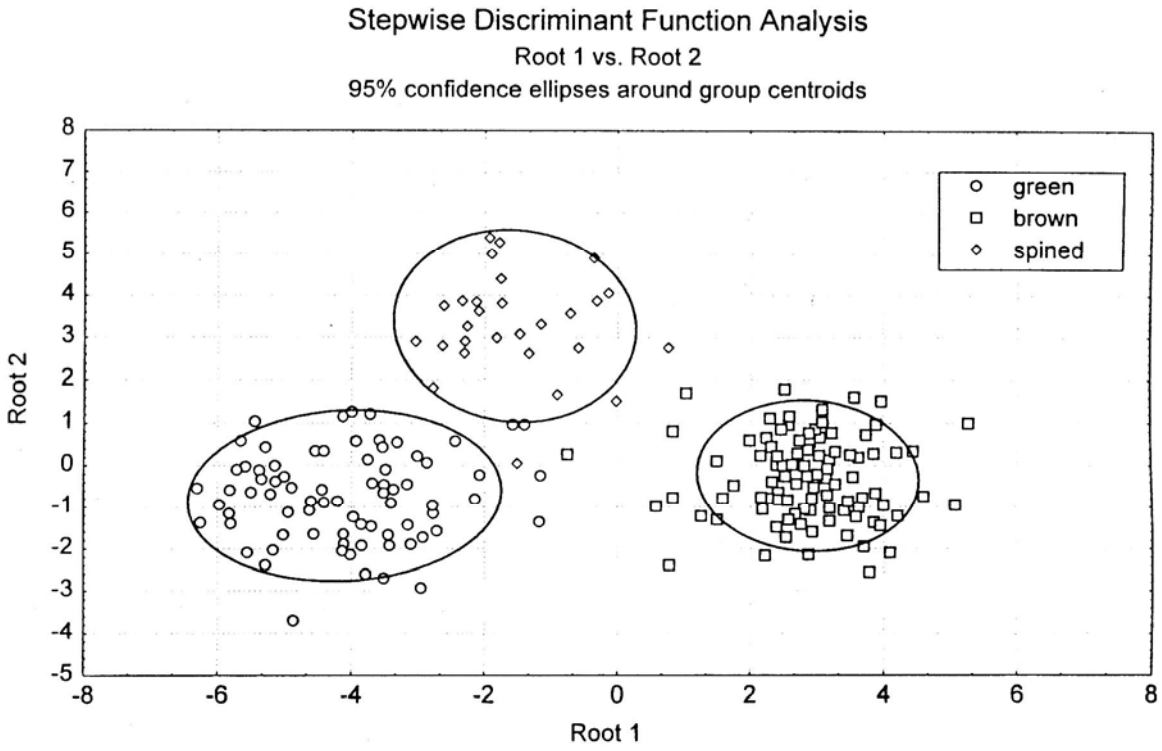
SPECIES	green	brown	spined
green	--	0.000	0.000
brown	0.000	--	0.000
spined	0.000	0.000	--

Classification Functions

Variable	green	brown	spined
FMSH/FW	2754.98	2389.79	2477.18
FMSS	-2.74	-12.41	-3.26
CICS/CPL	152.55	-882.62	-117.38
P3P	45.70	35.87	40.45
CPL/ICW	-88.59	-107.23	-56.04
CIPS/CPL	1100.18	1325.74	1413.26
CARAC	-4.09	-4.22	-5.54
FW/ICW	1905.20	2102.84	2109.58
SW/ICW	3826.49	3789.09	3675.59
AP	40.66	44.72	45.41
CHELC	-.61	-.61	.95
CML/CPL	297.12	266.84	305.10
PW5/5PL	1950.69	1884 01	1939.66

CPD/CPL	-625.02	-612.58	-788.97
CPW/CPL	1478.14	1528.51	1597.86
Constant	-2377.66	-2346.98	-2396.28

Figure 5.2 Graph of individual discriminant function scores with superimposed 95% confidence ellipses centred on each group centroid.



A similar analysis was conducted on the variables using sex as the grouping factor. The variables that were significant in this model were the ones that displayed the greatest amount of sexual dimorphism. The results of this discriminant function analysis are presented in Table 5.3.

Table 5.3 Results of forward Stepwise Discriminant Analysis by sex.

Last variable removed: CPL ICW	F (1, 231) = 2.030492	p < .15552
Wilks' Lambda: .1994691 approx.	F (9, 231) 103.0083	p <0.00000

Summary of Stepwise Analysis								
Variable Enter/Remove	**Step**	**F to entr / rem**	**df 1 df 2**	**p-level**	**vars. in**	**No of Lambda**	**F-value**	
AW SW-(E)	1	490.8823	1	239	0.000000	1.00000	.327450	490.8823
PW_PD_PL-(E)	2	48.6171	1	238	.000000	2.00000	.271907	318.6500
PWC_ICW-(E)	3	28.2135	1	237	.000000	3.00000	.242981	246.1281
SW_ICW-(E)	4	9.4588	1	236	.002349	4.00000	.233618	193.5492
CW_8CW-(E)	5	6.0783	1	235	.014401	5.00000	.227728	159.3869
CL_ICW-(E)	6	6.3369	1	234	.012495	6.00000	.221723	136.8950
FMSH_FW-(E)	7	3.4467	1	233	.064639	7.00000	.218491	119.0579
CICS_PL-(E)	8	9.3267	1	232	.002522	8.00000	.210047	109.0644
AP-(E)	9	4.5765	1	231	.033462	9.00000	.205966	98.9492
CPL_ICW-(E)	10	7.5594	1	230	.006444	10.00000	.199412	92.3390
PW_PD_PI-PL-(R)	11	.0655	1	230	.798173	9.00000	.199469	103.0083

Classification Matrix (summary2.sta) **Rows: Observed classifications** **Columns: Predicted classifications**			
Group	**Percent Correct**	**male p = .58506**	**female p = .41494**
male	98.58156	139	2
female	97.00000	3	97
Total	**97.92531**	**142**	**99**

7.4 Discussion

The most useful characters for distinguishing between species are, not surprisingly, characters that have been used by other authors to distinguish between their groupings (see Section 6). However, it is interesting to observe that some descriptive characters that have also been used are sexually dimorphic and their occurrence differs in the different species. The sexual variation in these characters has probably been one of the major sources of taxonomic confusion.

The most useful characters to distinguish between species (from Table 5.2), in order of significance are listed below.

1-	8.	Frontal median spine height (FMSH) / Frontal width (FW)
2-	35.	Frontal median spine shape (FMSS); 0 = blunt/rounded and shallow; 1 = triangular to slightly rounded and shallow; 2 = steep, rounded and deep
3-	23.	Inner carpus spine (CICS) / Propodus length (CPL)
4-	31.	Pattern on periopod 3 (P3P); 0 = none; 1 = fine; 2 = reticulated/polygonal
5-	16.	Propodus length (CPL) I Internal carapace width (ICW)
6-	21.	Inner propodus spine (CIPS) / Propodus length (CPL); 0 = no spine
7-	37.	Carapace colour (CaraC); 0 = green; 1 = browny green; 2 = greeny brown; 3 = brown
8-	6.	Carapace frontal width (FW) / Internal carapace width (ICW)
9-	13.	Sternum width (SW) / Internal carapace width (ICW)
10-	15.	Pattern on abdomen (AP); 0 = none; 1 = fine; 2 = reticulated/polygonal
11-	38.	Cheliped colour (ChelC) = background colour (other colour present); 0 = green; (brown); 1 = brown (green); 2 = orange (green); 3 = green (orange); 4 = brown (orange); 5 = purple/blue
12-	25.	Merus length (CML) / Propodus length (CPL)

13-	27.	5th periopod dactyl width (PW5) / 5th periopod dactyl length (5PL)
14-	19.	Propodus depth (CPD) I Propodus length (CPL)
15-	18.	Propodus width (CPW) / Propodus length (CPL)

Likewise, the characters that best described sexual differences and tended to confuse species differences (from Table 5.3), in order of significance are:

1-	14.	Abdomen width (AW) / Sternum width (SW)
2-	20.	Propodus width * Propodus depth (CPW*CPD*0.7854) / Propodus length (CPL)
3-	5.	Posterior width of carapace (PWC) / Internal carapace width (ICW)
4-	13.	Sternum width (SW) / Internal carapace width (ICW)
5-	2.	Carapace width (CW) I Carapace width at spine 8 (8CW)
6-	3.	Carapace length (CL) I Internal carapace width (ICW)
7-	8.	Frontal median spine height (FMSH) / Frontal width (FW)
8-	23.	Inner carpus spine (CICS) I Propodus length (CPL)
9-	15.	Pattern on abdomen (AP); 0 = none; 1 = fine; 2 =reticulated/polygonal
10-	16.	Propodus length (CPL) / Internal carapace width (ICW)

Of these characters, the last four are listed as being significant in discrimination between species. The most dimorphic character is No. 25., Merus length (CML) / Propodus length (CPL) for the "spined" and "brown" species (see Appendix 2).

8 Taxonomy

8.1 Introduction

Since Forsskal (1775) first named Cancer serratus from material collected from Jedah on the Red Sea, taxonomists have differed as to how many species of Scylla really exist. Table 6.1 lists all the available names in chronological order of their description. Forsskal died before he could return home from the Red Sea and his description, published posthumously, was of a crab without claws. The type material has never been located. This has undoubtedly caused much of the confusion that was to follow, however it is also true that the species of Scylla are remarkably similar, indicating relatively recent speciation. It is understandably difficult, without a large range of specimens from across the entire distributional range, to accurately define the point where intraspecific variation ends and real interspecific differences begin. Estampador (1949a) published an important paper recognising three species and a new subspecies from the Philippines. This was later supported by Serene (1952) who also recognised four forms in Vietnam. However the most recent revisionary work to be widely accepted was that of Stephenson & Campbell (1960), who felt that available evidence could only support the acceptance of a single species, Scylla serrata. Despite this a number of recent regional works have presented convincing arguments for the recognition of two or even three species (see Table 6.2). The present ACIAR funded project attempted to clarify this confusion by collecting material from the Red Sea (the original type locality of Scylla serrata), and also from as many other locations throughout the Indo-Pacific as possible. We have used genetic methods, both allozyme electrophoresis and mtDNA sequencing of COI and 16s genes, to unravel the mystery.

8.2 Materials and methods

The taxonomic status of mud crabs from throughout the range of the genus Scylla has been assessed through the use of two genetic methods and an analysis of morphological data. Specimens from the original collection location were obtained to provide a base reference point from which to proceed. This material was compared with the other material for allozyme patterns (Section 3), DNA sequences of two mitochondrial genes, COI and 16s RNA (Section 4), and morphological variation (Section 5). Comparative information was also obtained from recent literature and included to help show species differences.

8.3 Results

The results of all three methods used to characterise and differentiate the species (allozyme electrophoresis, mtDNA sequences and morphology) are complementary. These results indicate that there are three distinct species of mud crabs within the genus Scylla. Each species can be unambiguously classified by the possession of a distinctive array of alleles, unique mtDNA sequences and a range of morphological characteristics.

Analysis of morphological characters gives the least distinct results of all three classification methods. For many characters there are overlapping ranges in values and there is also significant sexual dimorphism. However for practical purposes several characters have been found that can be used with confidence. While genetic methods used in this study are unequivocal, they require specialised laboratory facilities.

