When morphology and molecular markers conflict: a case history of subterranean amphipods from the Pilbara, Western Australia

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Abstract

When morphology and molecular markers conflict: a case history of subterranean amphipods from the Pilbara, Western Australia.— Fifteen species of groundwater amphipods in the genus *Chydaekata* have been described from the Pilbara, Western Australia, each restricted to a single bore. Dewatering at a local mine site was halted while a second survey was undertaken. Newly collected samples were identified using the existing key, and allozyme analysis was used to test species boundaries. Allozymic diversity was not associated with single bores, and only two distinct genetic groups (one of which was very rare), were identified. Based on these results, and the finding that species were found to be more widespread, the Western Australian Environmental Protection Authority recommended that dewatering continue with caution at the site. This study provides an example of the problems associated with incongruent data sets, and the difficulties inherent in working with rare species, namely, interpreting the results of studies based on small samples or incomplete collections.

Key words: Conservation, Genetics, Morphology, Species, Amphipods.

Resumen

Cuando la morfología y los marcadores moleculares entran en conflicto: el ejemplo de los anfípodos subterráneos de la región de Pilbara, Australia Occidental.— Se han descrito quince especies de anfípodos de aguas subterráneas del género *Chydaekata* que habitan en la región de Pilbara, Australia Occidental, restringiéndose cada una de ellas a una única perforación. Mientras se elaboraba un segundo estudio, se interrumpió el desagüe que se estaba llevando a cabo en un emplazamiento minero local. Las nuevas muestras recolectadas se identificaron utilizando la clave existente, mientras que para verificar los límites de la especie se recurrió a un análisis alozimático. La diversidad alozimática no se asoció con perforaciones únicas y sólo se identificaron dos grupos genéticos bien diferenciados (uno de los cuales era muy poco común). Basándose en estos resultados y en el hallazgo de que la especie estaba más extendida, el Organismo de Protección Medioambiental de Australia Occidental recomendó que se actuara con cautela al proceder con el desagüe. El presente estudio brinda un ejemplo de los problemas asociados con conjuntos de datos incongruentes, así como las dificultades que conlleva trabajar con especies poco comunes, especialmente en lo que respecta a la interpretación de los resultados de estudios basados en pequeñas muestras o recolecciones incompletas.

Palabras clave: Conservación, Genética, Morfología, Especie, Anfípodos.

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Introduction

The groundwater of the Pilbara, Western Australia, is inhabited by a diverse and endemic assemblage of stygofauna (Humphreys, 1999, 2001). This fauna is dominated by crustaceans, although other groups such as annelids are occasionally represented. The region is particularly rich in amphipods, and recent morphological analyses of the ostracode and copepod fauna also suggest high levels of species diversity in these groups (Koranovic & Marmonier, 2003; Koranovic, 2004). Since 1995, five genera and 26 species of amphipods have been described from the Pilbara and Cape Range Peninsula, based on variation in morphological characters (Bradbury & Williams, 1996, 1997; Bradbury, 2000). The richness of the fauna is associated with the unique geology of the region (Humphreys, 2001). The Pilbara has an abundance of calcrete deposits, associated with current and paleo–river channels, within which form a network of fissures and chambers (Mann & Horwitz, 1979). Much of the region contains outcrops of banded iron ore and silica formations (Twidale et al., 1985), making the Pilbara an important producer of iron ore. High levels of species diversity and endemicity have major implications for mining industries in Western Australia, where much mining occurs beneath the local water table. Mining activity was halted during 2001 at one Pilbara mine site, Orebody 23 in the Ethel Gorge (23º 2' S, 119º 5' E), due to the discovery of a locally endemic stygofaunal community, and the concurrent introduction of the Environment Protection and Biodiversity Conservation Act in 1999, protecting threatened fauna and communities. This paper describes a genetic study and the associated series of events following the description of 15 species of *Chydaekata* from the Ethel Gorge and Fortescue Valley region, and highlights the difficulties involved in making decisions when sampling is incomplete and data sets are not congruent.

Morphological analysis indicated that the amphipod fauna of the Ethel Gorge is characterised by high levels of local endemism and narrow species distributions (Bradbury, 2000). Fourteen of the 15 species of the genus *Chydaekata* were described from single sampling locations (bores) over a distance of approximately 35 km along the Fortescue River catchment north of Newman, WA (23º 4' S, 119º 8' E), while the remaining species was described from a site more than 100 km downstream. Three bores were located within the region to be dewatered for the extraction of iron ore from the Orebody 23 site. One of these contained six species of *Chydaekata* (Bradbury, 2000). Many evolutionary, ecological, and practical issues arise from these findings. In particular, high levels of local species diversity lead to several areas of investigation, including identifying the mechanisms behind the generation of diversity, the ecological factors that maintain sympatric species, and, most relevant to this paper, the implications for human activities on an endemic fauna.

The present study used allozyme electrophoresis to provide an independent evaluation of species boundaries in the genus *Chydaekata* within the Ethel Gorge, in keeping with the directive from the Minister for the Environment in Western Australia to "assess the conservation significance" of the local fauna (Humphreys & Armstrong, unpublished report). The decision–making process for the future of the mine was dependent upon two key factors: whether genetic data supported the species deliniations described by Bradbury (2000), and whether species were recorded from areas both within and outside of the area of dewatering. This study is the first step towards bringing a combined morphological and genetic approach to conservation issues for the largely undescribed stygofauna of the extensive Pilbara region. It is also an example of an application of the "taxonomic revolution" (see *Trends in Ecology and Evolution*, vol. 18 for a summary), a growing trend toward reliance on molecular data in making taxonomic decisions.

