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Electrophoretic delineation of species boundaries within the genus *Chelodina* (Testudines: Chelidae) of Australia, New Guinea and Indonesia

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A total of 281 specimens of long-necked chelid turtles (*Chelodina*) were obtained from drainages of Australia, Papua New Guinea and the island of Roti in Indonesia. Ten diagnosable taxa were identified using allozyme profiles at 45 presumptive loci. *Chelodina expansa*, *C. parkeri*, *C. rugosa* and *C. burrungandjii* are in a Group A clade, *C. longicollis*, *C. novaeguineae*, *C. steindachneri*, *C. pritchardi* and *C. mccordi* are in a Group B clade, and *C. oblonga* is in a monotypic Group C clade, with each clade thought to represent a distinct subgenus. *Chelodina siebenrocki* is synonymised with *C. rugosa*. An eleventh taxon, *C. reimanni*, could not be distinguished from *C. novaeguineae* on the basis of allozyme profiles, but it is morphologically distinct. Its status is therefore worthy of further investigation. Three instances of natural hybridization were detected. *Chelodina rugosa* and *C. novaeguineae* hybridize in the Gulf country of Queensland, with evidence of backcrossing to *C. novaeguineae*. *Chelodina longicollis* and *C. novaeguineae* hybridize in central coastal Queensland, and *C. rugosa* and *C. burrungandjii* hybridize along their zone of contact in the plateau escarpment streams and pools. A phylogeny for the *Chelodina* is presented.

ADDITIONAL KEY WORDS: Natural hybridization – Australian chelid phylogeny – phylogenetic species – long-neck turtle – Chelonia.

INTRODUCTION

The freshwater turtle fauna of Australia and adjacent regions is dominated by the family Chelidae, which occurs only in Australia, New Guinea, the island of Roti in Indonesia and South America. Its fossil record extends back to the Upper Cretaceous of South America (de Broin, 1987) and the Miocene of Australia (Gaffney *et al.*, 1989), but no fossil chelids are known from outside the present range of the family (Williams, 1953, 1954; Gaffney, 1991). The family is therefore considered to be of Gondwanal origin (Burbidge *et al.*, 1974).

Chelodina is one of six chelid genera recognized from the Australian region, a clearly defined monophyletic group with a long list of shared-derived character states (Gaffney, 1977). Superficially, it is distinguished from other chelid genera of the region by possession of only four claws on the front feet, exceptionally long necks (often longer than the shell) and gular shields which typically meet in front of the intergular or nearly so in all species (Cogger, 2000). The genus was thought to have its closest extant relatives among the long-necked forms of South America (Gaffney, 1977), but the long necks and associated modifications of the head and shell of *Chelodina* are now thought to have been independently derived (Pritchard, 1984; Seddon *et al.*, 1997; Georges *et al.*, 1998).

Six species of *Chelodina* are regarded as endemic to Australia (Goode, 1967; Cogger *et al.*, 1983), four are endemic to New Guinea (Goode, 1967; Rhodin & Mittermeier, 1976; Philippen & Grossman, 1990; Rhodin, 1994a), *C. novaeguineae* is found in both Australia and New Guinea (Goode, 1967) and *C.*

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mccordi is endemic to the island of Roti in Indonesia (Rhodin, 1994b).

These species fall into three subgeneric groups (Burbidge *et al*., 1974). *Chelodina longicollis* and *C. steindachneri* of Australia, *C. pritchardi* and *C. reimanni* of New Guinea, *C. mccordi* of Roti (Indonesia) and *C. novaeguineae* belong to Group A with relatively narrow heads, shorter thinner necks and broader plastrons (Goode, 1967; Rhodin, 1994a). *Chelodina expansa*, *C. rugosa* and *C. burrungandjii* of Australia, and *C. siebenrocki* and *C. parkeri* of New Guinea belong to Group B with relatively broad heads, longer thicker necks and narrower plastrons (Goode, 1967; Rhodin & Mittermeier, 1976; Cann, 1998; Thomson *et al*., 2000). *Chelodina oblonga* of south-western Australia is superficially similar to species of Group B, and has often been placed in that group (Goode, 1967; Legler, 1981), however, we follow Burbidge *et al*. (1974) and place it in a third subgeneric group, Group C. It is distinguished from other described *Chelodina* by a consistent set of well-developed neural bones (Burbidge *et al*., 1974; Thomson & Georges, 1996). A brief historical account of the taxonomy of the *Chelodina* by Rhodin (1994a,b) reveals considerable confusion over the number of valid species and the correct designation of populations to species.

The above taxonomy has been formulated only recently and, while rectifying many long-standing deficiencies, several issues remain unresolved. There is uncertainty as to whether *C. rugosa* of northern Australia and *C. siebenrocki* of New Guinea are distinct species (Goode, 1967), whether populations of *C. novaeguineae* from northern Australia and New Guinea are conspecific (Rhodin, 1994a), whether *C. burrungandjii* and populations of *Chelodina* from the Kimberley of northern Australia are distinct (Thomson *et al*., 2000), and whether the coastal and inland forms of *C. expansa* represent two species or subspecies (Cann, 1998).

Allozyme electrophoresis offers an alternative to traditional morphological approaches to resolving issues such as these (Avise, 1975; Buth, 1984; Richardson *et al.*, 1986; Hillis, 1987; Buth & Rainboth, 1999). It provides a large number of quantitative characters that are genetically determined. The enzyme systems in use are sufficiently known to ensure that these characters are independent of each other and any morphological characters used, and they are usually expressed in all individuals regardless of age or sex. Moreover, when compared with morphological studies, fewer individuals need be sampled per population to identify diagnostic character states.

In this paper, we use allozyme electrophoresis to delimit species within the *Chelodina*, testing the genetic integrity of both currently recognized species and suspected new taxa. This study follows a similar one addressing the issues of species delimitation in the short-necked chelid genera of Australia (Georges & Adams, 1996). Our approach is to use an objective procedure to identify diagnosable taxa within the genus (Davis & Manos, 1991; Davis & Nixon, 1992), which can be regarded as phylogenetic species. Levels of divergence between these taxa, where they are allopatric, are used to make judgements on whether or not the phylogenetic species should be regarded as biological species. We also develop a phylogeny for the genus, and identify three instances of natural hybridization.

MATERIAL AND METHODS

Specimen Collection and Identification

A total of 281 specimens of *Chelodina* were collected from drainages in the five Australian mainland states and the Northern Territory, New Guinea and Indonesia (Fig. 1). Specimens of New Guinea species and *C. mccordi* of Roti Island were accessed through the collection of live turtles maintained by William McCord at the East Fishkill Animal Hospital in New York State. All recognized species except *C. kuchlingi*, and several forms thought to be distinctive, are represented (Table 1). The sampling strategy involved obtaining a minimum of five turtles (not always achieved) from each of several natural populations of each species, though only single populations were available for most New Guinea species. Samples for *C. longicollis* were supplemented from the Australian Biological Tissues Collection (ABTC), based at the South Australian Museum, and blood samples for *C. steindachneri* were kindly provided by Gerald Kuchling. The tissues used in this study have been lodged with the ABTC.

Turtles representing described Australian species were identified with the aid of keys provided by Cogger (2000). New Guinea forms, *C. burrungandjii* and *C. mccordi*, were identified by reference to original descriptions. Distinctive populations and undescribed species were assigned to species using these references and on the basis of locality of collection. *Chelodina kuchlingi* Cann (1997) is known from a single preserved specimen (WAM R29411) of doubtful origin, and was unavailable for allozyme analysis. Recent examination of the holotype of *C. oblonga* (BMNH 1947.3.5.89) shows that it is a form of *C. rugosa* (*C. oblonga* has precedence), as suspected by Cann (1998), and that *C. colliei* (Gray, 1856) should be the name applied to the distinctive long-necked turtle species of south-west Western Australia (Thomson, 2000). Thomson plans to review and amend some of the consequential changes in a submission to the ICZN, so the contemporary use of *C. oblonga* is retained here.