The type species, Scylla serrata, has been clearly identified. All the characteristic morphological features of mature male members of this species can be clearly seen in the drawing of Rüppell (1830) which is presented in a reduced form in Figure 6.1. This

drawing is of a male crab (20 cm carapace width) from the Red Sea, the type locality. We know from the allozyme and mitochondrial DNA data, that crabs that were collected from the type locality correspond with the group that has been termed "green" throughout this report. Similarly, Figure 6.2a is a copy of the illustration of Cancer olivaceous (Herbst 1796) and Figure 6.2b is a photograph of one specimen from the type series for Portunus tranquebaricus (Fabricius 1798). Morphological characteristics that can be used, in most cases, to determine the species identity are given in Table 6.3 and these characteristics can be clearly seen on the respective photographs in both Figure 6.2 and Figure 6.3.

8.4 Discussion

Table 6.1 presents a summary of the taxonomic work that has been conducted on Scylla species. The morphological characteristics given by Estampador (1949a) for his species allow us to allocate them to the "species" names used in this report, and an indication of the probable correct name is also given

Correct species name	This report	Estampador
Scylla serrata	*"green"*	*S. oceanica*
(?)Scylla olivaceous	*"brown"*	*S. serrata*
(?)Scylla tranquebarica	*"spined"*	*S. tranquebarica*

There are eight names available for use for Scylla species. These are listed in Table 6.1 below. Examination of type specimens, or designation of lectotypes or neotypes if needed, will be necessary before a decision can be made as to which of the names below can be correctly used, and which are to be treated as junior synonyms. The proposed designation of a neotype for Scylla serrata will anchor that name for the "green" species of this report. From the original figure (reproduced in Figure 6.2a) it appears that Scylla olivaceous will prove to be the same as our "brown" form. The type series of Scylla tranquebarica contains all three species recognised by us, and therefore a lectotype (the specimen photographed in Figure 6.2b) will be required to be designated to stabilise this name. This should allow the first three names that were used in the literature to be available for the three species recognised by our study. While this is the most likely outcome, the names should be used with caution, pending the finalisation of our study of the type material.

Table 6.1 List of described species of Scylla

Name	Type Specimen/s	Type locality
Cancer serratus Forsskâl, 1775: 90	lost	Djedda, Red Sea
Cancer olivaceous Herbst 1796157, p1.38, fig.3	Berlin?	Ostindien = East Indies which then stretched from the east coast of India through to the Indo-Malaysian region
Portunus tranquebaricus Fabricius, 1798: 366	Copenhagen Should be 4 syntypes lectotype designation will be necessary	"in Oceano Indico". Possibly from the eastern coast of India around Madras
Portunus leucodon Desmarest 1822: 86, pl. 6, figs 1-3	Paris, lost?	Unknown at time of writing
Lupa lobifrons H. Milne Edwards, 1834: 453	Paris, lost?	"les Indes orientales"= East Indies
Achelous crassimanus McLeay1838: 61	Not in the McLeay collection at the Australian Museum. Presumed lost	South Africa
Scylla tranquebarica var. *oceanica* Dana, 1852: 270	Lost	Navigator Islands (= Samoa)
Scylla serrata var. *paramamosain* Estampador, 1949a: 104	no type designated	In the region of Manila Philippines

Table 6.2 Summary of mud crab taxonomy

Estampador (1949a) Philippines	S. oceanica (bulik/banhawin)	polygonal pigmented areas on limbs, greenish to brownish grey, grow larger, roving life, 2 spines behind finger with outer one smaller, brush-like setae abundant over carapace, length of male chelipeds < 2 x carapace length
	S. tranquebarica (c.f. S. oceanica)	greenish background only showing on carapace and parts of legs generally deep purplish drab-green to lighter in shade, pigmented patterning only distinct on the last legs and not on female abdomen, male chelipeds length > 2 x carapace length, chelae larger in proportion to the body, male circumference of palm = C.W
	S. serrata (mamosain)	rust to dark brown colored, no real patterning on limbs or carapace, hole dwellers, mangrove swamp dwellers, outer of 2 spines behind finger is obsolescent or only a vestige, short setae on carapace confined to just below the orbit, more pores with hypodermal papillary tubes perforate the carapace, carapace more convex, "H" less distinct,
	S. serrata paramamosain (cf. S. serrata)	median pair of frontal teeth slightly more anteriorly produced than the 2 laterals (same for serrata), outer of 2 spines at base of finger smallest but not obsolescent
Serene (1952) Vietnam	assumed Estampador's classification wascorrec t	looked at coloration cheliped length/ carapace length external spine behind dactyl on cheliped median spines on front of carapace (sharpness and depth) anterolateral spine size and shape
Stimpson (ex-Estampador)	S. oceanica	frontal teeth blunt and level with median incision deepest, posterior teeth of the anterolateral margin longer than in the other, (same for S. tranquebarica)
Chayarat& Kaewridh(1978) Thailand	Type I - red crab	ground color greenish to greyish brown with rusty red on chelipeds, no clear stripes on paddle, obsolescent middle spine on wrist, smaller size than other types, ALS/ICW shortest, females ALS/ICW was larger for all types, FW/ICW shortest with females larger than males in all types
	T II - green crab	ground color purplish to greyish green with purplish shade on chelipeds, coarse stripes of purplish red on base of the paddle, middle spine on wrist elongated, ALS/ICW longest, FW/ICW medial.
	T III - white crab	spots on dorsal part and yellowish or orange on ventral part of chelipeds, fine dark green stripes on paddle, middle spine on wrist small and short, spawning season later than red crab, similar size to green crab, ALS/ICW medial, FW/ICW longest.
Joel and Raj (1980) India	S. tranquebarica	outer spine behind finger smaller than inner one but conspicuous and acuminate (sharp) and does not vary with age or sex, spine on outer anterolateral inferior border of carpus median frontal teeth sharper, outer basal lobe of the first pair of abdominal appendage of males less rounded with fewer spinules at outer midregion with sharp bend inwards from the basal lobe and no coloration on tip, wandering species on open muddy bottoms, burrows tend to have 2 openings, carapace light to dark greyish green, only abdomen of mature females with reticulation, outer surface of hands grey to dark brown, dactylus yellowish green with white tip and propodus brownish-yellow, anthrodial membrane opaque.
	S. serrata	no outer spine at base of finger or is dentiform (tooth-like, small) and vestigial however in juveniles it is sharp but relatively small, no spines at the outer anterolateral border of carpus, median frontal teeth blunt and level, outer basal lobe of the first pair of abdominal appendages of male more rounded and with denser spinules at outer midregion, chromatophores just below the tip of first pair of abdominal appendages (brownish-red color), hole dweller with greater affinity for shallower water, burrows with 1 opening,

		carapace ferruginous brown to dark greenish-brown, no reticulation, outer surface of hands pinkish-brown, dactylus greenish with pinkish tinge with brown tip and propodus pink, anthrodial membrane pinkish.
Radhakrishnan and Samual (1982) India	S. serrata	carapace dark green, unsmooth and less convex, H furrow deep, posterior border of carapace broad and less convex, anterolateral teeth not anteriorly truncated, ventral carapace white or cream, last pair of walking legs with numerous mosaic like light patches, chelae mostly green with numerous patches and yellowish lower margin, 2 stout spines on outer angle of carpus, abdomen of mature females with numerous yellow or white patches
	S. serrata serrata	carapace dark green, smooth and more convex, frontal lobe not pointed and level, H furrow less deep, posterior border of carapace narrower and more convex, anterolateral teeth anteriorly truncated, ventral carapace bluish with reddish tinge, last pair walking legs green or violet without patches, chelie orange with brownish green patches absent or subtle, only 1 spine on outer angle of carpus, abdomen of mature females with dark brownish black thick bands
Taylor (1984)	S. serrata	rusty-crown claws, deeper cosy, 1 spine on wrist and behind finger
W.A.	S. paramamosain	greenish with mottling, 2 spines on wrist and finger, larger max. CW
Kathirvel and Srinivasagam (1992)	S. oceanica	light green, polygonal markings on all limbs, frontal teeth sharp and level, 2 sharp spines behind finger, first maturity size 110mm
India	S. tranquebarica	light to dark greyish green, polygonal markings only on abdomen of mature females, frontal teeth sharp and median teeth more produced, 2 spines behind finger, first maturity size 123mm
	S. serrata	ferruginous brown to dark greenish brown, no patterning, carapace more convex than S. oceanica, frontal spines blunt and median pair slightly projected, 1 blunt tubercle behind finger, first maturity size 85mm
	S. serrata serrata	dark green (or as above), no patterning, frontal spines blunt and level?, 1 spine behind finger, first maturity size 98mm

Table 6.3 Morphological characteristics useful in determining species identity

species	frontal lobe spines		carpus spines	outer margin of carapace spines	cheliped spines
	shape	height			
"green"	pointed	high	both obvious	straight or slightly concave (narrow)	obvious
"spined"	blunted	moderate	both obvious	convex (broad)	obvious
"brown"	rounded	low	upper absent lower reduced	markedly convex (broad)	reduced

Figure 6.1 Drawing of a male Scylla serrata from the Red Sea by Rüppell (1830)

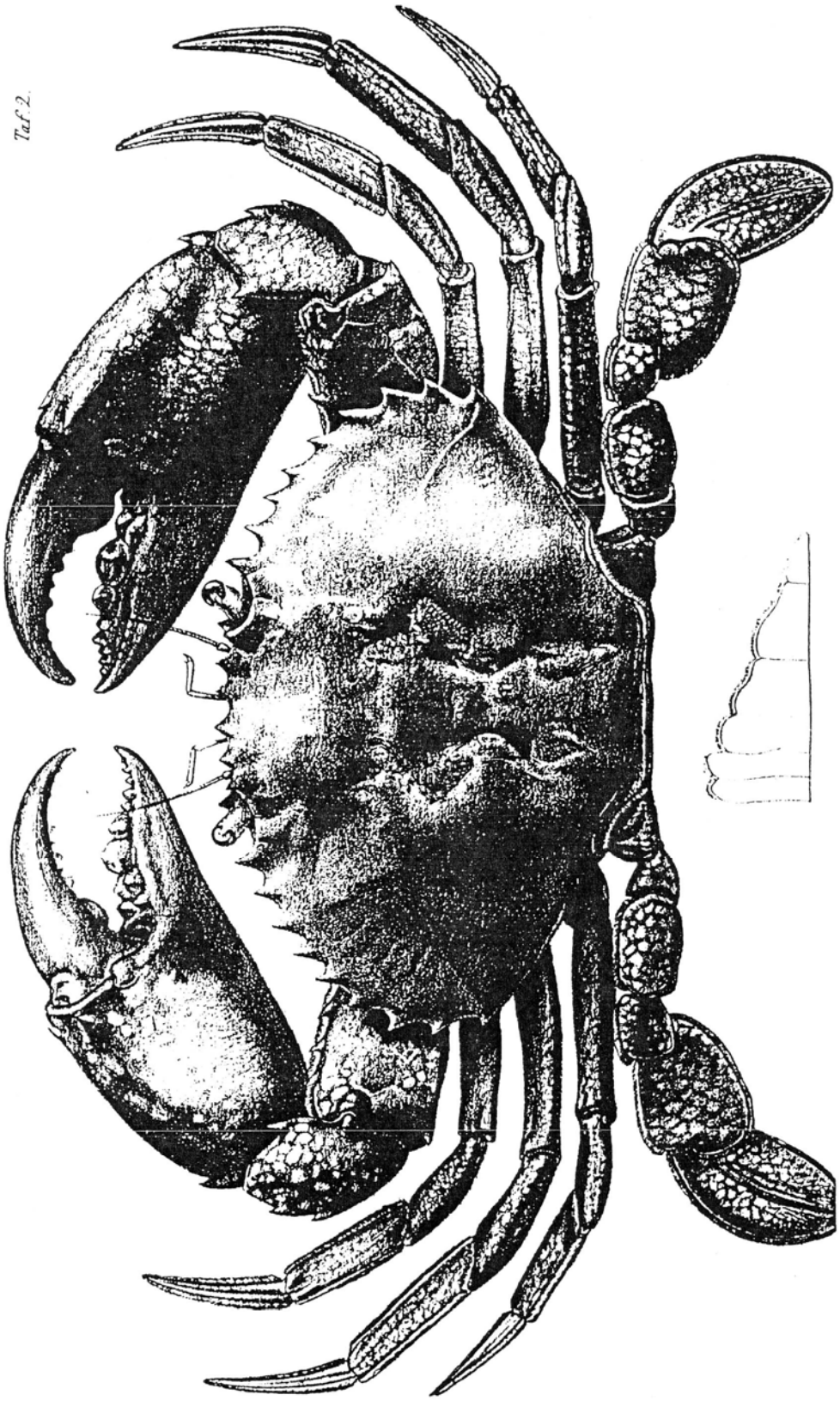


Figure 6.2 Reproductions of original material used in determining taxonomic nomenclature