Methods

Samples were obtained from ten bores in the vicinity of the mine site, two within, five outside, and one on the boundary of the area to be dewatered. Amphipods were collected with a plankton net of mesh size 200 μm fitted with a glass vial. Upon being brought to the surface, the samples were placed in an insulated container to maintain ambient water temperature (approximately 28ºC) and darkness. Effort was made to collect specimens of *Chydaekata* from their type localities, however only two (*C. acuminata* and *C. brachybasis*) were re–collected. In particular, bore WB23–4, the source of six of the described species, yielded no animals. Instead a sample was collected from a nearby bore in the same series of production bores (WB23–1) with similar depth and lithology.

Specimens were treated in three ways. Approximately two–thirds were frozen whole in liquid nitrogen for allozyme electrophoresis, and the others were processed in two ways: approximately half of these were placed into 70% ethanol for morphological identification using the key for *Chydaekata* (Bradbury, 2000), and the remainder were dissected. For each dissected specimen, a small piece of tissue from the abdomen was placed in liquid nitrogen for allozyme analysis, and the head, gnathopods, and the last three body segments (urosomes) and their associated uropods were placed in ethanol. In this way, allozyme phenotypes could be matched with morphological phenotypes. The sites and the number of samples used for allozyme electrophoresis are shown in fig. 1.

The amphipods were analysed for allozyme variation at 31 loci, using standard methods of cellulose acetate electrophoresis (Richardson et al., 1986), following a pilot study to identify polymorphic and readily interpretable loci. The present study included all 11 of the most commonly used enzymes compiled from more than 20 studies of allozyme variation in

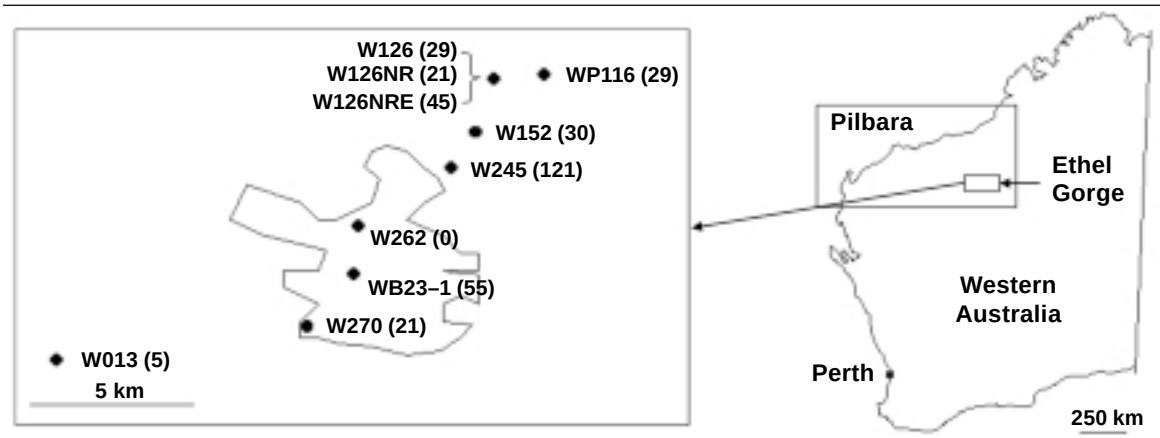

Fig. 1. Map showing the bores sampled in the present study and their placement in the mine site. Line indicates area of impact from dewatering activities; sample sizes for the allozyme analysis are in parentheses. Bore W262 yielded too few animals for electrophoresis.

Fig. 1. Mapa en el que se indican las perforaciones muestreadas durante la realización del presente estudio, así como su localización en el emplazamiento minero. La línea indica el área de impacto de las actividades de desagüe; los tamaños de las muestras para el análisis alozimático se indican entre paréntesis. La perforación W262 produjo muy pocos animales para electroforesis.

amphipods (Stewart, 1993). Because of their small size, individuals were not screened for all 31 loci, but samples from a bore were split and run in two sets. The majority of specimens were analysed for loci in set I, which contained the following 17 loci: Amino aspartate transferase (*Aat–m* and *Aat–s* loci), Alkaline phosphatase (*Alp*), Arginine kinase (*Apk1*, *Apk2*, and *Apk3* loci), Esterase (*Est*), Leucine amino peptidase (*Lap*), Malate dehydrogenase (*Mdh–1* and *Mdh–2* loci), Malate dehydrogenase NADP+ (*Me–1* and *Me–2* loci), Mannose phosphate isomerase (*Mpi*), Peptidase leu–ala (*Pep–1* and *Pep–2* loci), Phosphoglucose isomerase (*Pgi*), and Phosphoglucomutase (*Pgm*). Between five and 15 individuals from each bore were analysed for loci in set II, which contained the following 14 loci: Aconitase (*Acon–1, Acon–2* loci), Adenosine deaminase (*Ada*), Fructose–1,6–Diphosphatase (*Fdp–1, Fdp–2* loci), Glycerol–3–Phospate dehydrogenase (*Gpdh*), Glyceraldehyde–3–Phosphate dehydrogenase (*G3Pdh*), Glucose–6–Dehydrogenase (*G6pdh–1, G6pdh–2* loci), Isocitrate dehydrogenase (*Idh–1, Idh–2* loci), 6–Phosphogluconate dehydrogenase (*6Pgdh–1, 6Pgdh–2*), and Triose phosphate isomerase (*Tpi*). The only exception was in the case of W013, where only five individuals were collected; these were run for set I loci only. To maintain continuity between the two data sets, loci at which on average, the frequency of the most common allele was < 0.90 (*Aat, Mpi, Pgi, Pgm*) were analysed in both sets. A second locus appeared for 6–Phosphogluconate dehydrogenase, the origin of which is uncertain, and is listed here as *6Pgdh–2*. Alleles were scored relative to a common mobility standard of *Chydaekata* sp. from bore W245 in the Ethel Gorge.