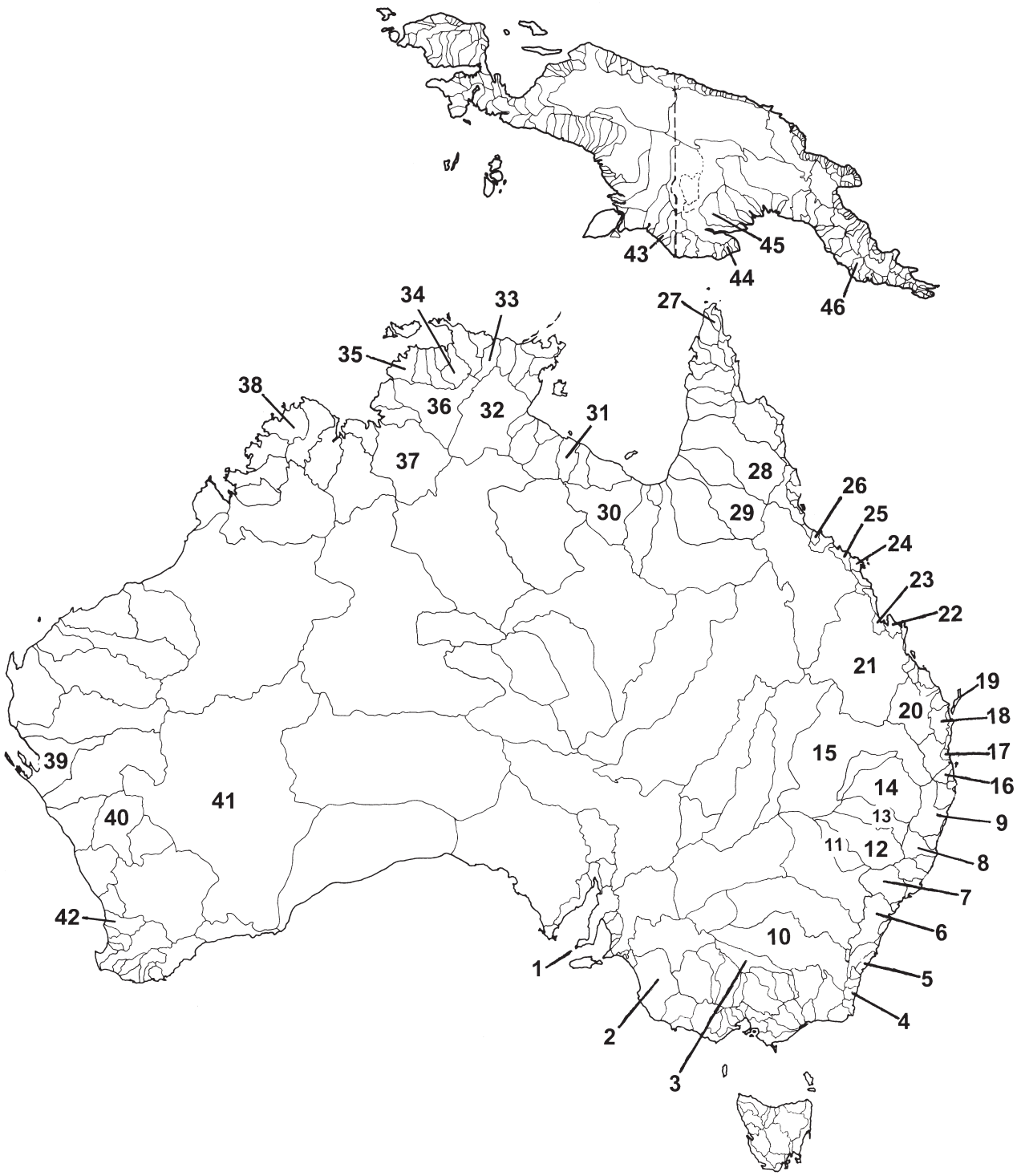

Figure 1. Australian and New Guinean drainage basins showing the 46 basins from which samples were collected. Drainage basins are numbered as follows. *South Australia*: **1.** Broughton (Clo); **2.** Millicent Coast (Clo). *Victoria*: **3.** Murray-Riverina (Cex, Clo); **4.** Bega (Clo). *New South Wales*: **5.** Clyde River–Jervis Bay (Clo); **6.** Hawkesbury (Clo); **7.** Hunter (Clo); **8.** Macleay (Clo); **9.** Clarence (Clo); **10.** Murrumbidgee (Clo); **11.** Castlereagh (Cex); **12.** Namoi (Cex, Clo); **13.** Gwydir (Clo); **14.** Border (Clo). *Queensland*: **15.** Condamine–Culgoa (Cex, Clo); **16.** Logan–Albert (Cex); **17.** Pine (Cex); **18.** Mary (Cex); **19.** Fraser Island (Cex, Clo); **20.** Burnett (Cex); **21.** Fitzroy (Cex); **22.** Shoalwater (Clo × Cno); **23.** Styx (Cno); **24.** Proserpine (Cno); **25.** Don (Cno, Clo × Cno); **26.** Ross (Cno); **27.** Jardine (Cru); **28.** Mitchell (Cru); **29.** Gilbert (Cno, Cru, Cno × Cru); **30.** Nicholson (Cno). *Northern Territory*: **31.** Robinson (Cru); **32.** Roper (Cno); **33.** Liverpool (Cbu); **34.** South Alligator (Cbu, Cru); **35.** Finniss (Cru); **36.** Daly (Cbu, Cru, Cbu × Cru); **37.** Victoria (Cbu). *Western Australia*: **38.** King Edward (Cbu); **39.** Wooramel (Cst); **40.** Yarra Yarra Lakes (Cst); **41.** Salt Lake (Cst); **42.** Swan Coast (Cob). *New Guinea*: **43.** Merauke (Cre, Csi); **44.** Binaturi (Cno, Csi); **45.** Aramia (Cno, Cpa); **46.** Kemp (Cpr). **47.** Roti Island, Indonesia (Cmc) is not shown.

Table 1. Described species and distinctive forms of *Chelodina* (Testudinata: Chelidae) from Australia, New Guinea and the island of Roti [10°45′S 123°10′E]. An initial reference, the drainage basins from which they were collected, and sample sizes are provided. Classification follows that of Cogger *et al*. (1983) and Rhodin (1994a,b), with the addition of *C. burrungandjii* (Georges & Adams, 1992; Thomson *et al*., 2000) and three undescribed forms identified by Cann (1998) and Thomson *et al*. (2000). *Chelodina kuchlingi* Cann (1997) is known from a single preserved specimen of uncertain origin, and was unavailable for study. Location numbers correspond to those used in Figure 1. Species groups are regarded as subgenera

Code	Species	Reference	Locations	Sample size
Species Group A				
Clo	*Chelodina longicollis*	(Shaw, 1794)	1–10, 12–15, 19	54
Cmc	*Chelodina mccordi*	Rhodin (1994b)	47	10
Cno (Aust)	*Chelodina novaeguineae*	(see Cann, 1998)	23–26, 29–30, 32	24
Cno (NG)	*Chelodina novaeguineae*	Boulenger (1888)	44–45	13
Cpr	*Chelodina pritchardi*	Rhodin (1994a)	46	9
Cre	*Chelodina reimanni*	Philippen & Grossman (1990)	43	11
Cst	*Chelodina steindachneri*	Siebenrock (1914)	39–41	17
Clo × Cno	*Chelodina* cf. *longicollis* [Don]	(see Cann, 1998)	22, 25	2
Species Group B				
Cbu	*Chelodina burrungandjii*	(Thomson *et al*., 2000)	33–34, 36	31
Csp	*Chelodina* cf. *rugosa* [Kimberley]	(Thomson *et al*., 2000)	37, 38	6
Cex	*Chelodina expansa*	Gray (1857)	3, 11–12, 15–21	33
Cpa	*Chelodina parkeri*	Rhodin & Mittermeier (1976)	45	6
Cru	*Chelodina rugosa*	Ogilby (1890)	27–29, 31, 34–36	45
Csi	*Chelodina siebenrocki*	Werner (1901)	43–44	12
Cno × Cru	*Chelodina* cf. *rugosa* [Gilbert]	see Cann (1998)	29	2
Species Group C				
Cob	*Chelodina oblonga*	Gray (1841)	42	7

With few exceptions, representative vouchers for the Australian specimens were lodged with museums and cross-referenced to tissue samples for future reference. Prior to 1990, most of the Australian specimens collected were returned to the laboratory alive where they were killed by intracranial injection of sodium pentobarbitone (Nembutal). Samples of liver, heart, muscle, kidney, whole blood and plasma were removed, immediately frozen by immersion in liquid nitrogen and stored at –70 °C prior to use. Only blood samples were available for Australian specimens collected since 1990 (most of the wild-caught animals used in this study) and for all specimens from the McCord's live collection.

The senior author (AG) and the third author (WMc) each examined all specimens drawn from the live collection at the East Fishkill Animal Hospital and identified them using original descriptions or published guides (Goode, 1967; Cogger, 2000). Representatives of each taxon were photographed to show diagnostic features, for future reference. The photographs are available on request. We are confident of the identity of the specimens bled in the live collection. Descriptions of the localities for these animals are accurate, but the precision of the locality data reflects uncertainty in their location of collection. In some cases, only a drainage basin could be given (e.g. Aramia River, PNG). In others, where the location was better known, or the drainage basin was very restricted, an approximate latitude and longitude is given (e.g. Merauke River, West Irian [*c*. 8°28′S 140°20′E]).

Allozyme analyses

As blood was the only tissue available for most animals, the allozyme study limited itself to loci expressed in this tissue. Fortunately, turtle blood displays a wider range of enzymes than does mammalian blood, and this limitation did not compromise the study. Blood lysates were screened electrophoretically on 'Cellogel' (M.A.L.T.A., Milan) using established techniques (Richardson *et al*., 1986). The principles used to assign mobility states, conduct allozymic interpretations and confirm electromorph identity are also detailed in Richardson *et al*. (1986). The enzyme products of 45 presumptive loci gave staining of sufficient intensity and resolution to be scored.

Budget and time restrictions meant that genetic characterization of every animal at all 45 loci could not be undertaken. Instead a two-stage process was

adopted, in line with standard practices employed for systematic revisions based on morphological assessments of a large number of specimens. In the overview stage, 6–10 individuals of each presumptive taxon were chosen from across the geographical range sampled. These individuals were analysed for the full 45 loci, allowing the provisional recognition of both taxa and loci yielding diagnostic characters. In Stage 2, all remaining animals were screened for all loci diagnostic for the putative taxon in the overview study plus all loci that were polymorphic for that taxon or group of related taxa. An important condition of this two-stage approach is that any animal found to be genetically anomalous using a subset of characters must then be examined for the full suite of characters. If such re-analysis leads to the recognition of additional taxa and therefore diagnostic characters, these additional characters must also be incorporated into to the second stage. In this study, all individuals not included in the overview stage were characterized at between 13 and 26 loci, chosen to critically test the provisional diagnosis obtained from morphology and assess the nature of within-taxon genetic variation; none required genetic re-assessment at additional loci.

Species delimitation

The first step towards delineating species involved the recognition of diagnosable taxa, defined herein as collections of populations whose individuals could all be distinguished from all individuals of other populations on the basis of unique allelic markers. To determine the diagnosable taxa, each local population (group of individuals collected at a particular site) was provisionally considered as a single biological population. If previous studies suggested that two morphologically distinguishable species were present in microsympatry, then the two distinguishable forms were regarded as separate biological populations. None of the resulting populations showed structure inconsistent with samples taken from a single panmictic population. Profiles of allele frequencies were determined for each population, and fixed differences between populations were tabulated. A fixed difference occurs between two populations when, at a given locus, the populations share no alleles. In practice, a fixed difference was scored at a given locus even when the two populations shared alleles, provided the cumulative frequency of all shared alleles was ≤5%. Such an operational definition is analogous with that used to identify diagnostic characters in morphological taxonomy (where characters are often not perfectly diagnostic for all individuals). Two populations were considered provisionally to belong to different diagnosable taxa if all individuals in one population could be distinguished from all of the individuals in the other by one or more fixed differences.

If two populations, two diagnosable taxa or a population and a diagnosable taxon shared all alleles at all presumptive loci, that is, lacked fixed differences, then they were considered to belong to the same diagnosable taxon and their profiles were combined. Note that by this procedure it is possible for two populations to differ by one or more fixed differences yet belong ultimately to the same diagnosable taxon. This occurs when, for example, the first population is fixed for allele *a*, the second population is fixed for allele *b* and a third population possesses both alleles. Following repeated application of this procedure to all populations and diagnosable taxa present at each step, the analysis yields either a single diagnosable taxon or two or more diagnosable taxa, each distinct from all others on the basis of one or more invariant characters.

There is a certain asymmetry in proof here. If two populations share alleles at all of their loci for the sample of individuals examined, then obviously the populations from which the individuals were taken share those alleles. The absence of fixed differences in the sample is sufficient to demonstrate absence of fixed differences between the populations, for the loci examined. Provided sufficient loci are examined, this is solid evidence that the populations are conspecific (notwithstanding the possibility of cryptic allozyme variation).

In contrast, an observed fixed difference between two populations may reflect either a true fixed difference or a sampling error, owing to the finite number of individuals examined. Statistical analysis is required to properly interpret fixed differences observed in electrophoretic studies of species boundaries. Unfortunately, no appropriate statistical test appears possible for any but the restrictive case of microsympatry, where a panmictic population can be assumed as the basis of a null hypothesis. In cases of allopatry and parapatry, formulation of a null hypothesis for statistical analysis requires knowledge of true allelic proportions for at least one of the populations being compared. Instead, we used a rule of thumb in the application of the above procedure to cases of allopatry and parapatry (Georges & Adams, 1996). We required at least two fixed differences when sample sizes were large (N_1 and $N_2 \geq 10$), and at least three fixed differences when sample sizes were small (N_1 or $N_2 < 10$) to regard populations as separate diagnosable taxa. This rule of thumb was applied only as a final step in the procedure, by which time diagnosable taxa included sufficient populations to raise sample sizes.