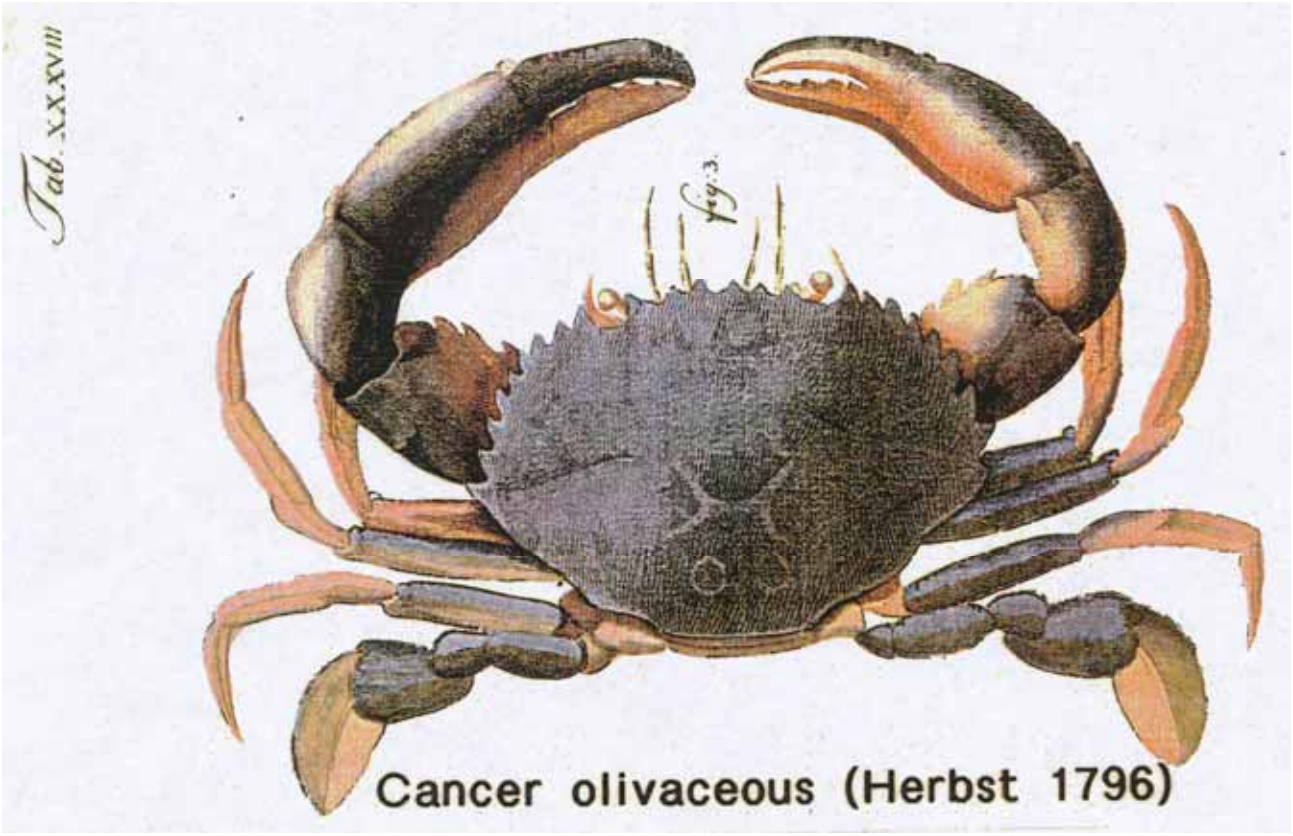


Figure 6.2a Cancer olivaceous (Herbst 1796)

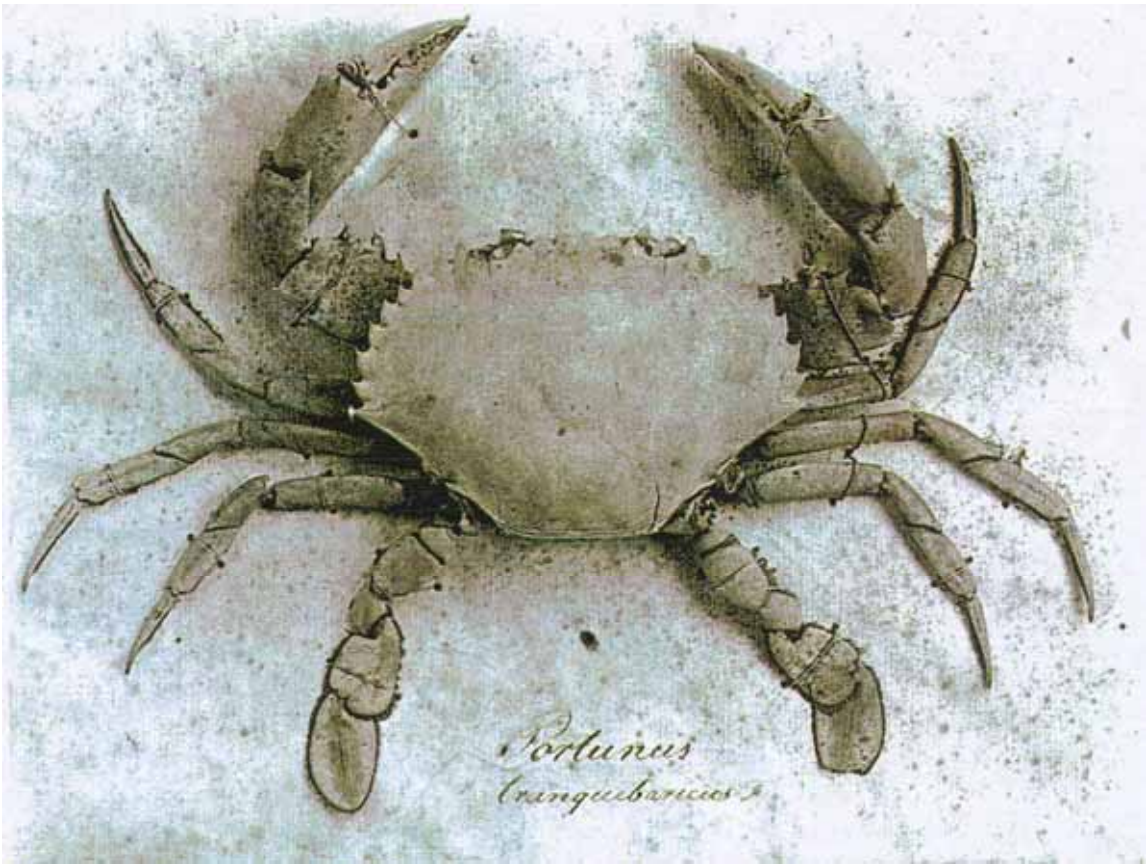


Figure 6.2b Portunus tranquebaricus (Fabricius 1798)

Figure 6.3 Dorsal and frontal photographs of examples of the three species of Scylla "green"

Figure 6.3a Scylla serrata (female, Moreton Bay, Qld "spined"

Figure 6.3b Scylla tranquebarica (male, Panay Is., Phil.) "brown"

Figure 6.3c Scylla olivaceous (male, Buntal, Sarawak)

9 Acknowledgements

With the assistance of many helpful people (acknowledged in Table 2.1) specimens were obtained from locations throughout the Indo/west-Pacific range of the genus. Without the contribution by these people this study would have been impossible. Mr Ted Burton deserves special mention for his assistance in contacting many of the people mentioned.

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11 Appendix I - mtDNA sequences

I. COI PCR mtDNA SEQUENCE

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                    111 111 111 122 222 222 223 333 333 333 444 444 444 455
          123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901
Ss4 COI   TTC TTT GGG CAT CCA GAA GTT TAC ATT CTT ATC TTA CCA GCA TTT GGT ATA
Ss6 COI   .?. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss44 COI  ??. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss114 COI ??. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss115 COI GG. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI   ..T ..? ..T ... ... ... ..C ... ... ... ..T C.. ..G ... ..C ... ...
Ss12 COI  GCT .GG ..T ..? ... ... ..C ... ... ... ..T ... ... ... ..C ... ...
Ss34 COI  ..T ..? ?.T ... ... ... ..C ... ... ... ..T C.. ..G ... ..C ... ...
Ss35 COI  ??T .?? ..T ... ... ... ..C ... ... ... ..T C.. ..G ... ..C ... ...
Ss36 COI  ..T ..? ..T ... ... ... ..C ... ... ... ..T C.. ..G ... ..C ... ...
Ss61 COIa ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss62 COIf ..T ..? ..? ..C ... ... ..C ... ... ... ..T ... ... ... .?C ... ...
Ss163 COI G?. ... ..T ... ... ... ... ..T ... ... ..T ... ... ... ..? ... ...
Ss206 COI GG. ... ..T ... ... ... ... ..T ... ... ..T ... ... ... ..C ... ...
Ss207 COI GG. ... ..T ... ... ... ... ..T ... ... ..T ... ... ... ..C ... ...
Ss211 COI GG. .?. ..T ... ... ... ... ..T ... ... ..T ... ... ... ..C ... ...