To identify the presence of co–occurring species, we first considered the possibility of multiple species within bores, then considered the issue of differences between bores. To look for the presence of more than one species in a sample, the data sets were sorted into multi–locus genotypes, using only those individuals with no missing data. The multi–locus genotypes were inspected for multiple allelic substitutions among individuals. Second, tests for Hardy–Weinberg equilibrium and linkage disequilibrium were run for samples containing ≥ 30 individuals. Hardy–Weinberg equilibrium was tested in the HDYWBG module in BIOSYS (Swofford & Selander, 1989), and linkage disequilibrium was tested using Option 2 in Genepop on the web (http://wbiomed.curtin.edu.au/genepop/ adapted from Raymond & Rousset, 1995). Significance values were assessed using the sequential Bonerroni technique as described by Rice (1989), in order to reduce the incidence of Type I errors. All populations were assessed for heterozygote deficiency or excess using the fixation index, F, calculated in the STATS module of GDA (Lewis & Zaykin, 2001). F was averaged across variable loci. We would anticipate deviations from Hardy–Weinberg expectations, association of alleles, and heterozygote deficits if more than one species were present in a sample. Next, the data were inspected for evidence of variation between bores. Genic differentiation between pairs of bores was tested for all populations with samples ≥ 30, using a contingency test on the total number of copies of each allele at each variable locus. This was done using Option 3 in Genepop, and Fisher's combined

probability method. Genetic distances and identities (Nei, 1972) were calculated among multi–locus genotypes and among bores in the DIST module of GDA (Lewis & Zaykin, 2001). Genetic distances were used in the MDS module in SYSTAT (Wilkenson, 1988). Multi–dimensional scaling was used to look for associations of genotypes with bores, indicating the presence of cryptic species. Data sets I and II were treated separately.

The dissected portions of 13 amphipods were suitable for morphological identification, the remainder being too damaged to permit proper study. These dissected specimens were identified as belonging to five species (*C. anophelma*, *C. acuminata*, *C. brachybasis*, *C. dolichodactyla*, and *C. tetrapsis*) and were sampled from four of the nine bores. The specimens were analysed for the 17 loci from set I. Nei's genetic identities and distances were calculated between each pair of individuals using the DIST module in GDA. This module was also used to produce a UPGMA dendogram of the distance values to investigate possible associations between genotypes and morphotypes.

Results

It was not possible to identify all specimens, because many appeared to possess mixtures of morphological characteristics of more than one species. However, some specimens could be identified, and in all, eight species were present in the ethanol preserved specimens (table 1). These examinations indicated that species had broader distributions than were indicated by the initial survey (Bradbury, 2000). Importantly, following the second survey, only one species, *C. transversa*, appeared to be restricted to the dewatering zone.

Thirteen of the 31 loci were found to be variable, and 12 of these were from data set I (table 2). The majority had only two or three alleles, but two loci (*Mpi*, *Pgi*) had five alleles. There were 75 multi–locus genotypes among the 242 individuals analysed for set I loci, and 21 genotypes among the 48 individuals analysed for set II loci (table 3). Some genotypes were very common and widespread. Eight of the nine bores possessed genotype "n" from set I, comprising 22% of all individuals. Indeed, 47% of all individuals were represented by just four genotypes (n, x, ag, aq). However, many genotypes (56/75 in set I and 13/21 in set II) were represented by single individuals. The number of unique genotypes within bores ranged from 0 to 17 in set I and from 0 to 5 in set II.

First we examined the possibility that more than one species was present in a bore. A single case was immediately detectable by allelic substitutions at six loci in the sample from W245 (table 2). Multi–dimensional scaling showed both the distinctiveness of one individual and the comparative cohesiveness of the others (fig. 2). The stress for this analysis was 0.106, indicating that the plot is a good representation of genetic variation. This population also showed linkage disequilibrium at six pairs of loci and Hardy–Weinberg disturbances at five loci. The divergent individual (W245–b) was removed from the data set, and the remaining individuals were examined for cryptic variation.