Applying this procedure to the overview dataset yielded an interim set of diagnosable taxa for the *Che-*

lodina. Diagnosable taxa identified in the overview stage that were marginal in their differentiation (i.e. less than four fixed differences from all other taxa), those that were drawn from a broad geographical range, or those that showed some indication of substructuring within, were then targeted as a series of subgroups in the second stage of the analysis. All additional individuals available from each of these subgroups were scored for a suite of appropriate diagnostic or polymorphic loci identified in the overview study, as separate analyses, and the process outlined above was repeated for each subgroup. In this way, a final set of diagnosable taxa was obtained, by refining the interim set obtained in the overview study.

Principal Co-ordinates Analysis (PCoA) (Gower, 1966) was applied using the computer package PATN (Belbin, 1993), using the complement of Roger's D (Rogers, 1972) as the measure of similarity. Results are presented in two dimensions, but where eigenvalues exceeded 1.0 for more than two dimensions, variation in these deeper dimensions was explored graphically to see if it would alter any conclusions drawn. In no case were the patterns observed in two dimensions substantially altered by viewing variation in deeper dimensions.

Enzymes examined

ACON, aconitate hydratase (Enzyme Commission No. 4.2.1.3); ACP, acid phosphatase (No. 3.1.3.2); ACYC, aminoacylase (No. 3.5.1.14); ADA, adenosine deaminase (No. 3.5.4.4); ADH, alcohol dehydrogenase (No. 1.1.1.1); AK, adenylate kinase (No. 2.7.4.3); ALB, albumin; CA, carbonate dehydratase (No. 4.2.1.1); CS, citrate synthase (No. 4.1.3.7); ENOL, enolase (No. 4.2.1.11); EST, esterases (No. 3.1.1); FUM, fumarate hydratase (No. 4.2.1.2); G6PD, glucose-6-phosphate dehydrogenase (1.1.1.49); GAPD, glyceraldehyde-3-phosphate dehydrogenase (No. 1.2.1.12); GLO, lactoylglutathione lyase (No. 4.4.1.5); GOT, aspartate aminotransferase (No. 2.6.1.1); GP, unknown plasma protein; GPI, glucose-phosphate isomerase (No. 5.3.1.9); GPX, glutathione peroxidase (1.11.1.9); GSR, glutathione reductase (No. 1.6.4.2); HB, haemoglobin; IDH, isocitrate dehydrogenase (No. 1.1.1.42); LAP, leucine amino-peptidase (No. 3.4.11.1); LDH, lactate dehydrogenase (No. 1.1.1.27); MDH, malate dehydrogenase (No. 1.1.1.37); ME, malic enzyme (No. 1.1.1.40); MPI, mannose-phosphate isomerase (No. 5.3.1.8); NDPK, nucleoside-diphosphate kinase (2.7.4.6); NP, purine nucleoside phosphorylase (No. 2.4.2.1); PEP-A, dipeptidase (val-leu, No. 3.4.13.11); PEP-B, tripeptidase (leu-gly gly, No. 3.4.11.4); PEP-D, prolidase (phe-pro, No. 3.4.13.9); PGAM, phosphoglycerate mutase (5.4.2.1); 6PGD, 6-phosphogluconate dehydrogenase (No. 1.1.1.44); PGK, phosphoglycerate kinase (No. 2.7.2.3); PGM, phosphoglucomutase (No. 2.7.5.1); PK, pyruvate kinase (No. 2.7.1.40); TPI, triose-phosphate isomerase (No. 5.3.1.1); XO, xanthine oxidase (No. 1.1.3.22). Conventions for naming loci and allozymes follow that of Adams *et al*. (1987). The electrophoretic phenotypes for HB were interpreted as if they were the products of a single locus. The f allozyme for *Acyc* displays no null activity in blood and reduced activity in liver; genotypes for *Acyc* have been determined assuming this null allele is present only in those taxa in which all individuals displayed the null phenotype.

Specimens examined

Chelodina expansa: Castlereagh River, Qld [31°43′S 148°39′E, *N* = 1]; Condamine–Culgoa Rivers, Qld [28°03′S 148°35′E, *N* = 1]; Fitzroy River, Qld [24°57′S 150°05′E, *N* = 1]; Fraser Island, Qld [25°20′S 153°10′E, *N* = 2], [25°27′S 153°04′E, *N* = 1]; Logan–Albert Rivers, Qld [28°13′S 153°02′E, *N* = 6]; Mary River, Qld [26°11′S 152°40′E, *N* = 2]; Murray River, NSW [36°05′S 146°56′E, *N* = 6], [36°06′S 147°00′E, *N* = 1]; Namoi River, NSW [30°45′S 150°43′E, *N* = 8], [28°44′S 151°59′E, *N* = 2]; South Pine River, Qld [27°22′S 152°56′E, *N* = 2]; Vouchers: QM J48005, J48014-5, J48018, J48020, J48032; AM R125066. *Chelodina longicollis*: Bega River, NSW [36°41′S 149°51′E, *N* = 2], [36°50′S 149°55′E, *N* = 1]; Broughton River, SA [33°36′S 138°23′E, *N* = 1]; Clarence River, NSW [33°36′S 138°23′E, *N* = 1]; Condamine–Culgoa Rivers, Qld [28°06′S 148°58′E, *N* = 1], [26°44′S 150°38′E, *N* = 1], [28°30′S 147°25′E, *N* = 5]; Fraser Island, Qld [25°13′S 153°10′E, *N* = 1]; Gwydir River, NSW [30°30′S 150°07′E, *N* = 3]; Hunter River, NSW [32°08′S 151°02′E, *N* = 2]; Jervis Bay, NSW [35°09′S 150°39′E, *N* = 4]; Macleay River, NSW [30°45′S 152°15′E, *N* = 3]; Millicent Coast, SA [36°16′S 140°04′E, *N* = 1]; Murray River, NSW/Vic [36°05′S 146°56′E, *N* = 3]; Murrumbidgee River, NSW/ACT [34°29′S 144°18′E, *N* = 2], [34°30′S 144°51′E, *N* = 1], [34°45′S 146°33′E, *N* = 1], [35°17′S 149°13′E, *N* = 7]; Namoi River, NSW [30°45′S 150°43′E, *N* = 2]; Nepean River, NSW [33°51′S 150°37′E, *N* = 8]; Severn River, Qld [28°48′S 151°50′E, *N* = 3]; Unspecified locality [*N* = 1]; Vouchers: SAM R46516, R49287; AM R123050, R123052-4, R12356-7, R123061; QM J48049. *Chelodina mccordi*: Roti Island, Indonesia [10°45′S 123°10′E, *N* = 10]. *Chelodina longicollis* × *novaeguineae* hybrids: Don River, Qld [20°01′S 148°15′E, *N* = 1]; Shoalwater Creek, Qld [22°55′S 150°08′E, *N* = 1]. *Chelodina novaeguineae* × *rugosa* hybrids: Gilbert River, Qld [18°17′S 143°33′E, *N* = 2]. *Chelodina novaeguineae*: Aramia River, PNG [*c*. 8°03′S 142°56′E, *N* = 10]; Binaturi River, PNG [*N* = 3]; Don River, Qld [20°01′S 148°15′E, *N* = 4]; Gilbert River,

Qld [18°17′S 143°33′E, *N* = 5]; Nicholson River, Qld [17°45′S 139°33′E, *N* = 1]; Proserpine River, Qld [20°24′S 148°35′E, *N* = 1]; Roper River, NT [16°08′S 133°36′E, *N* = 11]; Ross River, Qld [19°16′S 146°49′E, *N* = 1]; Styx River, Qld [22°21′S 149°32′E *N* = 1]; Vouchers: AM R135351. *Chelodina oblonga*: Swan River, WA [31°55′S 115°50′E, *N* = 7]; Vouchers: AM R125477. *Chelodina parkeri*: Aramia River, PNG [*N* = 6]. *Chelodina pritchardi*: Kemp River, PNG [*N* = 9]. *Chelodina reimanni*: Merauke River, West Irian [*c*. 8°28′S 140°20′E, *N* = 11]. *Chelodina rugosa* × *Chelodina burrungandjii* hybrids: Daly River, NT [14°15′S 132°38′E, *N* = 1]. *Chelodina rugosa*: Daly River, NT [14°05′S 131°13′E, *N* = 11]; Darwin Region, NT [12°27′S 130°50′E, *N* = 20]; Gilbert River, QLD [18°17′S 143°33′E, *N* = 3]; Jardine River, Qld [10°47′S 142°27′E, *N* = 1]; Mitchell River, Qld [*N* = 1]; Robinson River, NT [16°13′S 137°02′E, *N* = 4]; South Alligator River, NT [12°28′S 132°30′E, *N* = 5]; Vouchers: NTM R16334, R13430, R13434-5, R13437, R13439. *Chelodina seibenrocki*: Binaturi River, PNG [*N* = 2]; Merauke River, West Irian [*c*. 8°28′S 140°20′E, *N* = 10]. *Chelodina burrungandjii*: Daly River, NT [13°46′S 133°05′E, *N* = 9], [14°15′S 132°38′E, *N* = 12], [13°47′S 132°49′E, *N* = 4]; Liverpool River, NT [13°01′S 133°58′E, *N* = 1]; Mitchell River, WA [14°56′S 126°13′E, *N* = 2], [14°49′S 125°43′E, *N* = 4]; South Alligator River, NT [13°25′S 132°40′E, *N* = 4]; Victoria River, NT [15°45′S 129°37′E, *N* = 1]; Vouchers: NTM R13525, R16333, R22581-3; AM R13343-5. *Chelodina steindachneri*: Borodale Creek, WA [27°49′S 122°13′E, *N* = 2]; Kirkalocka River, WA [28°33′S 117°47′E, *N* = 6]; Wiluna, Salt Lake, WA [26°35′S 120°14′E, *N* = 1]; Wooramel River, WA [25°44′S 114°17′E, *N* = 8]; Vouchers: ANC R5058.

Museum abbreviations

AM, Australian Museum, Sydney; ANC, Australian National Wildlife Collection, CSIRO Gungahlin, Canberra; QM, Queensland Museum, Brisbane; NTM; Museums and Art Galleries of the Northern Territory, Darwin; SAM, South Australian Museum, Adelaide.