                                                                        111
          555 555 556 666 666 666 777 777 777 788 888 888 889 999 999 999 000
          234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012
Ss4 COI   ATT TCA CAC ATT GTA AGC CAA GAA TCA GGA AAA AAA GAA TCA TTC GGT ACA
Ss6 COI   ... ... ... ... ... ... ... ... ... ... ... ... ... ... .?. ... ...
Ss44 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss114 COI ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss115 COI ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI   ... ..T ..T ... ..G ..T ... ... ..T ... ... ... ... ... ... ..A ..C
Ss12 COI  ... ..T ... ... ..G ..T ... ... ..T ... ... ... ... ... ... ..A ..C
Ss34 COI  ... ..T ..T ... ..G ..T ... ... ..T ... ... ... ... ... ... ..A ..C
Ss35 COI  ... ..T ..T ... ..G ..T ... ... ..T ... ... ... ... ... .?. ..A ..C
Ss36 COI  ... ..T ..T ... ..G ..T ... ... ..T ... ... ... ... ... ... ..A ..C
Ss61 COIa ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss62 COIf ... ..T ... ... ..G ..T ... ... ..T ... ... ... ... ... .?. ..A ..C
Ss163 COI ... ..? ..T ... ... ..T ... ..G ..T ..G ... ... ... ... ..? ..A ..T
Ss206 COI ..? ... ..T ... ... ..T ... ..G ..T ..G ... ... ... ... ..G ..A ..T
Ss207 COI ..? ... ..T ... ... ..T ... ..G ..T ..G ... ... ... ... ?.G ..A ..T
Ss211 COI ..? ... ..T ... ... ..T ... ..G ..T ..G ... ... ... ... .?G ..A ..T

          111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111
          000 000 011 111 111 112 222 222 222 333 333 333 344 444 444 445 555
          345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123
Ss4 COI   TTA GGT ATA ATC TAT GCT ATA ATG GCC ATT GGT ATC TTG GGA TTC ATT GTC
Ss6 COI   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .?? ...
Ss44 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss114 COI ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss115 COI ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI   C.. ... ... ... ..C ... ... ... .?T ... ... ..T C.A ... ..T ... ...
Ss12 COI  C.G ... ... ... ... ... ... ... .?T ... ... ..T C.A ... ..T ... ...
Ss34 COI  C.. ... ... ... ..C ... ... ... ..T ... ... ..? C.A ... ..T ... ...
Ss35 COI  C.. ... ... ... ..C ... ... ... .?T ... ... ..T C.A ... ..T ... ...
Ss36 COI  C.. ... ... ... ..C ... ... ... ..T ... ... ..T C.A ... ..T ... ...
Ss61 COIa ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss62 COIf C.G ... ... ... ..C ... ... ... ..T ... ... ..T C.A ... ..T ... ...
Ss163 COI ??. ... ... ... ... ... ... ... ... ... ... ..T C.A ... ..T ... ..T
Ss206 COI C?? ... ... ... ... ... ... ... ... ..G ... ..T C.A ... ..T ... ...
Ss207 COI ?A. ... ?.. ... ... ... ... ... ... ..G ... ..T C.A ... ..T ... .?.
Ss211 COI CA? ... ?.. .?. ... .?. ... ... ... ..? ... ?.T C.A ... ..T ... ..?

          111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 122 222
          555 555 666 666 666 677 777 777 778 888 888 888 999 999 999 900 000
          456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234
Ss4 COI   TGA GCT CAC CAT ATG TTT ACA GTA GGT ATA GAC GTT GAT ACA CGA GCT TAC
Ss6 COI   ... ... ..T ... ..A .?? ... ... ... ... ... ... ... ..G ... ... ...
Ss44 COI  ... ... ..T ... ..A ... ... .?. ... ... ... ... ... ..G ... ... ...
Ss114 COI ... ... ..T ... ..A ... ... ... ... ... ... ... ... ..G ... ... ...
Ss115 COI ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI   ... ... ..T ..C ..A ... ... ..G ..G ... ... ... ... ..T ... ... ..T
Ss12 COI  ... ... ..T ..C ..A ... ... ..G ..A ... ... ... ... ..T ... ... ..T
Ss34 COI  ... ... ..T ..C ..A ... ... .?G ..G ... ... ... ... ..T ... ... ..T
Ss35 COI  ... ... ..T ..C ..A ... ... .?G ..G ... ... ... ... ..T ... ... ..T
Ss36 COI  ... ... ..T ..C ..A ... ... ..G ..G ... ... ... ... ..T ... ... ..T
Ss61 COIa ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss62 COIf ... ... ..T ..C ..A ... ... .?G ..A ... ... ... ... ..T ... ... ..T
Ss163 COI ... ... ..T ... ..A ... ... .?. ..A ... ..T ..? ... ... ... ... ..T
Ss206 COI ... ... ..? ..? ..A ..? ... .G. ..A ... ..T .?? ..? ... ... ... ?.T
Ss207 COI ... ... ..? ... ..A ... ... .G. ..A ... ..T ..G ... ... ... ... .?T
Ss211 COI ... ... ..? ... ..A ..? ... .G. ..A ... ..T ..G ... ... ... ... ..T
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COT PCR mtDNA SEQUENCE (cont'd)

```
           222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222
           000 001 111 111 111 222 222 222 233 333 333 334 444 444 444 555 555
           567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345
Ss4 COI    TTT ACA TCA GCA ACA ATA ATT ATT GCT GTC CCC ACG GGT ATC AAA ATT TTT
Ss6 COI    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss44 COI   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss114 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss115 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI    ... ... ... ... ... ..G ... ... ... ..T ... ..A ..A ..T ... ... ...
Ss12 COI   ... ... ..G ... ... ... ... ... ... ..T ... ..A ..A ..T ... ... ..C
Ss34 COI   ... ... ... ... ... ..G ... ... ... ..T ... ..A ..A ..T ... ... ...
Ss35 COI   ... ... ... ... ... ..G ... ... ... ..? ... ..A ..A ..T ..? ... ?.C
Ss36 COI   ... ... ... ... ... ..G ... ... ... ..T ... ..A ..A ..T ... ... ...
Ss61 COIa  ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss62 COIf  ... ... ..G .?. ... ... ... ... ... ..T ... ..A ..A ..T ..? ... ...
Ss163 COI  ... ... ... .?. ... ... ... ... ... .?. ..A ..A ..A ..T ... ... ...
Ss206 COI  ..? ... ... .?. ... .?. .?. ... ... ... ..A ..A ..A ..? ... .?. .?.
Ss207 COI  ..? ... ?.. .A. ... .?. ?.. ..? .?. ... ..A ?.A ..A ..? ... ... .??
Ss211 COI  ... ... ... .A. .?. .?. ... ... ... .?. ..A ??A ..A ..T ... ... .??

           222 222 222 222 222 222 222 222 222 222 222 222 222 222 223 333 333
           555 566 666 666 667 777 777 777 888 888 888 899 999 999 990 000 000
           678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456
Ss4 COI    AGA TGA CTT AGA ACC CTT CAT GGA ACT CAA ATC AAC TAC AGG CCC TCA ATG
Ss6 COI    ... ... ... ... ... ... ... ..? ... ... ... ... ... ... ... ... ...
Ss44 COI   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss114 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss115 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI    ... ... ... ... ..T ..C ... ... ..A ... ..T ..T ..T ..A ..T ..T ..A
Ss12 COI   ... ... ... ... ..T ..C ... ... ..A ... ..T ..T ..T ... ..T ..T ..A
Ss34 COI   ... ... ... ... ..T ..C ... ... ..A ... ..T ..T ..T ..A ..T ..T ..A
Ss35 COI   ... ... ... ... ..T ..C ... ... ..A ... ..T ..T ..T ..A ..T ..T ..A
Ss36 COI   ... ... ... ... ..T ..C ... ... ..A ... ..T ..T ..T ..A ..T ..T ..A
Ss61 COIa  ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ?.? ..T ?.? .?A
Ss62 COIf  ... ... ... ... ..T ..C ... ... ..A ... ..T ..? ..T ?.. ..T ?.T ?.?
Ss163 COI  ... ... ..C ... ... ..C ... ... ..C ... ..T ..T ..T ..A ..T ... ...
Ss206 COI  ... ... ?.? ... ... .?C ... ... ..C ... ..T ..T ..T ..A ..? ... ...
Ss207 COI  ... ... ..A ... ... ..C ..? ... .?C ... .?T ..? ..T ..A ..? ... ...
Ss211 COI  ... ... ..A ... ... ..C ..? ... ?.C ... ..T ..? ?.T ..A ..? ?.. ...

           333 333 333 333 333 333 333 333 333 333 333 333 333 333 333 333 333
           000 111 111 111 122 222 222 223 333 333 333 444 444 444 455 555 555
           789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567
Ss4 COI    CTT TGA GCC TTA GGT TTT ATT TTT TTA TTC ACT GTA GGC GGT CTT ACC GGA
Ss6 COI    ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ...
Ss44 COI   ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ...
Ss114 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ...
Ss115 COI  ... ... ... ... ... ..G ... ... ... ..T ... ... ... ... ... ... ...
Ss5 COI    T.A ..G ..T ... ..A ... ... ..C ... ... ... ..C ..T ... ... ..T ..G
Ss12 COI   T.A ..G ..T ... ..A ... ... ..C ... ... ... ..T ..T ... ... ..T ..G
Ss34 COI   T.A ... ..T ... ..A ... ... ..C ... ... ... ..C ..T ... ... ..T ...
Ss35 COI   T.A ... ..T ... ..A ... ... ..C ... ... ... .GC ..T ... ... ..T ...
Ss36 COI   T.A ... ..T ... ..A ... ... ..C ... ... ... ..C ..T ... ... ..T ...
Ss61 COIa  ??A ..G ..T ... ..A ??? .?? ?.C ... ... ... ..T ..T ... ... ..T ..G
Ss62 COIf  T.A ..G ..T .?? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss163 COI  ..A ... ..T ... ..A ... ..C ..C ... ..T ... ... ..A ... ... ..T ...
Ss206 COI  ..A ... ..T ... ..A ... ..C ..C ..G ?.T ... ... ..A ... ... ..T ...
Ss207 COI  ..A ... ..T ... ..A ... ..C ..C ..G ..T ... ..? ..A ... ... ..T ...
Ss211 COI  ..A ... ..T ... ..A ??? ..C ..C ..G ..T ... ... ..? ... ... ..T ...

           333 333 333 333 333 333 333 333 333 333 333 333 333 333 444 444 444
           556 666 666 666 777 777 777 788 888 888 889 999 999 999 000 000 000
           890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678
Ss4 COI    GTC GTT CTA GCT AAC TCA TCC ATC GAT ATT ATC CTA CAT GAC ACA TAC TAT
Ss6 COI    ... .?. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss44 COI   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss114 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss115 COI  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI    ..T ... T.. ... ..T ..G ..A ..T ... ... ..T ..T ... ... ... ..T ...
Ss12 COI   ..T ... T.. ... ..T ..G ..A ..T ... ... ..T ..T ... ... ... ..T ...
Ss34 COI   ..T ... T.. ... ..T ..G ..A ..T ... ... ..T ..T ... ... ... ..T ...
Ss35 COI   .?T .?? T.. ... ..? ..G ..A .?T ..? ... ..T .?T ... ... ... ..T ...
Ss36 COI   ..T ... T.. ... ..T ..G ..A ..T ... ... ..T ..T ... ... ... ..T ...
Ss61 COIa  ..T ... T.. ?.. ..T ..G ..A ..T ... ... ..T ..T ... ... ... ..T ...
Ss62 COIf  ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss163 COI  ..T ... T.. ... ..T ... ..A ..T ... ... ..T ..C ... ..T ... ..T ...
Ss206 COI  .?T ... T.. ... ..T ... ..A ..T ... ..? ..T ..C ... ..T ... ?.T ...
Ss207 COI  ..T ... T.. ... ..T ... ..A ..T ..? ... ..T ..C ... ..? ... ..T ...
Ss211 COI  ..T ... T.. ... ..T ... ..A ..T ..? ..? ..T ..C ... ..? ... ..T ...
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COT PCR mtDNA SEQUENCE (cont'd)

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            444 444 444 444 444 444 444 444 444 444 444 444 444 444 444 444 444 444
            011 111 111 112 222 222 222 333 333 333 344 444 444 445 555 555 555
            901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789
Ss4 COI     GTT GTA GCT CAC TTC CAC TAC G?G TTA TCC ATA GGA GCC GTA TTC GGT ATT
Ss6 COI     ... ... ... ... ... ... ... .T. ... ..T ... ... ... ... ... ... ...
Ss44 COI    ... ... ... ... ... ... ... .T. ... ..T ... ... ... ... ... ... ...
Ss114 COI   ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ...
Ss115 COI   ... ... .?. ... ... ... ... .T. ... ... ... ... ... ... ... ... ...
Ss5 COI     ... ... ..C ..T ... ..T ..T .TT C.T ..T ... ... ..T ... ..T ... ...
Ss12 COI    ... ... ..C ..T ... ..T ..T .TT C.T ..T ... ... ..T ... ..T ... ...
Ss34 COI    ... ... ..C ..T ... ..T ..T .TT C.T ..T ... ... ..T ... ..T ... ...
Ss35 COI    ... ... ..C ..T ... ..T ..T .TT C.T ..T ... ... ..T .?. .?T ... ...
Ss36 COI    ... ... ..C ..T ... ..T ..T .TT C.T ..T ... ... ..T ... ..T ... ...
Ss61 COIa   ... ... ..C ..T ... ..T ..T .TT C.T ..T ... ... ..T ... ..T ... ...
Ss62 COIf   ??? ??? ??? ??? ??? ??? ??? ?.? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss163 COI   ... ... ... ..T ... ... ..T .TT ... ..T ... ... ..T ... ..T ... ...
Ss206 COI   ... ... ... ..T ... ... ..T .TT ... ..T ... ... ..T ... ..T ... ...
Ss207 COI   ... ... ... ..? ... .?. ..T .TT ... ..T ... ... ..T ... ..T ... ...
Ss211 COI   ... ... ... ..? ... ... ..T .TT ... ..T ... ... ..T ... ..T ... ...

            444 444 444 444 444 444 444 444 444 444 444 444 444 444 455 555 555 555
            666 666 666 677 777 777 778 888 888 888 999 999 999 900 000 000 001
            012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890
Ss4 COI     TTC GCC GGT ATC GCC CAC TGA TTT CCA CTT TTT ACA GGT TTA TCC CTT AAT
Ss6 COI     ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...
Ss44 COI    ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...
Ss114 COI   ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...
Ss115 COI   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI     ..T ..A ... ..T ..A ..T ... ..C ..T ... ... ... ... ... ..T ... ...
Ss12 COI    ..T ..A ... ..T ..A ..T ... ..C ..T ... ... ... ... ... ..T ... ...
Ss34 COI    ..T ..A ... ..T ..A ..T ... ..C ..T ... ... ... ... ... ..T ... ...
Ss35 COI    ..T ..A ... ..T ..A ..T ... ..C ..T ... ... ... ... ... ..T ... ...
Ss36 COI    ..T ..A ... ..T ..A ..T ... ..C ..T ... ... ... ... ... ..T ... ...
Ss61 COIa   ..T ..A ... ..T ..A ..T ... ..C ..T ... ... ... ... ... ..T ... ...
Ss62 COIf   ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss163 COI   ..T ..T ..C ..T ..A ..T ... ... ... ... ... ..C ... ... ..T ... ...
Ss206 COI   ..T ..T ..C ..T ..A ..? ... ... ... ... ... ..C ... ... ..T ... ...
Ss207 COI   ..T ..T ..C ..T ..A ..? ... ... ... ... ... ?.C ..? ... ..T ... ...
Ss211 COI   ..T ..T ..C ..T ..A ..? ... ... ... ... ... ?.C ..? ... ..T ... ...

            555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555
            111 111 111 222 222 222 233 333 333 334 444 444 444 555 555 555 566
            123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901
Ss4 COI     CCT AAA ?GA ATA AAA ATT CAT TTC TCT ATT ?TA TTC GCA GGA GTA AAT ATT
Ss6 COI     ... ... T.. ... ... ... ... ... ... ... A.. ... ... ... ... ... ...
Ss44 COI    ... ... T.. ... ... ... ... ... ... ... A.. ... ... ... ... ... ...
Ss114 COI   ... ... T.. ... ... ... ... ... ... ... A.. ..T ... ... ... ... ...
Ss115 COI   ... ... T.. ... ... ... ... ... ... ... A.. ..T ... ... ... ... ...
Ss5 COI     ... ... T.. ... ... ... ... ..T ... ... A.G ..T A.T ... ..G ?.C ...
Ss12 COI    ... ... T.. ... ... ... ... ..T ... ... A.G ..T ..T ... ..G ?.C ...
Ss34 COI    ... ... T.. ... ... ... ... ..T ... ... A.G ..T A.T ... ..G ..C ...
Ss35 COI    ... ... T.. ... ... ... ... ..T ... ... A.G ..T A.T ... ..G ?.C ...
Ss36 COI    ... ... T.. ... ... ... ... ..T ... ... AAG ..T A.T ... .AG ..C ...
Ss61 COIa   ... ... T.. ... ... ... ... ..T ... ... A.G ..T ..T ... ..G ..C ...
Ss62 COIf   ??? ??? .?? ??? ??? ??? ??? ??? ??? ??? .?? ??? ??? ??? ??? ??? ???
Ss163 COI   ... ... T.. ... ... ... ... ..T ..C ... A.. ..T A.T ..T ..T ... ..C
Ss206 COI   ... ... T.. ... ... ... ... ..T ..C ... A.. ..T A.T ..T ..T ... ..C
Ss207 COI   ... ... T.. ... ... ... ... ..T ..C ... A.. ..T A.T ..T ..T ... ..C
Ss211 COI   ... ... T.. ... ?.. ... ... ..T ..C ... A.. ..T A.T ..T ..T ... ..C

            555 555 555 555 555 555 555 555 555 555 555 555
            666 666 667 777 777 777 888 888 888 899 999
            234 567 890 123 456 789 012 345 678 901 234
Ss4 COI     ACG TTC TTC CCC CAG CAT TTC TTA GGA CTT AAC
Ss6 COI     ..A ... ..? ... ... ... ... ... ... ... ...
Ss44 COI    ..A ... ... ... ... ... ... ... ... ... .?.
Ss114 COI   ..A ... ... ... ... ... ... ... ... ... ...
Ss115 COI   ... ... ... ... ... ... ... ... ... ... ...
Ss5 COI     ..T ... ... ... ..A ... ..T C.. ... ... ..T
Ss12 COI    ..T ... ... ... ..A ... ..T C.. ..G ... ..T
Ss34 COI    ..T ... ... ... ..A ... ..T C.. ... ... ..T
Ss35 COI    ..T ... ..? ?.. ..A ... ..T C.. ... ... ..T
Ss36 COI    ..T ... ... ... ..A ... ..T C.. ... ... ..?
Ss61 COIa   ..T ... ... ... ..A ... ..T C.. ..G ... ..T
Ss62 COIf   ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ???
Ss163 COI   ..C ..T ..T ..T ..A ... ..T C.. ... ... ..T
Ss206 COI   ..C ... ..? ..T ..A ..? ..T C.. ... ... ..T
Ss207 COI   ..C ... ... ..T ..A ... ..T C.. ... ... ..T
Ss211 COI   ..C ... ... ..T ..A ... ..T C.. ... ... ..T
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II. COI AMINO ACID SEQUENCE