Of the remaining 25 tests for HW equilibrium, there were no further deviations from expectations. Of 108 tests for linkage disequilibrium, there was only a single case of significant association between alleles. In W245–a, association was found between alleles at the *Pep–1* locus and the *Pgm* locus. The fixation index was positive in the majority of samples (table 2). An examination of the multi–locus genotype data does not coincide with the morphological patterns. The proportion of unique genotypes in samples containing single and multiple species were not substantially different. We might expect to see more unique genotypes in populations containing multiple species, but the opposite occurred. WP116 and W270 each were identified as containing single species, and had 50% and 53% unique genotypes, respectively. In contrast, WB23–1 and W245 were identified as containing multiple species, yet had 49% and 44% unique genotypes.

Next we addressed the possibility of differences among bores. Over all 31 loci, Nei's genetic identity among bores was very high, ranging from 0.984 to 0.999 (table 4). Multi–dimensional scaling of data set I showed no discrete clusters to indicate the presence of multiple species, i.e., there was no association of genotypes with bores, as individuals from single bores were distributed throughout the scatter plot (fig. 3, Set I, stress = 0.198). In contrast, multi–dimensional scaling of data set II appeared to show association of genotypes with bores (fig. 3, Set II, stress = 0.142). However, inspection of the data for this less polymorphic set of loci indicated that this divergence was attributable to allele frequency differences at single loci, such that the individuals clustered into homozygote and heterozygote classes. For example, WP116 appears distinct due to a high frequency of the Mpi^{4} allele. Of the four unique genotypes shown (the fifth was not unique to the bore and is indicated by a + in the plot), three were Mpi^{44} homozygotes, while one was an Mpi^{34} heterozygote. The other genotype was an Mpi^{33} homozygote. The Mpi^{44} homozygote is also present in bore WP126NRE. The distinctiveness of the genotypes from W270 is due to a high frequency of $Aat\text{–}s^{44}$ homozygotes. The $Aat\text{–}s^{4}$ allele appears in two other populations (range from 0.04 to 0.09; see table 2), however W270 lacks $Aat\text{–}s^{34}$ heterozygotes, despite the presence of $Aat\text{–}s^{33}$ homozygotes in the population. Although genetic identities were very high, frequency differences were detected in the contingency tests of genic differentiation. Using Fisher's combined probabilities, differences in allelic frequencies at a locus were significant between all three pairs of populations tested (W245, WB23–1, and WP126NRE). These differences were usually attributable to the presence of rare alleles in one of the pairs (table 2).

Although there were subtle genetic differences among bores, the genetic variation did not corre-

Table 1. Identifications based on the morphological key for *Chydaekata* (Bradbury, 2000) for specimens from ten bores in the Ethel Gorge, and their location with respect to the dewatering zone. Adapted from Humphreys (unpublished data).

Tabla 1. Identificaciones basadas en la clave morfológica correspondiente a Chydaekata *(Bradbury, 2000), para especímenes procedentes de diez perforaciones en Ethel Gorge, y su emplazamiento con respecto a la zona de desagüe. Adaptado de Humphreys (datos no publicados).*

Bore	Identification	Location
W013	Undetermined, showing characteristics of: *C. ovatosetosa, C. acuminata* and *C. brachybasis*	outside
W116	*C. ovatosetosa*	outside
W126	Undetermined, showing characteristics of: *C. diagonalis* and *C. carscutica*	outside
W126NR (type locality *C. brachybasis*)	Undetermined, showing characteristics of: *C. diagonalis* and *C. carscutica*	outside
W126NRE	Undetermined, showing characteristics of: *C. tetrapsis, C. anophelma* and *C. scopula*	outside
W152 (type locality *C. acuminata*)	*C. acuminata*	outside
WB23–1	*C. dolichodactyla, C. scopula, C. diagonalis* and *C. transversa*	inside
W245	*C. brachybasis, C. anophelma* and Undetermined, showing characteristics of: *C. scopula* and *C. nudula*	outside
W262	Undetermined, showing characteristics of: *C. acuminata* and *C. brachybasis*	inside
W270	*C. dolichodactyla*	boundary

spond to morphology. Not all samples were unequivocally assigned to species, but where definitive identifications were made, there was no corresponding genetic support. For example, WP116 appeared to contain a single species, *C. ovatosetosa*, a species not definitively found in any other bore, yet four of the five genotypes in WP116 were distributed amongst six other bores. Likewise, *C. dolichodactyla* was the sole species identified from the ethanol collections in bore W270, appearing in only one other bore (WB23–1; with three other species), yet five of the eight genotypes found in W270 also occurred in six other bores.

Five species were identified from the dissected portions of specimens. UPGMA clustering of genetic distances between dissected specimens showed three main clusters (fig. 4A). However, the genetic distances between clusters were not large, ranging from 0.0 to 0.14 (identities ranged from 0.78 to 1.0), and the clusters did not correspond to morphological species. For example, the most distinct cluster (A) contained two individuals whose morphology placed them with *C. anophelma* and *C. brachybasis*. The two other major clusters, B and C, also contained individuals that keyed to *C. anophelma*. Cluster B consisted of several smaller clusters that showed no correspondence to morphology —all five morphospecies were represented in this cluster. Cluster C contained two morphospecies that were also found in clusters A and B. To look for frequency differences among species instead of individuals, multilocus genotypes belonging to the same species were pooled and genetic distances were reanalysed. Genetic distances were even lower among groups, ranging from 0.012 to 0.072 (fig. 4B). Identities ranged from 0.91 to 0.98.