RESULTS

Species boundaries

The raw data obtained from the overview study comprised the allozyme genotypes from 102 specimens of *Chelodina* representing 45 populations overall. These data are summarized in Table 2 as allele frequencies for each diagnosable taxon (including the morphologically diagnosable *C. reimanni* plus the various geographical/putative forms of the *C. rugosa* group). Table 3 gives details of the subgroups that were subjected to further screening in Stage 2 of the analysis. The genotypic data for both the overview and various Stage 2 analyses are not presented due to considerations of space, but are available from the authors upon request.

It was clear from the outset that four individuals in the overview study and one in the Stage 2 analyses had hybrid origins. These specimens were omitted from the species boundary analyses. The analysis of their hybrid origins is presented separately, later in the results.

A matrix of fixed differences between the 45 populations was constructed using the raw allozyme data from the overview study and reduced to a matrix of differences between diagnosable taxa only by the procedure outlined in the Material and Methods. The following taxa were strongly supported as distinctive by this overview analysis: *Chelodina expansa* (6–20 fixed differences from other taxa, *N* = 8), *C. oblonga* (14–22 fd, *N* = 7), *C. parkeri* (6–24 fd, *N* = 6) and *C. steindachneri* (4–20 fd, *N* = 6). Additional specimens of *C. expansa* and *C. steindachneri*, the two taxa which were sampled from multiple localities across their entire range, were examined for a suite of diagnostic and polymorphic loci in Stage 2 (Table 3). The genetic profiles of these additional animals confirmed both their provisional identification and the utility of the diagnostic loci employed. Of the two taxa, only *C. expansa* showed any genetic substructuring across its range. Populations from the Murray–Darling drainage to the west of the Great Dividing Range showed some differentiation from those in coastal eastern Queensland (Fig. 2). However in the absence of any fixed differences, these two groupings could not be regarded as diagnosable taxa.

Among the remaining taxa, forms currently assigned *C. rugosa* and *C. siebenrocki* split into three interim diagnosable taxa that did not correspond with existing species boundaries. These three interim taxa were combined as one subgroup for further detailed analysis using all available specimens (Table 3). Similarly, forms currently assigned to *C. novaeguineae*, *C. reimanni*, *C. mccordi*, *C. pritchardi* and *C. longicollis* were combined as a separate subgroup (Table 3).

A summary of the number of fixed differences between taxa that were diagnosable at one or more loci is provided in Table 4. This table was constructed by integrating the overview analysis and the various subgroup analyses on the assumption that the monomorphic loci from the overview analysis that were not further analysed would have remained largely monomorphic (they certainly would have failed to become in any way diagnostic) had they been subject to greater scrutiny in the subgroup analyses.

Principal co-ordinates analysis (PCoA) applied to the *C. rugosa*/*seibenrocki* forms revealed three major

Table 2. Allozyme frequencies at 45 loci for all previously recognized forms of *Chelodina* examined in the overview study. Two additional loci, *Hb* and *Gp*, could not be scored across all taxa but were included in various Stage 2 analyses. Sample sizes for each OTU are presented in parentheses, with the first value representing the number included in the overview study and any second value representing the number subsequently examined in the Stage 2 analyses. Where a locus is polymorphic within a population, the frequency of each common allozyme is given as a percentage; the frequency of the rarest allozyme can be calculated by subtraction. Taxon abbreviations are given in Table 1

Locus	Cex (8; 36)	Clo (7; 54)	Cmc (6; 10)	Cno (Aus) (11; 24)	Cno (PNG) (5; 13)	Cob (7)	Cpa (6)	Cpr (6; 9)	Cre (6; 11)	Csp (4; 6)	Cbu (11; 31)	Cru (NT) (7; 40)	Cru (QLD) (5)	Csi (7; 12)	Cst (6; 17)
Acon1	b = 88; c	d	d	d = 90; e	d	d	f	d	d	b = 75; d	b = 86; d = 9; f	d = 71; f = 15; a	f = 50; d	d = 86; f	d
Acon2	c = 94; f	b = 71; e = 14; d = 8; a	b	b	b	b	b	c	b	b = 87; a	b = 64; a	b = 86; a	b	b = 86; c	b
Acp	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Acyc	e = 75; b	f	f	f	f	f	e	f	f	c = 50; e = 25; a = 13; d	c = 95; e	c = 50; e = 43; b	e = 50; c = 40; a	a = 64; b = 21; e	f
Ada	g = 75; i	c	b	a = 82; b	a = 50; b	f	g	a	b = 83; a	g	g	g = 86; h	g = 90; d	g	c = 83; e
Adh	b	b = 93; d	b	b = 73; c	b = 90; c	a = 92; b	b	b	b	b	b	b = 93; d	b = 70; c	c = 64; b	b = 67; c
Ak	a	a	a	a	a	b	a	a	a	a	a	a	a	a	b
Alb	a = 75; b	g	h	g = 95; h	h	c = 93; d	d	f	h	d	d	d	d	d	e
Ca1	a = 81; c	b = 97; e	b	b	b	d	g	b	b	b	b	c = 93; d	c = 50; b = 40; d	b = 93; c	f
Ca2	b	a	a	a	a	b	b	a	a = 92; b	b	b	b = 50; c	b	b	a
Cs	b = 94; a	c = 57; b	b	b	b	c	b	b	b	b	b	b = 93; c	b	b	b
Enol	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Est	c	c = 93; a	b	b	b	c	c	c	b	c	c	c	c	c	c
Fum	a	a	a	a	a	a	a	a	a	a	a	a	a = 70; b	a = 79; b	a
G6pd	a	a	a	a	a	b	b	a	a	b	b = 86; a	a	a	a	a
Gapd	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Glo	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b = 75; a
Got1	b = 94; d	b = 93; e	b	b	b	a	c	b	b	b	b	b	b	b	b

Got2	c		c = 71; a	c	c = 95; b	c	e	c	c	c = 83; b	c	c	c	c	c = 93; d	c
Gpi	b		b	b	b	b	c	b	b	b = 83; a	b	b	b = 93; a	b	b	b
Gpx	a		a	a	a	a	a	a	a	a	a	a	a	a	a	a
Gsr	c		c	c	c	c = 90; a	c	c	c	c	b	b = 82; c	b = 43; c = 29; d = 21; a	c = 80; b = 10; d	c = 64; b	c
Idh	c = 88; d	b	b	c = 54; b = 32; a	a	b	b	c	a = 83; b	b = 75; c	b = 86; c	c = 71; b	b	b	b = 67; c
Lap	b	a	a	a	a	a	b	a	a	b	b	b	b	b	b	a
Ldh1	c = 94; a	d	d	d = 86; e	d	b = 79; e	c	d	d	c	c	c	c	c	c	d
Ldh2	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b = 79; a	b
Mdh1	b	b	b	b	b	b = 86; d	c	b	b	c	c = 95; a	c	c	c	c	b
Mdh2	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Me1	b	a = 79; b	b	b	b	a = 79; d	d	b	b = 92; d	d = 75; c	d	d = 71; b	d = 90; c	d = 86; c	d = 86; c	b
Me2	b	b	b	b	b	c	c	b	b	c	c	c = 93; d	c	c	c	b
Mpi	b	b	b	b	b	b = 86; c	a	b	b	b	b	b	b	b	b	b
Ndpk	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Np	g = 44; j = 31; i = 19; c	f = 86; b	b	b = 41; d = 32; f = 14; a = 9; h	a = 80; b	b	g	b = 83; e	a = 83; b	g = 75; j	g	g	g	g	g	b = 83; a
PepA	c	c	b	b = 91; c	b	d = 93; f	c	b = 92; a	b	e	e	h = 86; g	e	e	e	b
PepB	b = 94; c	b = 86; c	b	b = 55; c	b = 90; c	b = 86; d	c	b	b = 67; a = 25; c	b	b	b	b	b	b	b
PepD1	d	b	b	b	b	d = 93; e	c	b	b = 92; a	d	d	b = 79; d	d = 90; b	d	b	b
PepD2	c	c	c	c	c	c	c = 92; b	c	c	c	c	c	a = 50; c	c = 64; a	c	c
Pgam	b	a	a	a = 95; c	a	b	a	a	a	a	a	a	a	a	a	a
6Pgd	b	b	b	b	b	b = 93; c	b	b	b	b	b	b = 95; a	b = 79; a	b	b	b = 92; c
Pgk	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Pgm1	c	c = 71; b	b	b = 91; c	b	c	b	b	b	c	c = 95; a	c = 93; a	c	c	b = 83; c	c
Pgm2	d	d	e	d	d = 90; e	d = 93; c	d	d	d	d	d	d = 79; a	d = 80; b	d = 86; b	d = 92; b	b
Pk	b	a = 93; b	a	a	a	b = 93; c	b	a	a	b	b	b	b	b	b	b
Tpi	a	a	b	a	a	a	a	a	a	a	a	a	a	a	a	a
Xo	a	a	a	a	a	c	b = 92; c	a	a	c = 75; a	c = 82; a	c	c	c	c	a

Table 3. Subsets of taxa and allozyme loci examined in the Stage 2 analysis. Species abbreviations are as for Table 1

Subgroup	No. loci	Loci examined
Cex (extra $N = 28$)	17	*Acon1*, *Acon2*, *Acyc*, *Ada*, *Alb*, *Ca1*, *Ca2*, *Est*, *Got1*, *Got2*, *Gp*, *Hb*, *Idh*, *Ldh1*, *Ldh2*, *Np*, *PepB*
Cst (extra $N = 11$)	13	*Ada*, *Adh*, *Alb*, *G6pd*, *Glo*, *Gp*, *Hb*, *Idh*, *Np*, *PepB*, *6Pgd*, *Pgm1*, *Pgm2*
Cru/Cbu/Csp/Csi (extra $N = 60$)	24	*Acon1*, *Acon2*, *Acyc*, *Ada*, *Adh*, *Ak*, *Ca1*, *Ca2*, *Fum*, *G6pd*, *Gpi*, *Gsr*, *Hb*, *Idh*, *Me1*, *Me2*, *Np*, *PepA*, *PepD1*, *PepD2*, *6Pgd*, *Pgm1*, *Pgm2*, *Xo*
Clo/Cno/Cpr/Cre/Cmc (extra $N = 80$)	26	*Acon1*, *Acon2*, *Ada*, *Adh*, *Alb*, *Ca1*, *Ca2*, *Cs*, *Est*, *Got1*, *Got2*, *Gp*, *Gpi*, *Hb*, *Idh*, *Ldh*, *Me1*, *Me2*, *Np*, *PepA*, *PepB*, *Pgam*, *Pgm1*, *Pgm2*, *Pk*, *Tpi*