```
                    1 1111111112 2222222223 3333333334 4444444445 5555555556
           1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss4 COI    FFGHPEVYIT ILPAFGIISH IVSQESGKKE SFGTLGIIYA IMAIGILGFI VWAHHMFTVG
Ss6 COI    ?......... .......... .......... .?........ .........? .....I?...
Ss44 COI   ?......... .......... .......... .......... .......... .....I..?.
Ss114 COI  ?......... .......... .......... .......... .......... .....I....
Ss115 COI  G......... .......... .......... .......... .......... ..........
Ss5 COI    .?......... .T........ .......... ....T..... ..?...T... .....I....
Ss12 COI   AW.?....... .......... .......... ....T..... ..?...T... .....I....
Ss34 COI   .??........ .T........ .......... ....T..... .....?T... .....I..?.
Ss35 COI   ??......... .T........ .......... .?..T..... ..?...T... .....I..?.
Ss36 COI   .?......... .T........ .......... ....T..... ......T... .....I....
Ss61 COIa  ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
Ss62 COIf  .??........ ....?..... .......... .?..T..... ......T... .....I..?.
Ss163 COI  ?......... ....?...?. .......... .?..?..... ......T... .....I..?.
Ss206 COI  G......... .......?.. .......... .L..?..... ...M..T... ...??I?.G.
Ss207 COI  G......... .......?.. .......... .?..?.?... ...M..T... ?..?.I..G.
Ss211 COI  G?........ .......?.. .......... .?..?.??.? ...?.?T... ?..?.I?.G.

                                                      1 1111111111 1111111111
           6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
           1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss4 COI    IDVDTRAYFT SATIIIAVPT GIKIFRWTRT THGTQINYRP SMTWALGFIF LFTVGGTTGV
Ss6 COI    .......... .......... .......... ..?....... .......... ..........
Ss44 COI   .......... .......... .......... .......... .......... ..........
Ss114 COI  .......... .......... .......... .......... .......... ..........
Ss115 COI  .......... .......... .......... .......... .......L.. ..........
Ss5 COI    .......... ...M...... .......... .......... .IL....... ..........
Ss12 COI   .......... .......... .......... .......... .IL....... ..........
Ss34 COI   .......... ...M...... .......... .......... .IL....... ..........
Ss35 COI   .......... ...M...?.. ..?.?..... .......... .IL....... ...G.....?
Ss36 COI   .......... ...M...... .......... .......... .IL....... ..........
Ss61 COIa  ?????????? ?????????? ?????????? ?????????. ???....??? ..........
Ss62 COIf  .......... .?........ ..?..... ......?.?. ??L..????? ??????????
Ss163 COI  ..?....... .?.....?.. .......... .......... .......... ..........
Ss206 COI  ..??...??. .?.??..... .?.??..?.. ?........? .......... .?.......?
Ss207 COI  .......??. ?E.????..? .?..?..... .?.?.??..? .......... ...?......
Ss211 COI  .......... .E??...?.? ....?..... .?.?..??.? ?......?.. ....?.....

           1111111111 1111111111 1111111111 1111111111 1111111111 1111111111
           2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
           1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss4 COI    VTANSSIDII THDTYYVVAH FHY?LSIGAV FGIFAGIAHW FPTFTGLSTN PK?IKIHFSI
Ss6 COI    ?......... .......... ...V...... .......... .......... ..W.......
Ss44 COI   .......... .......... ...V...... .......... .......... ..W.......
Ss114 COI  .......... .......... ...V...... .......... .......... ..W.......
Ss115 COI  .......... ........?. ...V...... .......... .......... ..W.......
Ss5 COI    .L........ .......... ...VT..... .......... .......... ..W.......
Ss12 COI   .L........ .......... ...VT..... .......... .......... ..W.......
Ss34 COI   .L........ .......... ...VT..... .......... .......... ..W.......
Ss35 COI   ?L.?..??.. ?......... ...VT....? ?......... .......... ..W.......
Ss36 COI   .L........ .......... ...VT..... .......... .......... ..W.......
Ss61 COIa  .L?....... .......... ...VT..... .......... .......... ..W.......
Ss62 COIf  ?????????? ?????????? ???.?????? ?????????? ?????????? ??.???????
Ss163 COI  .L........ .......... ...V...... .......... .......... ..W.......
Ss206 COI  .L......?. ....?..... ...V...... ........?. .......... ..W.......
Ss207 COI  .L.....?.. ..?......? .?.V...... ........?. ....??.... ..W.......
Ss211 COI  .L.....??. ..?......? ...V...... ........?. ....??.... ..W.?.....