Discussion

The patterns of variation produced by analysis of allozyme markers differed from that of the original morphology–based taxonomy. In contrast to high levels of localised diversity and narrow distributions, i.e. endemism associated with individual bores, we found a high degree of overlap among individual genotypes from different bores. However,

Table 2. Allele frequencies for samples of amphipods from nine bores in the Pilbara, Western Australia: * Type locality for *C. brachybasis*; ** Type locality for *C. acuminata;* N. Number of individuals screened for loci in set I and set II, respectively; F. Mean fixation index, averaged across loci. Allele frequencies were pooled for loci common to both surveys; n.d. No data. The following loci were monomorphic for all individuals screened: *Acon–1, Acon–2, Ada, Apk–2, Fdp–1, Fdp–2, Gpdh, G6pdh–1, G6pdh–2, Idh–1, Idh–2, Lap, Mdh–1, Mdh–2, Me–2, 6Pgdh–1, 6Pgdh–2, Pgm–2*.

*Tabla 2. Frecuencia de alelos para muestras de anfípodos procedentes de nueve perforaciones en la región de Pilbara, Australia Occidental: * Localidad tipo para* C. brachybasis*; ** Localidad tipo para* C. acuminata*; N. Número de individuos investigados para cada loci en los conjuntos de datos I y II respectivamente; F. Índice de fijación media, variación entre los loci. Las frecuencias de alelos se agruparon para aquellos loci que apaarecieron en ambos muestreos; n.d. Sin datos. Los siguientes loci fueron monomórficos para todos los individuos analizados:* Acon–1, Acon–2, Ada, Apk–2, Fdp–1, Fdp–2, Gpdh, G6pdh–1, G6pdh–2, Idh–1, Idh–2, Lap, Mdh–1, Mdh–2, Me–2, 6Pgdh–1, 6Pgdh–2, Pgm–2.

Site	W013	WP116	W126	W126NR	W126NRE*	W152**	WB23–1	W245–a	W245–b	W270
N	5 / 0	20 / 9	24 / 5	11 / 10	30 / 15	20 / 10	47 / 8	104 / 16	1 / 0	11 / 10
F	0.000	0.378	0.047	–0.052	0.150	0.130	0.100	0.101	0.000	0.376
Aat–m										
2	****	0.12	****	****	****	****	****	0.01	****	****
3	1.00	0.88	1.00	1.00	1.00	1.00	1.00	0.98	1.00	0.93
4	****	****	****	****	****	****	****	0.01	****	0.07
Aat–s										
2	****	****	****	****	****	****	****	0.01	1.00	****
3	1.00	0.91	1.00	1.00	0.96	1.00	1.00	0.99	****	0.81
4	****	0.09	****	****	0.04	****	****	****	****	0.19
Alp										
2	****	****	****	****	****	0.05	****	****	****	0.05
3	1.00	1.00	0.87	0.91	0.89	0.85	0.93	0.98	1.00	0.95
4	****	****	0.13	0.09	0.11	0.10	0.07	0.02	****	****
Apk–1										
3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	****	1.00
5	****	****	****	****	****	****	****	****	1.00	****
Apk–3										
3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	****	1.00
5	****	****	****	****	****	****	****	****	1.00	****
Est										
2	****	****	****	****	****	****	****	0.05	****	****
3	1.00	1.00	0.97	1.00	0.98	0.95	0.96	0.95	1.00	1.00
4	****	****	0.03	****	0.02	0.05	0.04	****	****	****
Me–1										
2	****	****	****	****	0.04	****	0.01	****	****	****
3	1.00	1.00	0.97	1.00	0.96	1.00	0.99	1.00	****	1.00
4	****	****	0.03	****	****	****	****	****	1.00	****
Mpi										
2	****	****	****	****	****	****	0.02	0.01	****	****
3	1.00	0.29	0.76	0.91	0.87	0.70	0.69	0.81	****	0.81
4	****	0.71	0.24	0.09	0.13	0.26	0.25	0.18	1.00	0.19
5	****	****	****	****	****	0.02	0.03	****	****	****
7	****	****	****	****	****	0.02	0.01	****	****	****

Table 2. (Cont.)

Site		W013	WP116	W126	W126NR	W126NRE*	W152**	WB23–1	W245–a	W245–b	W270
Pep–1											
	2	0.10	****	****	****	****	****	****	0.07	****	****
	3	0.90	1.00	1.00	1.00	1.00	1.00	0.99	0.93	1.00	1.00
	4	****	****	****	****	****	****	0.01	****	****	****
Pep–2											
	2	****	****	****	****	****	****	0.07	0.02	****	****
	3	1.00	1.00	1.00	1.00	1.00	1.00	0.94	0.98	1.00	1.00
Pgi											
	1	****	****	****	****	****	****	****	****	1.00	****
	2	****	****	****	****	****	****	0.01	****	****	0.03
	3	0.90	1.00	0.84	0.91	0.98	0.67	0.88	0.97	****	0.80
	4	0.10	****	0.16	0.09	0.02	0.33	0.09	0.03	****	0.17
	5	****	****	****	****	****	****	0.02	****	****	****
Pgm											
	2	****	****	0.13	0.05	0.02	****	0.06	0.01	0.50	****
	3	1.00	0.53	0.53	0.77	0.26	0.73	0.61	0.75	0.50	0.73
	4	****	0.47	0.34	0.18	0.72	0.27	0.33	0.24	****	0.27
Tpi											
	2	n.d.	****	****	****	****	****	0.06	****	n.d.	****
	3		0.71	1.00	1.00	1.00	1.00	0.94	1.00		1.00
	4		0.29	****	****	****	****	****	****		****

Table 3. Multi–locus genotypes found in each bore for data sets I and II: * Unique genotype.