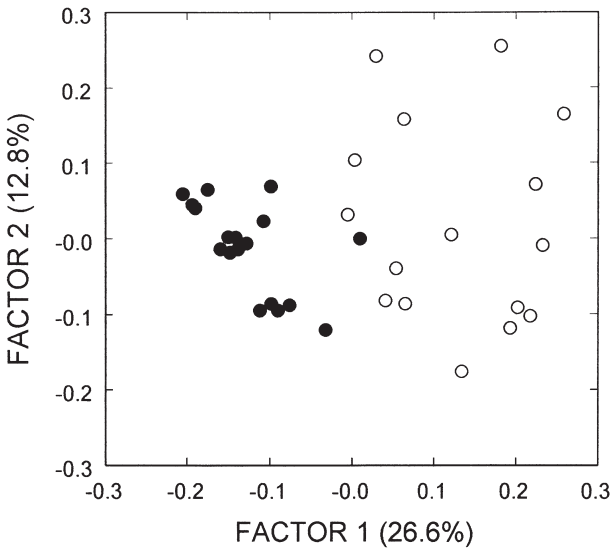

Figure 2. Principal co-ordinates analysis applied to a matrix of Roger's D genetic distances between all individuals of *Chelodina expansa*. The plot shows a degree of differentiation between coastal Queensland forms (Albert River in the south to Fitzroy–Dawson River in the north, including Fraser Island) (○) and those of the Murray-Darling system (●), but these differences have not moved to fixation at any locus (45 loci).

groupings (Fig. 3). Specimens from the Kimberley and Arnhem Land formed one group, corresponding to *C. burrungandjii* (= *Chelodina* sp. aff. *rugosa* (Mann] of Georges & Adams (1992)). A second group comprised lowland forms of *C. rugosa* from the Northern Territory east to the Macarthur River in the Gulf of Carpentaria. The third group comprised lowland forms of *C. rugosa* from Cape York (the Type locality), rivers of the eastern Gulf of Carpentaria in Queensland, and *C. siebenrocki* of New Guinea. These forms are very closely related, differing by only one fixed difference in each instance which, in cases of allopatry, is insufficient to establish a diagnostic taxon by our rule of thumb on sample size and statistical significance. Under this rule, there is insufficient evidence to regard *C. rugosa* from the Northern Territory, *C. rugosa* from Queensland, and *C. siebenrocki* from New Guinea as more than a single diagnosable taxon. The Kimberley and Arnhem Land forms are a single diagnosable taxon which is in sympatry with *C. rugosa* in the Victoria, Daly and South Alligator rivers of the Northern Territory (only the latter two drainages are included in our sampling of *C. rugosa*). One fixed difference and the level of divergence illustrated in Fig. 3 (equivalent to Rogers D = 0.16, reflecting the additional presence of several near-fixed differences) in sympatry is regarded as sufficient evidence of two discrete taxa, particularly as the two taxa are also morphologically diagnosable (Thomson *et al*., 2000).

PCoA applied to the second subunit of taxa (*C. novaeguineae* and related species or forms) revealed three distinctive groupings of populations that corresponded to the recognized taxa *C. mccordi*, *C. pritchardi* and *C. longicollis* (Fig. 4). Each differed from the other by 6–8 fixed differences and so represent clear diagnosable taxa. Two additional groupings corresponded to the Australian *C. novaeguineae* vs. a cluster comprising *C. reimanni* plus New Guinea *C. novaeguineae*. These groupings differed from *C. mccordi*, *C. pritchardi* and *C. longicollis* by three fixed differences in each instance, but from each other by only one fixed difference. Again, this is insufficient evidence in allopatry to establish separate diagnosable taxa using the allozyme data alone.

HYBRIDIZATION

Two forms of *Chelodina*, initially thought to be distinct species (Cann, 1998), appear to be hybrids based on their allozyme profiles. The first is a morphologically distinctive form from the Fitzroy–Dawson drainage on the boundary of the distributions of *C. longicollis* and *C. novaeguineae*. It is a large animal, with shell attributes most closely resembling *C. novaeguineae*

Table 4. Raw fixed differences (lower matrix) and Roger's D (upper matrix) for all forms of *Chelodina* that were diagnosable at one or more loci in our sample of populations. Species abbreviations are as for Table 1

Taxon	Cex	Clo	Cmc	Cno (Aus)	Cno (PNG)/Cre	Cob	Cpa	Cpr	Cbu	Cr (NT)	Cru (Qld)/Csi	Cst
Cex	–	0.36	0.43	0.37	0.39	0.43	0.33	0.32	0.26	0.27	0.28	0.37
Clo	14	–	0.24	0.19	0.21	0.42	0.45	0.21	0.40	0.38	0.38	0.21
Cmc	20	7	–	0.15	0.14	0.51	0.53	0.18	0.46	0.43	0.44	0.20
Cno (Aus)	15	3	3	–	0.10	0.49	0.49	0.14	0.43	0.39	0.41	0.18
Cno (PNG)/Cre	18	5	3	1	–	0.50	0.51	0.17	0.43	0.39	0.42	0.19
Cob	19	15	22	18	20	–	0.48	0.50	0.43	0.46	0.42	0.41
Cpa	13	17	24	19	21	18	–	0.51	0.23	0.27	0.25	0.48
Cpr	15	6	8	4	5	21	23	–	0.42	0.38	0.42	0.17
Cbu	7	12	17	14	15	14	7	15	–	0.16	0.13	0.42
Cru (NT)	7	12	17	14	15	13	7	15	1*	–	0.13	0.37
Cru (Qld)/Csi	8	13	18	15	16	15	7	16	1	1	–	0.39
Cst	15	4	8	6	6	16	20	6	14	13	15	–

*Comparison in sympatry, but not microsympatry.

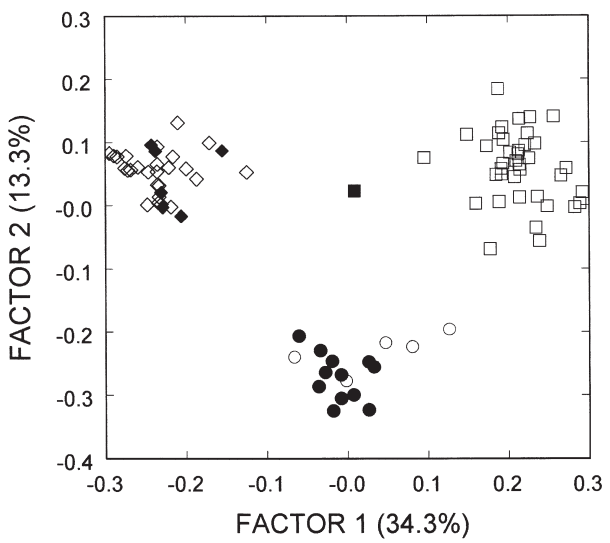

Figure 3. Principal co-ordinates analysis applied to a matrix of Roger's D genetic distances between all individuals of *Chelodina rugosa* (Queensland form) (○), *C. rugosa* (Northern Territory form) (□), *C. siebenrocki* (●), *Chelodina burrungandjii* (◇) and an undescribed form from the Kimberley plateau of Western Australia (◆). A hybrid between *Chelodina burrungandjii* and *C. rugosa* (Northern Territory form) is included (■). Three groups are evident. *Chelodina burrungandjii* and the undescribed form from the Kimberley plateau are undifferentiated and probably represent a single taxon. *Chelodina seibenrocki* and the Queensland form of *C. rugosa* are undifferentiated, and also probably represent a single taxon. The Northern Territory form of *C. rugosa* represents the third group. These three groups differ each by only one fixed difference.

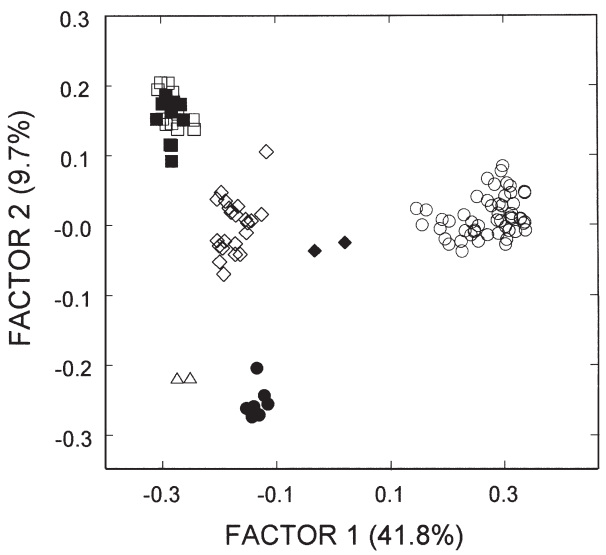

Figure 4. Principal components analysis applied to a matrix of Roger's D genetic distances between all individuals of *Chelodina longicollis* (○), *C. mccordi* (△), *C. pritchardi* (●), *C. novaeguineae* (New Guinea form) (■), *C. novaeguineae* (Australian form) (◇) and *C. reimanni* (□). Two individuals which are hybrids between *C. longicollis* and *C. novaeguineae* (Australian form) are included (◆). Five groups are evident. *Chelodina longicollis*, *C. mccordi* and *C. pritchardi* are well differentiated. *Chelodina novaeguineae* (New Guinea form) and *C. reimanni* are undifferentiated and may represent a single taxon (but see text). The Australian and New Guinea forms of *C. novaeguineae* are clearly differentiated, but only one difference has moved to fixation.

and external attributes of the head and jaws most closely resembling *C. longicollis* (see Cann, 1998, 98–99, for photographs and an historical account of its discovery). The two individuals we obtained of this form appear to be hybrids between *C. longicollis* and *C. novaeguineae* (Table 5). Both animals displayed the heterozygous genotypes expected for a *C. longicollis* × *C. novaeguineae* F_1 cross at the three loci (*Ada*, *Est* and *Hb*) that were unequivocally diagnostic for these two species. In addition, a comparison of their allozyme profiles at the other 42 loci found only two loci displaying any allele not detected in one or both parents, and in both cases (*Me-2*^{a} and *PepA*^{f}) these were unique alleles and present only in the heterozygous state. Independent support for their F_1 hybrid status can be seen from the PCoA of this subgroup, which places them intermediate between the *C. longicollis* and Australian *C. novaeguineae* clusters (Fig. 4).

The second instance of hybridization involves two species that are quite distant phylogenetically, *C. novaeguineae* and *C. rugosa* (see Fig. 7), and may one day be placed in separate genera (Legler, 1981). It involves two specimens collected from the Gilbert River, near Georgetown in Queensland. The first specimen is morphologically distinctive, possessing an admixture of attributes from each of *C. novaeguineae* and *C. rugosa*, but it too was initially regarded as a separate species (see Cann, 1998, 96 for photographs). The allozyme profile obtained for this individual clearly demonstrates its status as an F_1 hybrid between *C. novaeguineae* and *C. rugosa*, as it displays the heterozygous genotypes expected for an F_1 hybrid at all of the 14 loci that display absolute or effective fixed differences between the parental species, and no genotypic inconsistencies at any other locus (Table 5).