           1111111111 11111111
           8888888889 99999999
           1234567890 12345678
Ss4 COI    ?FAGVNITFF PQHFLGTN
Ss6 COI    I........? ........
Ss44 COI   I......... .......?
Ss114 COI  I......... ........
Ss115 COI  I......... ........
Ss5 COI    M.T..?.... ....T...
Ss12 COI   M....?.... ....T...
Ss34 COI   M.T....... ....T...
Ss35 COI   M.T..?...? ?...T...
Ss36 COI   K.T.E..... ....T..?
Ss61 COIa  M......... ....T...
Ss62 COIf  .????????? ????????
Ss163 COI  I.T....... ....T...
Ss206 COI  I.T......? ..?.T...
Ss207 COI  I.T....... ....T...
Ss211 COI  I.T....... ....T...
```

III. 16S PCR mtDNA CODON SEQUENCE

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                    1 1111111112 2222222223 3333333334 4444444445 5555555556
              1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss6 16s       CTTTTATAGC TGCTGCACTA TAAAGACATT TTAATTCAAC ATCGAGGTCG ?AAACTCCTT
Ss114 16s     .........? ..?..... .........?. .......... .......... C.........
Ss12 16sAR    ?????????? ?????????? ?????????? ?????????? ?????????? .?????????
Ss35 16s      .......... .T..... .......... .......... .......... C.....T..C
Ss36 16s      .......... .T..... .......... .......... .......... C.....T..C
Ss61 16s      .......... .T..... .......... .......... .......... C.....T..C
Ss50 16s      .......... .T..... .......... .......... .......... C.....T...
Ss163 16s     .......... .T..... .......... .......... .......... C.....T...
Ss164 16s     ...?...... .T..... .......... ...?...... .......... C.....T...
Ss206 16s     .......... .T..... ......T... .......... .......... C.....T...
Ss207 16s     .......... .T..... .......... .......... .......... C.....T...
Ss211 16s     .........? .T..... .......... .......... .......... C.....T...

                                                                1 1111111111 1111111111
              6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
              1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss6 16s       CTTTGATAAG AACTCTTAAA AGAAATTACG CTGTTATCCC TAAAGTAACT TGATCTTTTA
Ss114 16s     .......... .......... .......... .......... .......... .......??.
Ss12 16sAR    ?????????? ?????????? ???.?..?.? ....??.... .??..??... .A........
Ss35 16s      .......... .......... .......... .......... .......... .A........
Ss36 16s      .......... .......... .......... .......... .......... .A........
Ss61 16s      .......... .......... .......... .......... .......... .A........
Ss50 16s      .......... .......... .......... .......... .......... ..........
Ss163 16s     .......... .......... .......... .......... .......... ..........
Ss164 16s     .......... .......... .......... .......... .......... ..........
Ss206 16s     ......?... .......... .....?.... .......... .......... ...?......
Ss207 16s     ......?... ......?... .......... .......... .......... ..........
Ss211 16s     .......... .......... .......... .......... .......... ..........

              1111111111 1111111111 1111111111 1111111111 1111111111 1111111111
              2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
              1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss6 16s       ATCTTTATTA AGGATCATTT AAATT-TTTA TCAAATATAA TTGTTTAAAT TTAATAGCAG
Ss114 16s     .?..G..... ........?. ...?...... .......... ........?. ..........
Ss12 16sAR    ...CA..GCG ?..?...A.? ?C.?..CCA? C.....?..? ....C..?CC ?..?....?.
Ss35 16s      ...CA..GCG .......A.. .C.A...CA. C.......... ....C...CC ..........
Ss36 16s      ...CA..GCG .......A.. .C.A...CA. C.......... ....C...CC ..........
Ss61 16s      ...CA..GCG .......A.. .C.A..CCA. C.......... ....C...CC ..........
Ss50 16s      ...CA..A.. .......A.. .T.A.T..A. .......... ........T. ..........
Ss163 16s     ...CA..A.. .......A.. .T.A.T..A. .......... ........T. ..........
Ss164 16s     ...CA..A.. .......A.. .T.A.T..A. .......... ?......?T. ......?..
Ss206 16s     .?.CA..A?. ....?..A.. .T.A.T?.A. ?.......... ........T. ....?..?..
Ss207 16s     .?.CA..A.. ....?..A.. .T.A.T?.A. ?.......... .......?T. ..........
Ss211 16s     ...CA..A.. .......A.. .T.A.T..A. .......... ........T. ...?......

              1111111111 1111111112 2222222222 2222222222 2222222222 2222222222
              8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
              1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss6 16s       TTATTTAACA TTATACATTT GTCGCCCCAA CCAAATAAAT CTTCCAAATT TACTTTTATT
Ss114 16s     .......... .......... .?........ .......... .......... ..........
Ss12 16sAR    ..?....?TT ....?.CC.. .......... .T......GC T.?.T..?.. ..........
Ss35 16s      ........TT ......CC.. .......... .T......GC T.A.T..... ..........
Ss36 16s      ........TT ......CC.. .......... .T......GC T.A.T..... ..........
Ss61 16s      ........TT ......CC.. .......... .T......GC T.A.T..... ..........
Ss50 16s      ........-T ......CA.. .......... .......... A.ATT..... ...C......
Ss163 16s     ........-T ......CA.. .?........ .......... A.ATT..... ...C......
Ss164 16s     ?.......-T ......CA.. .......... .......... A.ATT..... ...C......
Ss206 16s     .....?..-T ......CA.. .G........ ..........? A.ATT...G. ...C.G....
Ss207 16s     ........-T ......CA.. .G........ .......... A.ATT...G. ...C......
Ss211 16s     .....?..-T ......CA.. .......... .......... A.ATT..... ...C......

              2222222222 2222222222 2222222222 2222222222 2222222222 2222222223
              4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
              1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss6 16s       CTAAAAAATT GG-TTTAATC CACTCAATCT ATAAAGCTTT ATAGGGTCTT ATCGTCCCTC
Ss114 16s     .......... ........?. .......... .......... .........? ..........
Ss12 16sAR    ...-...?.C C?..C....T T.T.T..CT. ..G....... .......... ..........
Ss35 16s      ...-.....C CA..C....T T.T.T..CT. ..G....... .......... ..........
Ss36 16s      ...-.....C CA..C....T T.T.T..CT. ..G....... .......... ..........
Ss61 16s      ...-.....C CA..C....T T.T.T..CT. ..G....... .......... ..........
Ss50 16s      ...T...... T.T.C...CT T...T...T. .......... .......... ..........
Ss163 16s     ...T...... T.T.C...CT T...T...T. ..G....... .......... ..........
Ss164 16s     ...T...... T.T.C...CT T...T...T. ..G....... .......... ..........
Ss206 16s     ...T...... T.T.C...CT T...T..?T. ..G?...... .......... ..........
Ss207 16s     ...T...... T.T.C...CT T...T...T. .......... .......... ..........
Ss211 16s     ...T...... T.T.C..GCT T...T..?T. ..G....... .......... ..........
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16S PCR mtDNA CODON SEQUENCE (cont'd)

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            3333333333 3333333333 3333333333 3333333333 3333333333 3333333333
            0000000001 1111111112 2222222223 3333333334 4444444445 5555555556
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss6 16s     TAGAAAATCT AAGCCTTTTC ACTTAGAAGT TAATTTCAAT TTTAATAGAA GAGACAGCTT
Ss114 16s   .......... ........?. .......... ..?....... ?......... ...?....?.
Ss12 16sAR  .......... .......... .......... ........?. .......A.. .......TC.
Ss35 16s    .......... .......... ......?... .......... .......A.. .......TC.
Ss36 16s    .......... .......... .......... .......... .......A.. .......TC.
Ss61 16s    .......... .......... .......... .......... .......A.. .......TC.
Ss50 16s    .......... .......... .......... .......... .......A.. ..........
Ss163 16s   .......... .......... .......... .......... .......A.. ..........
Ss164 16s   .......... .......... .......... ..?....... .......A.. ...?......
Ss206 16s   .......... .......... .......... .......... .......A.. ..........
Ss207 16s   .......... .......... .......... .......... .......A.. ..........
Ss211 16s   .......... .......... .......?.. .......... .......A.. ..........

            3333333333 3333333333 3333333333 3333333334 4444444444 4444444444
            6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
Ss6 16s     TTTCTTTGTC CAACCATTCA TACAAGTTTT CAATTAAAAA ACTAATGATT ATGCTACCTT
Ss114 16s   .........? .??....... ......?.?. ...?.???.. ...C...?.. ..........
Ss12 16sAR  .......... .....?.... .........C ..?....... .......?.. .....?....
Ss35 16s    .......... .....?...? .........C ..?....... .......?.. .....?....
Ss36 16s    .......... .......... .........C .......... .......... ..........
Ss61 16s    .......... .......... .........C .......... .......... ..........
Ss50 16s    .......... .......... .........C .......... .......... ..........
Ss163 16s   .......... .......... .........C .......... ...?...?.. .....?....
Ss164 16s   .......... ..?....... .........C ......??.. .......?.. ..........
Ss206 16s   .......... .......... .........C .......... .......?.. ..........
Ss207 16s   .......... ..?....... .........C .......... ?......?.. ..........
Ss211 16s   .......... .......... .........C .......... ?...?..?.. ..........

            4444444444 4444444444 4444444444 4444444444 4444444444 4444
            2222222223 3333333334 4444444445 5555555556 6666666667 7777
            1234567890 1234567890 1234567890 1234567890 1234567890 1234
Ss6 16s     TGCACGGTCA AAATACCGCG GCTATTTAAC ATT-CTTGTC AGTGAGCAGG CTAG
Ss114 16s   ...?.....? ??.??...?. ?....?.... .......... .......... ....
Ss12 16sAR  .......... .......... .......... .C.TT..... .......... ....
Ss35 16s    .......... ..?....... ...?....?. ?..TT..... .......... ....
Ss36 16s    .......... .......... .......... ...TT..... .......... ....
Ss61 16s    .......... .......... .......... .C.TT.?... .......... ..?.
Ss50 16s    .......... .......... .......... ..C.T.?... .......... ..?.
Ss163 16s   .......... .......... ?......... ?.C.T..... .......... ....
Ss164 16s   .......... ....?..... ...?...... ?.C.T..... .......... ...?
Ss206 16s   .......... ..?....... ?......... ?.C.T..... .......... ....
Ss207 16s   .......... .......... .......... ?.C.T..... .......... ....
Ss211 16s   .......... .......... .......... ..C.T..... .......... ....
```

12 Appendix II - morphological data

I. Morphometric variable specifications:

No	Name	Long Label
5	SEX	
6	MAT	maturity
7	LSH/ICW	Lateral spine height (LSH) / Internal carapace width (ICW)
8	CW/8CW	External carapace width (CW) / Carapace width at spine 8 (8CW)
9	CL/ICW	Carapace length (CL) / ICW
10	BD/ICW	Body depth (BD) / ICW
11	PWC/ICW	Posterior width of carapace (PWC) / ICW
12	FW/ICW	Carapace frontal width (FW) / ICW
13	PWC/FW	PWC / FW
14	FMSH/FW	Front median spine height (FMSH) / FW
15	DFMS/FW	Distance between front median spines (DFMS) / FW
16	FMSHDFMS	Front median spine height (FMSH)/ DFMS
17	DFMSDFLS	Distance between front median spines (DFMS) / DFLS
18	DFLS/FW	Distance between front lateral spines (DFLS) / FW
19	CPL/ICW	Cheliped propodus length (CPL) / ICW
20	CDL/CPL	Cheliped dactyl length (CDL) / CPL
21	CPW/CPL	Cheliped propodus width (CPW) / CPL
22	CPD/CPL	Cheliped propodus depth (CPD) / CPL
23	PW/PD/PL	CPW*CPD*0.7854 / CPL
24	CIPS/CPL	Cheliped Inner propodus spine (CIPS) / CPL
25	COPS/CPL	Cheliped Outer propodus spine (COPS) / CPL
26	CICS/CPL	Cheliped Inner carpus spine (CICS) / CPL
27	COCS/CPL	Cheliped Outer carpus spine (COCS) / CPL
28	CML/CPL	Cheliped Merus Length (CML) / CPL
29	PW5/5PL	5th periopod dactyl width (5PW) / 5th periopod dactyl length (5PL)
30	P3ML/ICW	3rd periopod merus length (P3ML) / ICW
31	SW/ICW	Sternum width (SW) / ICW
32	AW/SW	Abdomen width (AW) / SW
33	FMSS	Frontal median spine shape (FMSS)
34	ALSS	Antero-lateral spine shape (ALSS)
35	CARAC	Carapace ground colour (CARAC)
36	CHELC	Cheliped colour (CHELC)
37	CTC	Cheliped tip colour (CTC)
38	CPP	Reticulated/polygonal pattern on Cheliped propodus (CPP)
39	P1P	periopod 1 patterning(P1P)
40	P2P	Periopod 2 patterning (P2P)
41	P3P	Periopod 3 patterning (P3P)
42	P4P	Periopod 4 patterning (P4P)
43	P5P	Periopod 5 patterning (P5P)
44	AP	Abdomen patterning (AP)