*Tabla 3. Genotipos multilocus hallados en cada perforación para los conjuntos de datos I y II: * Genotipo único.*

Bore	N	Set I	N	Set II
W013	5	n, s	0	
WP116	10	q, aa, ax, bn, bt*	10	d, f, h*, n*, q*
WP126	20	a*, c, e*, n, q, r*, s, aa, ag, ah, ai*, ak, aq, bn, bq*	5	b, e, g, k, p*
W126NR	11	c, n, t*, y, ag, as*	10	a*, b, e, i*, k, m*
WP126NRE	28	g, m*, n, aa, ag, am*, ap*, aq, bb*, bh, bi*, bj*, bk*, bl*, bm, bn, bu*	5	c, f, j*, l*, o
W152	20	n, s, v, x, y, z*, ac*, ad*, ak, ar*, at*, au*, av, ax, bw*	5	b, c, e
WB23–1	45	b*, c, d*, g, h*, i*, j*, n, o, p*, s, u*, v, x, ag, ah, aj*, an*, aq, av, aw*, ay*, ba*, bd*, be*, bm, bn, bo*, bp*, bs*	8	c, d, e, f, g
W245	91	f*, g, k*, l*, n, o, s, w*, x, aa, ab*, af*, ag, ak, al*, ao*, aq, ax, az*, bc*, bf*, bg*, bh	15	b, c, e, k
W270	11	n, x, ae*, ag, ak, aq, br*, bv*	7	b, k, r*, s*, t*, u*, v*

there was also considerably more overlap in morphological characters among specimens collected for this study, blurring species boundaries, and making species identifications difficult. The group shows extreme morphological diversity. The original taxonomy was based on a limited amount of material, and many of the specimens were juveniles. This precluded a study of character variation within species. Additional sampling associated with this study, while increasing the number of specimens examined, also revealed greater complexity and new combinations of characters.

The most common multi–locus genotype was found in eight of the nine bores, representing nearly 25% of all individuals sampled. The presence of an apparently common, widespread species contradicts the most recent morphological identifications, where no species was definitively found in more than two bores. The same common alleles were present in all samples, and genetic identities among samples were very high. These values are typical of those associated with conspecifics, both for animals in general (Thorpe, 1982) and amphipods in particular (Stewart, 1993). Samples of *Chydaekata*, with identity values ranging from 0.984 to 0.999, are even less differentiated than typical allopatric populations of other amphipod species, showing differentiation that might be expected among individuals sampled from the same population. While there were small frequency differences among bores at some loci, including the occurrence of a few unique rare alleles, we could not find evidence to support the hypothesis that each bore contained a distinct species. The only exception was the presence of a single individual in bore W245 that showed substitutions at six of 17 loci, and large frequency shifts at two additional loci. However, without a morphological specimen, it is difficult to ascertain whether this represents a case of sympatry between species of *Chydaekata*, or between species in different genera.

Of potentially greater relevance is the trend toward deficits of heterozygotes within bores, which suggests the mixing of genetically different groups. Notable diversity was detected in bore W270, where no

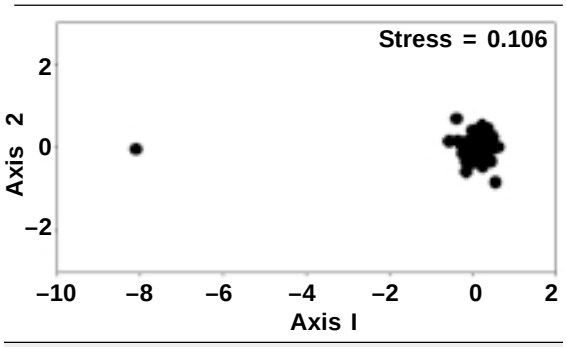

Fig. 2. Multi–dimensional Scaling (MDS) plot of genetic distances among multilocus genotypes, showing distinctiveness of a single genotype at W245.

Fig. 2. Representación gráfica mediante escalamiento multidimensional (MDS) de distancias genéticas entre genotipos multilocus, indicándose el carácter distintivo de un solo genotipo en W245.

Table 4. Matrix of Nei's (1972) genetic identities (above the diagonal) and distances (below the diagonal) among bores, excluding the genetically distinctive individual W245–b.

Tabla 4. Matriz de identidades genéticas (por encima de la diagonal) y de distancias (por debajo de la diagonal) de Nei (1972) entre las diversas perforaciones, sin incluir los individuos genéticamente diferentes W245–b.