Unlike the previous case of hybridization between *C. longicollis* × *C. novaeguineae*, the large number of diagnostic markers available to distinguish *C. novaeguineae* from *C. rugosa* allow us to distinguish the allozyme profiles expected for an F_1 hybrid vs. those which would characterize a second generation hybrid (e.g. F_2, F_1 backcrossed with *C. novaeguineae*, F_1 backcrossed with *C. rugosa*, etc.). Thus an F_1 hybrid would be heterozygous at all 14 diagnostic markers, whereas a backcross animal will on average be heterozygous for only half of the diagnostic loci, displaying a genotype expected for one of the parental species at the remaining diagnostic loci. A second specimen from the Gilbert River, initially assigned to *C. novaeguineae* in the field, provides strong evidence of backcrossing between hybrid individuals and the parent species *C. novaeguineae*. Of the 14 key diagnostic loci, this individual displayed an expected F_1 genotype at seven loci and an expected *C. novaeguineae* genotype at the remaining seven loci (Table 5). The genotypes at the other 31 loci were also in line with the hypothesis that this individual has resulted from a genetic cross between an F_1 (*C. novaeguineae* × *C. rugosa*) and *C. novaeguineae*. The allozyme data therefore provide direct evidence for the partial-fertility of F_1 hybrids, opening up the possibility of introgression between these distantly related species.

The third instance of hybridization involves *C. rugosa* and *C. burrungandjii*, occasionally sympatric in the Northern Territory. These two species are genetically similar, displaying a single fixed difference plus major differences in allele frequency at several other loci. PCoA for the *C. rugosa* subgroup (Fig. 3) revealed a single individual from the Katherine River which was genetically intermediate between *C. rugosa* and *C. burrungandjii*, and the allozyme profile of this individual is consistent with it being an F_1 hybrid between the two species (Table 5). The individual concerned displayed the heterozygous genotype expected for an F_1 at the diagnostic locus *Ca1* and the nearly diagnostic loci *Acon1* and *G6pd*, plus no genotypic inconsistencies at any other locus. Nevertheless, given the lack of multiple fixed differences between the parental species, our data cannot rule out alternative scenarios involving second-generation hybridization events for this animal, nor can they determine whether introgression is occurring between these two species. Such an analysis would require additional genetic markers and detailed survey of populations in sympatry, parapatry and allopatry across several drainages.

Phylogenetic reconstruction

Figure 5 shows a neighbour-joining tree applied to the matrix of Roger's D distances (Rogers, 1972) of Table 4. It did not differ topologically from the maximum likelihood Fitch–Margoliash or in any substantial way from trees generated using percentage fixed differences as the distance measure. The tree is shown as an unrooted network because without an outgroup for this study, it was not possible to root the tree using data presented here. However, a number of previous molecular studies that have included *Chelodina* have been unanimous in placing the root on the branch between *C. oblonga* and *C. expansa* (Georges & Adams, 1992; Seddon *et al*., 1997; Georges *et al*., 1998).

DISCUSSION

Species delimitation

The genus *Chelodina* comprises a number of taxa that are diagnosable using allozyme profiles, most of which

Table 5. Parental allele frequencies and hybrid genotypes at the 21 loci where the percentage difference between any two parental taxa is greater than 50%. Each of the two alleles displayed by a hybrid animal is indicated with a cross (+). Parental profiles for Clo and Cno have largely been taken from the overview study (Table 2; data for *Hb* from Stage 2) whereas those for Cru (combining the NT and Qld populations) and Cbu (NT populations only) were obtained from the Stage 2 analyses. None of the 24 loci not presented show genotypes inconsistent with a hybrid origin. An asterisk indicates that the presence of the null allele *Acyc*^{f} has been assumed, given the observed electrophoretic phenotype (the same mobility as allozyme *e* but a much-reduced activity). Superscripts are used to indicate which loci are diagnostic for the various parental taxa: ^{A}Clo vs. Cno; ^{B}Cno vs. Cru; ^{C}Cru vs. Cbu (or effectively diagnostic if Cru is also represented by the NT populations only). (na = genotype was not determined)

Locus allele	**Parent Clo**	Clo × Cno	Clo × Cno	**Parent Cno**	Cno × Cru	Cno × (Cno × Cru)	**Parent Cru**	Cru × Cbu	**Parent Cbu**
Acon1									
f					+		**19**		**5**
e				**10**					
d	**100**	+ +	+ +	**90**	+	+ +	**79**	+	**5**
b								+	**90**
a							**2**		
Acyc^{B}									
f	**100**	+ +	+ +	**100**	+*	+*			
e					+	+	**38**		**2**
c							**44**	+	**93**
b							**4**		**2**
a							**14**	+	**3**
Ada^{A,B}									
h							**4**		
g					+	+	**95**	+ +	**100**
d							**1**		
c	**100**	+	+						
b		+		**18**					
a			+	**82**	+	+			
Alb^{B}									
h				**5**	+				
g	**100**	+ +	+ +	**95**		+			
d					+	+	**100**	na	**100**
Ca1^{B,C}									
e	**7**								
d						+	**8**		
c					+		**87**	+	**3**
b	**93**	+ +	+ +	**100**	+	+	**4**	+	**97**
a							**1**		
Ca2^{B}									
c							**19**	+	
b					+	+	**81**	+	**100**
a	**100**	+ +	+ +	**100**	+	+			
Cs									
c	**57**		+				**4**		
b	**43**	+ +	+	**100**	+ +	+ +	**96**	na	**100**
Est^{A,B}									
c	**93**	+	+		+		**100**	na	**100**
b		+	+	**100**	+	+ +			
a	**7**								
G6pd									
b								+	**91**
a	**100**	+ +	+ +	**100**	+ +	+ +	**100**	+	**9**
Hb^{A}									
d							**19**	+	**83**
c	**100**	+	+				**12**		**17**

Table 5. (*Continued*)

Locus allele	**Parent Clo**	Clo × Cno	Clo × Cno	**Parent Cno**	Cno × Cru	Cno × (Cno × Cru)	**Parent Cru**	Cru × Cbu	**Parent Cbu**
b							**2**		
a		+	+	**100**	+ +	+ +	**67**	+	
Idh									
c		+		**54**		+	**62**	+	**5**
b	**100**	+	+ +	**32**	+ +		**38**	+	**95**
a				**14**		+			
Lap^{B}									
b					+		**100**	na	**100**
a	**100**	+ +	+ +	**100**	+	+ +			
Ldh1^{B}									
e				**14**					
d	**100**	+ +	+ +	**86**	+	+ +			
c					+		**100**	na	**100**
Mdh1^{B}									
c					+		**100**	na	**95**
b	**100**	+ +	+ +	**100**	+	+ +			
a									**5**
Me1									
d					+		**93**	+ +	**98**
c							**1**		**2**
b	**21**	+ +	+ +	**100**	+	+ +	**6**		
a	**79**								
Me2^{B}									
d							**1**		
c					+		**99**	+ +	**100**
b	**100**	+	+ +	**100**	+	+ +			
a		+							
Np^{B}									
h				**4**					
g					+	+	**100**	+ +	**100**
f	**86**	+	+	**14**		+			
d				**32**					
b	**14**	+	+	**41**					
a				**9**	+				
PepA^{B}									
i							**2**		
h							**78**		**13**
g							**7**		
f		+							
e					+		**13**	+ +	**87**
c	**100**	+	+ +	**9**					
b				**91**	+	+ +			
Pgm1									
d							**1**		
c	**71**			**9**	+		**98**	+ +	**98**
b	**29**	+ +	+ +	**91**	+	+ +			
a							**1**		**2**
Pk^{B}									
b	**7**				+	+	**100**	na	**100**
a	**93**	+ +	+ +	**100**	+	+			
Xo^{B}									
c					+		**100**	+ +	**92**
a	**100**	+ +	+ +	**100**	+	+ +			**8**

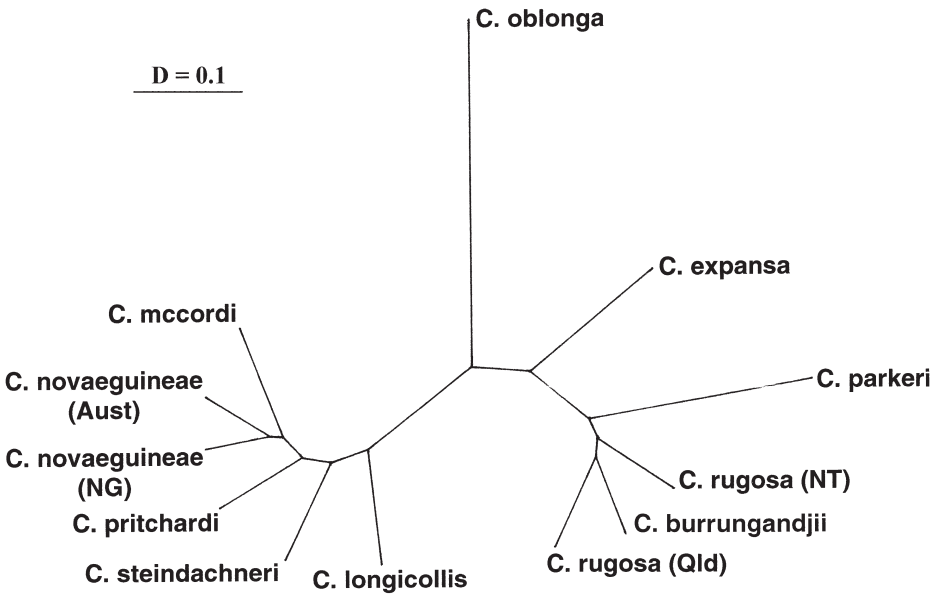

Figure 5. An unrooted neighbour-joining tree showing the phylogenetic relationships among taxa that were diagnosable in our sample. This includes forms whose diagnosis was tentative: that is, involving one fixed difference in allopatry.

correspond to described species. *Chelodina expansa*, *C. parkeri*, *C. oblonga*, *C. longicollis*, *C. steindachneri*, *C. pritchardi* and *C. mccordi* are each supported as single, well-defined diagnosable taxa. *Chelodina burrungandjii* from Arnhem Land (including the undescribed form from the Kimberley) is sufficiently differentiated from *C. rugosa* in sympatry to regard it as a diagnosable taxon. *Chelodina novaeguineae* from New Guinea and Australia are weakly differentiated, and by our rule of thumb on statistical significance, insufficiently so in allopatry to declare the two as separate diagnosable taxa. *Chelodina reimanni* also groups within this single diagnosable taxon, on the electrophoretic evidence. Similarly, *C. rugosa* from Queensland, *C. rugosa* from the Northern Territory and *C. siebenrocki* are weakly differentiated in allopatry, and are regarded as a single diagnosable taxon.