II. Means, sample sizes, standard deviations and variances of morphometric variables for each species

	Mean	N	Std.Dv.	Variance
5. SEX				
green	1.562500	80	.499208	.249209
brown	1.341085	129	.475922	.226502
spined	1.321429	28	.475595	.226190
All Grps	1.413502	237	.493504	.243546
6. MAT				
green	.578947	38	.500355	.250356
brown	.386364	44	.492545	.242600
spined	.625000	8	.517549	.267857
All Grps	.488889	90	.502677	.252684
7. LSH/ICW				
green	.031561	77	.005976	.000036
brown	.023510	123	.005009	.000025
spined	.030960	28	.005339	.000029
All Grps	.027144	228	.006663	.000044
8. CW/8CW				
green	1.014135	75	.010981	.000121
brown	1.000117	121	.009804	.000096
spined	1.013339	26	.010518	.000111
All Grps	1.006401	222	.012356	.000153
9. CL/ICW				
green	.694692	80	.010119	.000102
brown	.699549	126	.009421	.000089
spined	.694921	28	.010214	.000104
All Grps	.697335	234	.010009	.000100
10. BD/ICW				
green	.384211	42	.014013	.000196
brown	.405468	50	.009185	.000084
spined	.388892	18	.011102	.000123
All Grps	.394639	110	.015237	.000232
11. PWC/ICW				
green	.331426	79	.016636	.000277
brown	.321203	125	.016269	.000265
spined	.321957	28	.016494	.000272
All Grps	.324775	232	.017039	.000290

	Mean	N	Std.Dv.	Variance
12. FW/ICW				
green	.376681	80	.019696	.000388
brown	.420988	129	.016995	.000289
spined	.412357	28	.016235	.000264
All Grps	.403949	237	.033270	.001107
13. PWC/FW				
green	.882886	79	.075167	.005650
brown	.764718	125	.054114	.002928
spined	.782891	28	.064102	.004109
All Grps	.817209	232	.183372	.033625
14. FMSH/FW				
green	.059317	79	.010274	.000106
brown	.028520	127	.006540	.000043
spined	.043011	27	.005949	.000035
All Grps	.041351	233	.020485	.000420
15. DFMS/FW				
green	.144338	75	.008687	.000075
brown	.129461	119	.011106	.000123
spined	.139352	27	.009411	.000089
All Grps	.137345	221	.027873	.000777
16. FMSHDFMS				
green	.407746	75	.063777	.004068
brown	.219659	119	.046060	.002122
spined	.309310	27	.042419	.001799
All Grps	.294443	221	.100734	.010147
17. DFMSDFLS				
green	1.074744	74	.077606	.006023
brown	.948278	117	.087339	.007628
spined	1.055479	27	.084358	.007116
All Grps	1.004484	218	.103280	.010667
18. DFLS/FW				
green	.134630	79	.005806	.000034
brown	.136763	125	.007218	.000052
spined	.132880	28	.007988	.000064
All Grps	.136994	232	.022899	.000524

	Mean	N	Std.Dv.	Variance
19. CPL/ICW				
green	.713590	73	.078674	.006190
brown	.755166	126	.068858	.004741
spined	.790853	27	.096060	.009227
All Grps	.746000	226	.079437	.006310
20. CDL/CPL				
green	.445301	72	.018142	.000329
brown	.452235	125	.017961	.000323
spined	.452654	27	.014967	.000224
All Grps	.450057	224	.017918	.000321
21. CPW/CPL				
green	.420485	73	.027251	.000743
brown	.413110	126	.040159	.001613
spined	.430753	27	.029274	.000857
All Grps	.417600	226	.035600	.001267
22. CPD/CPL				
green	.266133	73	.013904	.000193
brown	.271614	126	.020703	.000429
spined	.258904	27	.014862	.000221
All Grps	.268325	226	.018541	.000344
23. PW/PD/PL				
green	8.232537	73	2.656453	7.056743
brown	6.710033	126	2.610391	6.814143
spined	7.898576	27	2.251733	5.070300
All Grps	7.343809	226	2.673541	7.147820
24. CIPS/CPL				
green	.031116	69	.008844	.000078
brown	.023918	123	.009332	.000087
spined	.030408	27	.008096	.000066
All Grps	.026986	219	.009649	.000093
25. COPS/CPL				
green	.019222	69	.006042	.000037
brown	.007827	122	.005441	.000030
spined	.016016	27	.006332	.000040
All Grps	.012448	218	.007806	.000061

	Mean	N	Std.Dv.	Variance
26. CICS/CPL				
green	.019234	70	.006600	.000044
brown	.000417	125	.001507	.000002
spined	.014696	27	.005506	.000030
All Grps	.008087	222	.009819	.000096
27. COCS/CPL				
green	.020606	71	.005623	.000032
brown	.011477	124	.004996	.000025
spined	.015650	27	.005772	.000033
All Grps	.014904	222	.006703	.000045
28. CML/CPL				
green	.455986	71	.060237	.003628
brown	.462409	120	.053923	.002908
spined	.469041	26	.052303	.002736
All Grps	.461102	217	.055781	.003112
29. PW5/5PL				
green	.620504	62	.026301	.000692
brown	.610099	101	.021141	.000447
spined	.626626	20	.020139	.000406
All Grps	.615431	183	.023624	.000558
30. P3ML/ICW				
green	.379026	56	.035702	.001275
brown	.417774	101	.032966	.001087
spined	.422760	15	.024584	.000604
All Grps	.405593	172	.037942	.001440
31. SW/ICW				
green	.527209	67	.013143	.000173
brown	.535720	129	.011831	.000140
spined	.523851	28	.013168	.000173
All Grps	.531690	224	.013252	.000176
32. AW/SW				
green	.677767	67	.169798	.028831
brown	.577967	129	.140871	.019845
spined	.603478	28	.186454	.034765
All Grps	.611007	224	.161588	.026111

	Mean	N	Std.Dv.	Variance
33. FMSS				
green	1.974684	79	.158096	.024994
brown	.843750	128	.405426	.164370
spined	1.821429	28	.390021	.152116
All Grps	1.340426	235	.643063	.413531
34. ALSS				
green	.265823	79	.673850	.454073
brown	.325581	129	.470419	.221294
spined	.285714	28	.712697	.507937
All Grps	.300847	236	.574789	.330382
35. CARAC				
green	2.153846	78	1.129037	1.274725
brown	1.751938	129	1.132137	1.281734
spined	.928571	28	1.245096	1.550265
All Grps	1.787234	235	1.197151	1.433170
36. CHELC				
green	1.985714	70	1.765144	3.115735
brown	2.031496	127	.331830	.110111
spined	3.296296	27	1.539601	2.370370
All Grps	2.169643	224	1.215656	1.477819
37. CTC				
green	.953125	64	.213042	.045387
brown	.926829	123	.291146	.084766
spined	.750000	28	.585314	.342593
All Grps	.911628	215	.330116	.108976
38. CPP				
green	1.338028	71	.476405	.226962
brown	1.000000	128	.125497	.015750
spined	1.111111	27	.320256	.102564
All Grps	1.119469	226	.338458	.114553
39. P1P				
green	1.333333	69	.474858	.225490
brown	1.047244	127	.213001	.045369
spined	1.185185	27	.395847	.156695
All Grps	1.152466	223	.360281	.129802

	Mean	N	Std.Dv.	Variance
40. P2P				
green	1.600000	50	.494872	.244898
brown	.978723	94	.145079	.021048
spined	1.062500	16	.250000	.062500
All Grps	1.181250	160	.417719	.174489
41. P3P				
green	1.689655	58	.466675	.217786
brown	.990099	101	.099514	.009903
spined	1.066667	15	.258199	.066667
All Grps	1.229885	174	.435457	.189623
42. P4P				
green	1.963636	55	.188919	.035690
brown	1.000000	88	.151627	.022991
spined	1.769231	13	.438529	.192308
All Grps	1.403846	156	.505185	.255212
43. P5P				
green	2.000000	64	.002520	.000006
brown	1.627273	110	.504277	.254295
spined	2.000000	22	.004364	.000019
All Grps	1.790816	196	.420160	.176535
44. AP				
green	.686567	67	.956777	.915423
brown	.000000	129	.000001	.000000
spined	.357143	28	.780042	.608466
All Grps	.250000	224	.662919	.439462

III Means, sample sizes, standard deviations and variances of morphometric variables for each species x sex combination.

	Mean	N	Std.Dv.	Variance
6. MAT				
green, M	1.000000	1	0.000000	0.000000
green, F	.567568	37	.502247	.252252
brown, M	1.000000	1	0.000000	0.000000
brown, F	.372093	43	.489083	.239203
new, M	--	0	0.000000	0.000000
new, F	.625000	8	.517549	.267857
All Grps	.488889	90	.502677	.252684
7. LSH/ICW				
green, M	.029781	34	.006556	.000043
green, F	.032968	43	.005125	.000026
brown, M	.021750	83	.003989	.000016
brown, F	.027162	40	.004976	.000025
new, M	.029264	19	.004249	.000018
new, F	.034539	9	.005860	.000034
All Grps	.027144	228	.006663	.000044
8. CW/8CW				
green, M	1.009905	33	.008862	.000079
green, F	1.017458	42	.011430	.000131
brown, M	.996700	82	.008608	.000074
brown, F	1.007300	39	.008213	.000067
new, M	1.009034	18	.008027	.000064
new, F	1.023025	8	.009153	.000084
All Grps	1.006401	222	.012356	.000153
9. CL/ICW				
green, M	.692890	35	.010491	.000110
green, F	.696093	45	.009706	.000094
brown, M	.697460	83	.008717	.000076
brown, F	.703581	43	.009516	.000091
new, M	.697541	19	.010841	.000118
new, F	.689390	9	.006080	.000037
All Grps	.697335	234	.010009	.000100
10. BD/ICW				
green, M	.386856	16	.009783	.000096
green, F	.382582	26	.016043	.000257
brown, M	.405622	31	.009490	.000090
brown, F	.405217	19	.008913	.000079
new, M	.389231	13	.010872	.000118
new, F	.388011	5	.012956	.000168
All Grps	.394639	110	.015237	.000232