	W013	WP116	WP126	WP126NR	WP126NRE	W152	WB23–1	W245	W270
W013	*****	0.981	0.993	0.997	0.984	0.992	0.991	0.997	0.991
WP116	0.016	*****	0.989	0.987	0.989	0.990	0.994	0.991	0.988
WP126	0.006	0.009	*****	0.999	0.997	0.997	0.999	0.997	0.996
WP126NR	0.002	0.009	0.000	*****	0.994	0.997	0.997	0.998	0.996
WP126NRE	0.015	0.011	0.003	0.005	*****	0.992	0.997	0.994	0.993
W152	0.006	0.009	0.002	0.002	0.008	*****	0.997	0.996	0.995
WB23–1	0.008	0.004	0.001	0.002	0.002	0.002	*****	0.998	0.994
W245	0.002	0.007	0.002	0.001	0.006	0.004	0.002	*****	0.994
W270	0.008	0.010	0.003	0.003	0.006	0.004	0.005	0.005	*****

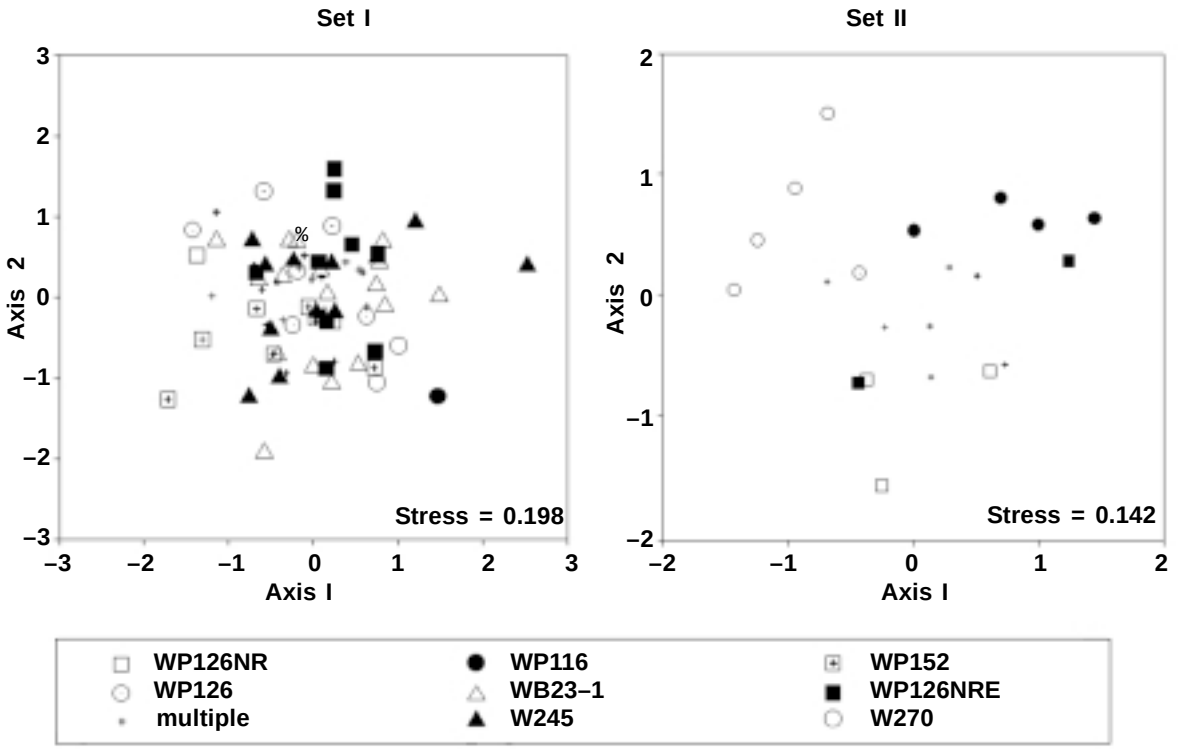

Fig. 3. Multi–dimensional Scaling (MDS) plot of genetic distances among multilocus genotypes (divergent genotype, W245–b, removed).

Fig. 3. Representación gráfica mediante escalamiento multidimensional (MDS) de distancias genéticas entre genotipos multilocus (genotipo divergente, W245–b, eliminado).

heterozygotes for *Aat–s* were found. One explanation of the trend towards deficits of heterozygotes could be the co–occurrence of subtly different species. Sampling from bores could also result in the artificial mixing of genetically divergent conspecific populations from different aquifers. However, sample sizes were small, particularly for set II loci, which may have affected our ability to pick up polymorphisms. Thus, although we cannot exclude the possible presence of multiple species, neither the minor genetic differences among samples nor the possible deficits of heterozygotes provide convincing evidence for multiple species.

Hence, the allozyme data do not support the current taxonomy of endemism associated with individual bores. Even though mixture of genetically similar species would be more difficult to detect, especially in small samples, the bores purported to contain multiple species contained similar proportions of unique genotypes to those purportedly containing only one species. While small samples and limitations in the collections (i.e. lack of type specimens and definitive species identifications) mean these conclusions are provisional, these results make sense in light of the recent morphological ambiguities, as well as the hydrological structure of the aquifer. The Fortescue River transects the borefield in the Ethel Gorge. A thin layer of alluvium overlays an extensive dolomite–calcrete deposit, which contains the aquifer. It is considered to be a continuous flow system, with recharge from the Fortescue River and rainfall (Barnett & Commander, 1985). Thus, hydrologically it behaves as a single, alluvial aquifer, and this connectivity would support the explanation of the presence of a common, widely distributed species.