Whether these diagnosable taxa should be regarded as biological species is a difficult question (Georges & Adams, 1996), particularly given the evidence of hybridization within *Chelodina*. Our approach has been to regard the diagnosable taxa as phylogenetic species, by definition (Nixon & Wheeler, 1990), and to regard them as biological species by inference (Mayr, 1969 : 197; Richardson *et al*., 1986 : 51) with the weight given to evidence depending on the context: sympatry, parapatry or allopatry (Georges & Adams, 1996). In cases of sympatry, evidence of status as separate biological species is direct; fixed allozyme differences between two forms or substantial genetic divergence without genetic intermediates are direct evidence of reproductive incompatibility. When turtle populations occupy adjacent drainages (parapatry, or shallow allopatry), there is presumably opportunity over time for exchange of individuals between populations, so fixed differences are also regarded as evidence of reproductive incompatibility.

In cases of allopatry, the biological species concept is particularly problematic, as has long been recognized. It depends theoretically on the notion of reproductive incompatibility but, in practice, a decision needs to be made as to whether the observed level of genetic divergence (whether assessed via morphological or molecular data) among allopatric forms is great enough for the two forms to be regarded as reproductively incompatible. For *C. oblonga* (>28.9% fixed differences from all other diagnosable taxa), *C. expansa* (>15.6%) and *C. parkeri* (>15.6%), levels of divergence are sufficiently great to support inference of reproductive incompatibility (after Georges & Adams, 1996). For *C. longicollis* (>6.7%), *C. steindachneri* (>8.9%), *C. pritchardi* (>8.9%) and *C. mccordi* (>6.7%), levels of divergence are sufficient not to seriously challenge their current status as biological species. We therefore regard these each as good biological species.

The allozyme data for species currently regarded as *C. rugosa*, *C. siebenrocki*, *C. novaeguineae* and *C. reimanni* require more detailed interpretation. *Chelodina rugosa* (Ogilby, 1890: Type locality Cape York) and *C. siebenrocki* (Werner, 1901: New Guinea) have a long and confused history, compounded by conflicting opinion on the allocation of specimens to *C.*

oblonga (Gray, 1841) (see Thomson (2000) for a reinterpretation of *C. oblonga*). *Chelodina rugosa* and *C. seibenrocki* have been variously regarded as distinct species (Wermuth & Mertens, 1961; Burbidge *et al*., 1974; Cogger *et al*., 1983) or the same (Siebenrock, 1909, 1915; Goode, 1967). Morphological evidence in support of their separation is scant. Burbidge *et al*. (1974 : 393) cite only 'consistent, if minor, differences' between the two as their basis for recognizing them as separate species. Rhodin & Mittermeier (1976) failed to reliably distinguish *C. rugosa* and *C. siebenrocki* following a morphological analysis, and chose to refer to them collectively as the *C. rugosa* complex. In the absence of any substantive diagnostic morphological differences and any fixed allozyme differences, we synonymise *C. siebenrocki* with *C. rugosa* (the latter has precedence). *Chelodina rugosa* is one of three species whose distribution in Australia and New Guinea was once contiguous, centred on Lake Carpentaria, now the Gulf of Carpentaria, with Australian populations separated from those of New Guinea only when the sea levels rose again approximately 12 000 years ago (Torgersen *et al*., 1988). *Chelodina novaeguineae* is a second such species, and *Emydura subglobosa* is a third (Georges & Adams, 1996).

The Australian forms of *C. rugosa* and the closely related *C. burrungandjii* show some differentiation, but as this differentiation is not great its interpretation depends upon the context in which it is detected (sympatry, parapatry or allopatry) (Georges & Adams, 1996). The presence of one fixed difference and the level of differentiation evident in Figure 3 (D = 0.16) between *C. burrungandjii* and *C. rugosa* of the South Alligator and Daly River systems is sufficient evidence *in sympatry* to support the designation of two biological species. The two are in contact along the escarpment, a hybrid individual was detected in our analyses, yet the genetic differentiation is maintained. They are morphologically distinct (Thomson *et al*., 2000). In contrast, our electrophoretic evidence indicates that the recently described *C. burrungandjii* has a distribution in the sandstone plateau country of both Arnhem Land and the Kimberley, not just Arnhem Land as proposed by Thomson *et al*. (2000).

A similar level of differentiation occurs between the populations of *C. rugosa* in the Northern Territory and those of *C. rugosa* of Queensland and New Guinea (including *C. siebenrocki*) (D = 0.13, 1 fixed difference, 2.2%). Unfortunately, we lacked samples from the intervening rivers discharging into the southern coast of the Gulf of Carpentaria, and the single fixed difference may not have been sustained had we collected there. One fixed difference in allopatry is regarded as insufficient to establish the two as clearly diagnosable taxa (see Material and Methods), and certainly insufficient in allopatry to infer that they are biological species. Divergence of 2.2% is well within that expected of populations of the same species (0–9%, Georges & Adams, 1996) and outside the range of percent fixed differences typical between species in the same genus. In the absence of any concurrent morphological evidence, we regard the lowland form of *C. rugosa* from the Northern Territory, the form of *C. rugosa* of Queensland, including the type locality, to be a single taxon.

Similar difficulties occur in the definition of *C. novaeguineae* and *C. reimanni*. *Chelodina novaeguineae* from Australia and New Guinea differ by only one fixed difference and a Roger's D of 0.10 in strict allopatry, insufficient evidence to regard them as anything other than a single taxon. These genetic data are not surprising by themselves, but do raise some intriguing questions when *C. reimanni* is included in the comparison. *Chelodina reimanni* and the PNG population of *C. novaeguineae* not only show no fixed differences but actually share common alleles at all loci. These two taxa are morphologically distinct (Philippen & Grossman, 1990; Rhodin, 1994a) and have geographical distributions which abut close to the border between Papua New Guinea and Irian Jaya (Rhodin, 1994a), suggesting that they may occur in parapatry or even sympatry. The allozyme data are consistent with any of three possible explanations. It may be that *C. reimanni* and *C. novaeguineae* have speciated very recently (from an ancestral population in PNG which was nevertheless already genetically distinctive from Australian populations of *C. novaeguineae*), providing insufficient time to accumulate genetic differences that are detectable by allozyme analysis. Alternatively, the morphological differences associated primarily with the jaws and triturating surfaces may be environmentally induced, in response to a molluscivorous diet (Rhodin, 1994a), as is suspected in macrocephalic individuals of *Emydura* (Legler, 1981). A third possibility is that the Australian and non-Australian forms are separate species, diagnosable at one fixed difference, in which case the morphological character(s) apparently diagnostic for *C. reimanni* actually represent within-species variation in the PNG species. The status and relationships of *C. reimanni* and *C. novaeguineae* clearly require further scrutiny.

In summary, we recognize 12 species of *Chelodina* (Table 6). *Chelodina reimanni* is recognized on the basis of published morphological evidence only (Philippen & Grossman, 1990) and *C. kuchlingi*, unavailable for electrophoretic comparisons, has been described from a single specimen of uncertain origin (Cann, 1997). Both deserve further scrutiny. So too does the distinction between *C. novaeguineae* of Australia and New Guinea, and between *C. rugosa* of

Table 6. A list of recognized species of *Chelodina* (Testudinata: Chelidae) from Australia, New Guinea and the island of Roti. *Chelodina rugosa* is synonymised with *C. siebenrocki; C. burrungandjii* incorporates the form from the Kimberley Plateau of Western Australia tentatively regarded by Thomson *et al*. (2000) as a distinct species. *Chelodina reimanni* is recognized on the basis of published morphological evidence only (Philippen & Grossman, 1990), being indistinguishable from *C. novaeguineae* using electrophoresis. Common names are after those of Cogger (2000) and Iverson (1992). *Chelodina kuchlingi* is known from a single preserved specimen of uncertain origin, and was unavailable for study. Species groups are regarded as subgenera

Species	Reference	Common name
Species Group A		
Chelodina longicollis	(Shaw, 1794)	Eastern snake-necked turtle
Chelodina mccordi	Rhodin (1994b)	Roti Island snake-necked turtle
Chelodina novaeguineae	Boulenger (1888)	New Guinea snake-necked turtle
Chelodina pritchardi	Rhodin (1994a)	Pritchard's snake-necked turtle
Chelodina reimanni	Philippen & Grossman (1990)	Reimann's snake-necked turtle
Chelodina steindachneri	Siebenrock (1914)	Helmet-shelled turtle
Species Group B		
Chelodina burrungandjii	(Thomson *et al*., 2001)	Sandstone snake-necked turtle
Chelodina expansa	Gray (1857)	Broad-shelled river turtle
Chelodina kuchlingi	Cann (1997)	Kuchling's snake-necked turtle
Chelodina parkeri	Rhodin & Mittermeier (1976)	Parker's snake-necked turtle
Chelodina rugosa	Ogilby (1890)	Northern snake-necked turtle
Species Group C		
Chelodina oblonga	Gray (1841)	Oblong turtle

Queensland (including *C. siebenrocki*) and *C. rugosa* of the Northern Territory.

HYBRIDIZATION

Hybridization presents particular problems for any species concept, as substantial gene flow between taxa acts against their divergent evolution and blurs their separation as discrete entities. Traditionally, hybridization is interpreted as contributing to a final stage in speciation, whereby introgression leads to reduced fitness and positive selection for traits limiting further hybridization (Dobzhansky, 1940, 1970). When two diagnosable taxa are in long-standing allopatry, the possibility exists that despite substantial genetic divergence, reproductive incompatibility may not be fully effected, the final stage of reproductive character displacement requiring a period in sympatry. Our results demonstrate that reproductive compatibility, a pleisiomorphic trait, can persist for substantial periods in a phylogeny. *Chelodina rugosa* and *C. novaeguineae* are distant phylogenetically (see below), and may be placed in separate genera (Legler, 1985), yet they hybridize in the Gulf region of Queensland with evidence of introgression. *Chelodina longicollis* and *C. novaeguineae* are not sister taxa, yet they hybridize in central coastal Queensland. In practice, all species concepts must be relaxed to include the possibility that species can form natural hybrids (Arnold, 1997 : 13), and that those hybrids can be partially fertile. We do not regard these instances of natural hybridization to demand modification of the list of species that we have identified in the present study (Table 6).

The existence of hybridization raises a number of other issues however. First of all, it serves to highlight the value of nuclear molecular markers in studies of species boundaries, for without the electrophoretic analysis, the hybrid forms would probably have been described as distinct species (Cann, 1998). How widespread is hybridization among other chelid taxa? We have direct evidence that the hybrids are at least partly fertile in one cross (*C. novaeguineae* × *C. rugosa*, the two most divergent lineages), which opens the possibility of introgression. Is introgression occurring? The F_1 hybrids are morphologically distinctive in the cases of *C. novaeguineae* × *C. longicollis* and *C. novaeguineae* × *C. rugosa.* Yet in the latter case, the backcrossed individual between the hybrid and parent species *C. novaeguineae* was not morphologically distinctive, and was detected only from the electrophoretic data. If a second-generation hybrid can not be distinguished morphologically from its parent species, then how much undetected introgression might be occurring in other taxa?