	Mean	N	Std.Dv.	Variance
11. PWC/ICW				
green, M	.318165	35	.011710	.000137
green, F	.341974	44	.011687	.000137
brown, M	.313535	82	.012658	.000160
brown, F	.335826	43	.011777	.000139
new, M	.312297	19	.008196	.000067
new, F	.342352	9	.008800	.000077
All Grps	.324775	232	.017039	.000290
12. FW/ICW				
green, M	.382857	35	.013557	.000184
green, F	.371878	45	.022372	.000501
brown, M	.421050	85	.016631	.000277
brown, F	.420867	44	.017873	.000319
new, M	.419930	19	.013179	.000174
new, F	.396371	9	.008660	.000075
All Grps	.403949	237	.033270	.001107
13. PWC/FW				
green, M	.832197	35	.045902	.002107
green, F	.923206	44	.069485	.004828
brown, M	.745815	82	.044593	.001989
brown, F	.800764	43	.052750	.002783
new, M	.744575	19	.035020	.001226
new, F	.863781	9	.016153	.000261
All Grps	.817209	232	.183372	.033625
14. FMSH/FW				
green, M	.058103	35	.008604	.000074
green, F	.060282	44	.011436	.000131
brown, M	.028157	83	.006766	.000046
brown, F	.029204	44	.006107	.000037
new, M	.041321	18	.004450	.000020
new, F	.046393	9	.007323	.000054
All Grps	.041351	233	.020485	.000420
15. DFMS/FW				
green, M	.143353	35	.008208	.000067
green, F	.145199	40	.009101	.000083
brown, M	.129867	78	.011511	.000133
brown, F	.128688	41	.010384	.000108
new, M	.136123	18	.007037	.000050
new, F	.145811	9	.010593	.000112
All Grps	.137345	221	.027873	.000777

	Mean	N	Std.Dv.	Variance
16. FMSHDFMS				
green, M	.405212	35	.054988	.003024
green, F	.409964	40	.071211	.005071
brown, M	.216977	78	.047103	.002219
brown, F	.224763	41	.044124	.001947
new, M	.304791	18	.039962	.001597
new, F	.318346	9	.048130	.002317
All Grps	.294443	221	.100734	.010147
17. DFMSDFLS				
green, M	1.069500	35	.080069	.006411
green, F	1.079450	39	.076063	.005786
brown, M	.952750	76	.090586	.008206
brown, F	.939988	41	.081408	.006627
new, M	1.038452	18	.086667	.007511
new, F	1.089534	9	.072180	.005210
All Grps	1.004484	218	.103280	.010667
18. DFLS/FW				
green, M	.134332	35	.006132	.000038
green, F	.134867	44	.005593	.000031
brown, M	.136430	81	.007310	.000053
brown, F	.137375	44	.007086	.000050
new, M	.132335	19	.007877	.000062
new, F	.134032	9	.008575	.000074
All Grps	.136994	232	.022899	.000524
19. CPL/ICW				
green, M	.781156	30	.076983	.005926
green, F	.666451	43	.031350	.000983
brown, M	.791788	82	.055879	.003122
brown, F	.686916	44	.024025	.000577
new, M	.838037	18	.081505	.006643
new, F	.696484	9	.029063	.000845
All Grps	.746000	226	.079437	.006310
20. CDL/CPL				
green, M	.435336	30	.020370	.000415
green, F	.452419	42	.012324	.000152
brown, M	.446952	82	.017192	.000296
brown, F	.462309	43	.014968	.000224
new, M	.446944	18	.012937	.000167
new, F	.464074	9	.012340	.000152
All Grps	.450057	224	.017918	.000321

	Mean	N	Std.Dv.	Variance
21. CPW/CPL				
green, M	.431800	30	.024940	.000622
green, F	.412591	43	.026236	.000688
brown, M	.430016	82	.037565	.001411
brown, F	.381604	44	.021664	.000469
new, M	.441112	18	.029701	.000882
new, F	.410034	9	.013640	.000186
All Grps	.417600	226	.035600	.001267
22. CPD/CPL				
green, M	.270889	30	.012876	.000166
green, F	.262814	43	.013765	.000189
brown, M	.281077	82	.017160	.000294
brown, F	.253978	44	.014223	.000202
new, M	.263622	18	.011685	.000137
new, F	.249467	9	.016658	.000277
All Grps	.268325	226	.018541	.000344
23. PW/PD/PL				
green, M	9.205000	30	2.966637	8.800934
green, F	7.554074	43	2.207641	4.873677
brown, M	7.732396	82	2.670099	7.129430
brown, F	4.804720	44	.819047	.670837
new, M	8.672692	18	2.387453	5.699930
new, F	6.350343	9	.566698	.321146
All Grps	7.343809	226	2.673541	7.147820
24. CIPS/CPL				
green, M	.024749	29	.007472	.000056
green, F	.035732	40	.006656	.000044
brown, M	.020277	80	.008067	.000065
brown, F	.030693	43	.007635	.000058
new, M	.027003	18	.006919	.000048
new, F	.037217	9	.005751	.000033
All Grps	.026986	219	.009649	.000093
25. COPS/CPL				
green, M	.015072	28	.004210	.000018
green, F	.022055	41	.005461	.000030
brown, M	.005670	80	.004802	.000023
brown, F	.011934	42	.004069	.000017
new, M	.013300	18	.004342	.000019
new, F	.021447	9	.006359	.000040
All Grps	.012448	218	.007806	.000061

	Mean	N	Std.Dv.	Variance
26. CICS/CPL				
green, M	.014838	27	.005126	.000026
green, F	.021995	43	.005922	.000035
brown, M	.000229	81	.000958	.000001
brown, F	.000765	44	.002157	.000005
new, M	.012718	18	.003601	.000013
new, F	.018650	9	.006676	.000045
All Grps	.008087	222	.009819	.000096
27. COCS/CPL				
green, M	.016284	28	.004496	.000020
green, F	.023421	43	.004374	.000019
brown, M	.009687	81	.003805	.000014
brown, F	.014850	43	.005263	.000028
new, M	.012599	18	.002971	.000009
new, F	.021754	9	.005162	.000027
All Grps	.014904	222	.006703	.000045
28. CML/CPL				
green, M	.452176	29	.058288	.003398
green, F	.458616	42	.062108	.003857
brown, M	.457455	79	.057667	.003325
brown, F	.471953	41	.044987	.002024
new, M	.474504	17	.058548	.003428
new, F	.458723	9	.038856	.001510
All Grps	.461102	217	.055781	.003112
29. PW5/5PL				
green, M	.625723	25	.025034	.000627
green, F	.616978	37	.026882	.000723
brown, M	.610645	68	.023591	.000557
brown, F	.608975	33	.015147	.000229
new, M	.630360	14	.017933	.000322
new, F	.617911	6	.023976	.000575
All Grps	.615431	183	.023624	.000558
30. P3ML/ICW				
green, M	.411495	23	.022791	.000519
green, F	.356395	33	.023406	.000548
brown, M	.433026	68	.022941	.000526
brown, F	.386344	33	.027949	.000781
new, M	.436517	10	.016064	.000258
new, F	.395246	5	.010735	.000115
All Grps	.405593	172	.037942	.001440

	Mean	N	Std.Dv.	Variance
31. SW/ICW				
green, M	.530580	29	.009250	.000086
green, F	.524637	38	.015088	.000228
brown, M	.536800	85	.009864	.000097
brown, F	.533633	44	.014828	.000220
new, M	.526114	19	.010010	.000100
new, F	.519072	9	.017942	.000322
All Grps	.531690	224	.013252	.000176
32. AW/SW				
green, M	.524664	29	.012181	.000148
green, F	.794610	38	.137561	.018923
brown, M	.492225	85	.014523	.000211
brown, F	.743605	44	.126703	.016054
new, M	.485010	19	.015325	.000235
new, F	.853578	9	.114467	.013103
All Grps	.611007	224	.161588	.026111
33. FMSS				
green, M	2.000000	34	.002462	.000006
green, F	1.955556	45	.208409	.043434
brown, M	.845238	84	.424946	.180579
brown, F	.840909	44	.369989	.136892
new, M	1.842105	19	.374634	.140351
new, F	1.777778	9	.440959	.194444
All Grps	1.340426	235	.643063	.413531
34. ALSS				
green, M	.088235	34	.378806	.143494
green, F	.400000	45	.809040	.654545
brown, M	.423529	85	.497050	.247059
brown, F	.136364	44	.347142	.120507
new, M	.315789	19	.749269	.561404
new, F	.222222	9	.666667	.444444
All Grps	.300847	236	.574789	.330382
35. CARAC				
green, M	2.060606	33	1.223197	1.496212
green, F	2.222222	45	1.063632	1.131313
brown, M	2.011765	85	1.052142	1.107003
brown, F	1.250000	44	1.123222	1.261628
new, M	1.157895	19	1.384965	1.918129
new, F	.444444	9	.726483	.527778
All Grps	1.787234	235	1.197151	1.433170

	Mean	N	Std.Dv.	Variance
36. CHELC				
green, M	2.214286	28	1.892620	3.582011
green, F	1.833333	42	1.680834	2.825203
brown, M	2.012048	83	.246635	.060829
brown, F	2.068182	44	.452267	.204545
new, M	3.500000	18	1.617914	2.617647
new, F	2.888889	9	1.364225	1.861111
All Grps	2.169643	224	1.215656	1.477819
37. CTC				
green, M	.923077	26	.271746	.073846
green, F	.973684	38	.162221	.026316
brown, M	.888889	81	.316228	.100000
brown, F	1.000000	42	.220863	.048780
new, M	.684211	19	.582393	.339181
new, F	.888889	9	.600925	.361111
All Grps	.911628	215	.330116	.108976
38. CPP				
green, M	1.285714	28	.460044	.211640
green, F	1.372093	43	.489083	.239203
brown, M	1.000000	84	.001552	.000002
brown, F	1.000000	44	.215666	.046512
new, M	1.055556	18	.235702	.055556
new, F	1.222222	9	.440959	.194444
All Grps	1.119469	226	.338458	.114553
39. P1P				
green, M	1.259259	27	.446576	.199430
green, F	1.380952	42	.491507	.241580
brown, M	1.048193	83	.215475	.046430
brown, F	1.045455	44	.210707	.044397
new, M	1.166667	18	.383482	.147059
new, F	1.222222	9	.440959	.194444
All Grps	1.152466	223	.360281	.129802
40. P2P				
green, M	1.590909	22	.503236	.253247
green, F	1.607143	28	.497347	.247354
brown, M	.984375	64	.125000	.015625
brown, F	.966667	30	.182574	.033333
new, M	1.000000	9	0.000000	0.000000
new, F	1.142857	7	.377964	.142857
All Grps	1.181250	160	.417719	.174489

	Mean	N	Std.Dv.	Variance
41. P3P				
green, M	1.750000	24	.442326	.195652
green, F	1.647059	34	.485071	.235294
brown, M	.985294	68	.121268	.014706
brown, F	1.000000	33	.002500	.000006
new, M	1.000000	10	0.000000	0.000000
new, F	1.200000	5	.447214	.200000
All Grps	1.229885	174	.435457	.189623
42. P4P				
green, M	1.958333	24	.204124	.041667
green, F	1.967742	31	.179605	.032258
brown, M	1.000000	62	.181071	.032787
brown, F	1.000000	26	.002828	.000008
new, M	1.833333	6	.408248	.166667
new, F	1.714286	7	.487950	.238095
All Grps	1.403846	156	.505185	.255212
43. P5P				
green, M	2.000000	27	.002774	.000008
green, F	2.000000	37	.002357	.000006
brown, M	1.621622	74	.488293	.238430
brown, F	1.638889	36	.542627	.294444
new, M	2.000000	15	.003780	.000014
new, F	2.000000	7	.005774	.000033
All Grps	1.790816	196	.420160	.176535
44. AP				
green, M	0.000000	29	0.000000	0.000000
green, F	1.210526	38	.990711	.981508
brown, M	.000000	85	.000000	.000000
brown, F	.000000	44	.000002	.000000
new, M	.000000	19	.000000	.000000
new, F	1.111111	9	1.054093	1.111111
All Grps	.250000	224	.662919	.439462