PHYLOGENY

Burbidge *et al*. (1974) were the first to develop a phylogeny for the Australian chelidae. Using a combination of serology and morphology, they recognized three clades within the *Chelodina*. *Chelodina longicollis*, *C. steindachneri* and *C. novaeguineae* (Group A) formed an unresolved trichotomy, *Chelodina expansa*, *C. rugosa* and *C. siebenrocki* (Group B) formed a second unresolved trichotomy, and *C. oblonga* (Group C) was regarded as the sister taxon to the remaining *Chelodina*. Since then, considerable evidence for these three clades has mounted from allozyme electrophoresis (Georges & Adams, 1992) and gene sequence data (Seddon *et al*., 1997; Georges *et al*., 1998), but with *C. oblonga* as sister to the *C. longicollis* clade. This arrangement, incorporating the subsequent refinements of Rhodin (1994a,b) for the *C. longicollis* group and recently described species (Rhodin & Mittermeier, 1976; Philippen & Grossman, 1990; Rhodin, 1994a,b; Thomson *et al*., 2000), is the basis of the phylogeny best supported by data prior to our study (Fig. 6). It is regarded as the currently accepted phylogeny for the *Chelodina*, against which our phylogeny can be compared.

A phylogeny consistent with our data is given in Figure 7. The major points of disagreement between the currently accepted phylogeny and the phylogeny supported by our data are in the relationships among the Group A species. We would have *C. mccordi* as sister taxon to *C. novaeguineae* and *C. reimanni*, with this clade having *C. pritchardi* as its sister. Rhodin (1994b) grouped *C. mccordi* and *C. pritchardi* together on the basis of a shared partial reduction in parietal roof width and slightly widened triturating surfaces. He also observed that the skulls of *C. mccordi* and *C. pritchardi* were strikingly similar, and differentiated from *C. novaeguineae* by their relative lack of robusticity. He inferred from this a close phylogenetic relationship between the two (Fig. 6).

Under our phylogeny, we would interpret this shared lack of robusticity as a shared primitive character, held in common with the outgroup taxon *C. steindachneri* (an outgroup in both our phylogeny and that of Rhodin). We regard the more robust skulls of

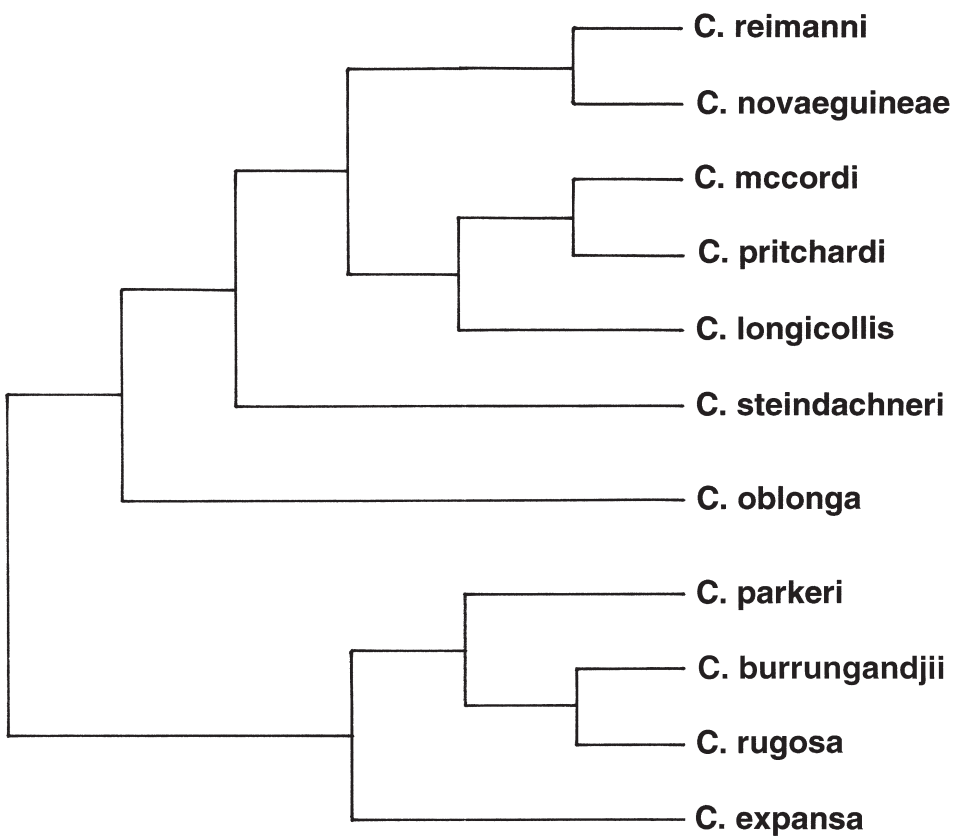

Figure 6. The best-supported phylogeny for the *Chelodina* prior to the present electrophoretic study (Burbidge *et al*., 1974; Rhodin & Mittermeier, 1976; Georges & Adams, 1992; Rhodin, 1994a,b). The root was chosen on the basis of evidence presented by Georges & Adams (1992), Seddon *et al*. (1997) and Georges *et al*. (1998). This phylogeny serves as the working hypotheses against which to compare our data. Only those taxa we regard to be species are included.

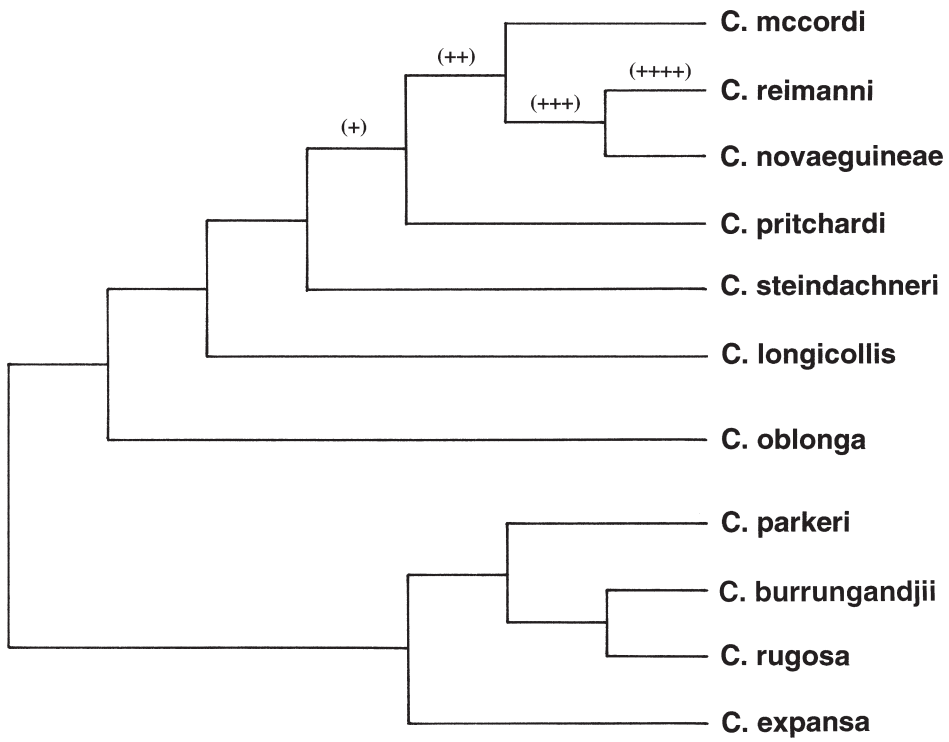

Figure 7. The phylogeny most strongly supported by our electrophoretic data. Note that *C. reimanni* and *C. novaeguineae* could not be separated electrophoretically, but we retain them as separate on the basis of morphological evidence (Philippen & Grossman, 1990; Rhodin, 1994a). The symbols + show the progressive development of robusticity in both the skull and triturating surfaces.

C. novaeguineae and *C. reimanni* as derived, as did Rhodin (1994a) in an earlier paper. Similarly, we regard the development of the wide and robust maxillary and mandibular triturating surfaces that reach their highest development in *C. reimanni*, to be a progression to a derived state (see Fig. 7). Under the phylogeny of Rhodin (1994b), the development of robust triturating surfaces would have had to occur twice, once in the *C. novaeguineae/reimanni* clade, and a second time to a lesser degree in the *C. pritchardi/mccordi* clade. Alternatively, the robust surfaces might have been lost in *C. longicollis*, but independent of their absence in *C. steindachneri*. Our arrangement of taxa is more parsimonious with respect to skull robustness and development of the triturating surfaces (Fig. 7), and consistent with the new allozyme data. Similarly, we would interpret the presence of a posterior pterygoid foramen as a primitive state retained in *C. stiendachneri* and variably in *C. longicollis*, but lost on the branch leading to the remaining Group A taxa. We therefore put our phylogeny forward as the one most parsimoniously consistent with all current information.

The greatest genetic distance between species of *Chelodina* was 53% in terms of fixed differences and 0.53 Roger's D (*C. mccordi* × *C. parkeri*; Table 4). This is comparable to the level of divergence among short-necked species across the genera *Elseya*, *Elusor*, *Emydura* and *Rheodytes*, which attains a level of 57% in fixed differences and 0.55 Roger's D (*Elusor macrurus* × *Elseya georgesi*) (Georges & Adams, 1992). Legler (1985) has foreshadowed his intention to split the genus *Chelodina* into two genera corresponding to Goode's (1967) Group A (*C. longicollis* and related forms) and Group B taxa (*C. rugosa* and related forms, plus *C. oblonga*). Under the revised position of *C. oblonga*, as the sister taxon to Group A species (Georges & Adams, 1992; Seddon *et al*., 1997; Georges *et al*., 1998), such a move would create an unacceptable paraphyly. If we separate *C. oblonga* out into a Group C, as suggested by Burbidge *et al*. (1974), then levels of divergence among the three prospective genera (mean Roger's D = 0.43, range = 0.32–0.53) is comparable to that among five short-necked genera (mean = 0.46, range = 0.37–0.55, Georges & Adams, 1992) and among 20 batagurine genera (Cryptodira: Emydidae) (mean = 0.44, range = 0.18–0.70, Sites *et al*., 1984). We have used allozyme data to recommend splitting the genus *Elseya* into two genera (Georges & Adams, 1992), but this was to resolve an unacceptable

paraphyly. In the case of *Chelodina*, no such paraphyly exists, so our inclination is to maintain the status quo at generic level, and regard the genus as comprising three subgenera corresponding to Groups A, B and C of Burbidge *et al*. (1974). This is consistent with our view that higher level taxonomic categories are essentially morphological constructs, constrained by the proviso only that they be consistent with phylogeny.

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