Still, we must consider the possibility that the allozyme data were not adequate for detecting species differences. *Chydaekata* may be a recent radiation, and may not have evolved allozymic divergence that corresponds to morphological variation. Shared polymorphisms do occur between closely related species due to common ancestry or introgression (Clark, 1997; Clarke et al., 1996; Morrow et al., 2000). Models predict that random drift will drive polymorphic sites to fixation. This can happen relatively quickly in small populations (Clark, 1997), but these polymorphisms may persist in large populations. The use of a large number of allozyme

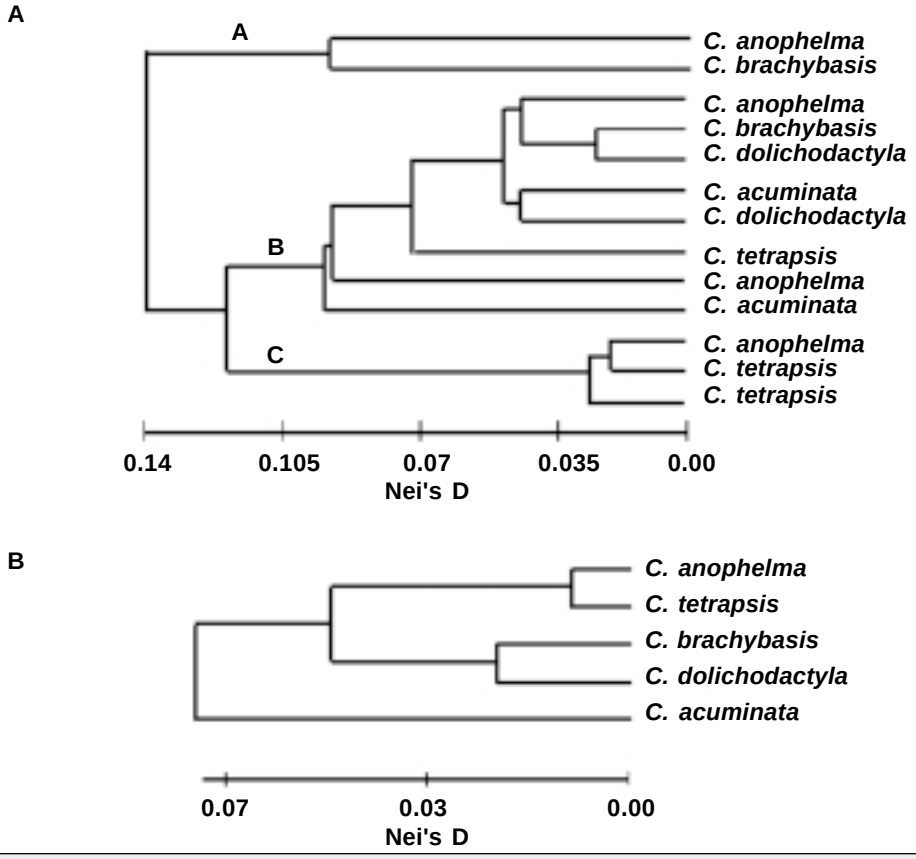

Fig. 4. UPGMA (Unweighted Pair Group Method with Arithmetic Average) diagram of Nei's genetic distances among 13 individuals of *Chydaekata* with complementary allozyme phenotypes and morphological identifications: A. All individuals treated separately; B. Pooling of individuals belonging to the same species.

Fig. 4. Diagrama UPGMA (Método de agrupamiento por parejas no ponderado con media aritmética) de las distancias genéticas de Nei entre 13 individuos de Chydaekata *con fenotipos alozimáticos complementarios e identificaciones morfológicas: A. Todos los individuos tratados por separado; B. Conjunto de individuos pertenecientes a la misma especie.*

loci should however, be able to overcome the signal of shared polymorphisms (Ting et al., 2000).

Finally, morphological variation needs to be re–examined in this group. Many crustaceans, including amphipods, are known to exhibit variable morphological and life history characters (Dickson, 1977; Holsinger & Culver, 1970; Culver et al., 1990). Incongruous combinations of morphological characters and blurring of species boundaries in the present collections suggest that this may be the case, and that a taxonomic revision may be called for. Other approaches, such as a thorough investigation of character variation within species, and the use of more sensitive markers (e.g. rapidly evolving mtDNA genes), need to be undertaken in order to investigate further the relationship between genetic and morphological variation.

Despite ambiguities among data sets, a decision on the issue was requested by the mine operators, because of the cost to industry associated with an interruption to operations. The Department of Conservation and Land Management, the state government body responsible for advising the Environmental Protection Authority (EPA), took the position that dewatering would not lead to the extinction of a species. This was based on the following three factors: (1) most species previously thought to be restricted to the dewatering zone were found to have broader distributions outside the area of impact; (2) morphological boundaries between species were ambiguous; and (3) the allozymes provided no supporting evidence for distinct species. Consequently, the EPA advised the Minister for the Environment to allow mining activity to resume at Orebody 23, with the

condition that regular monitoring of the bores for stygofauna would continue.

This case is an example of the difficulties scientists and managers face when studying rare and endangered fauna, that of small samples and incomplete collections. It highlights the dilemma posed by non–congruent data sets, underlining the need for guidelines for action when such circumstances arise. There are several examples where uncertainties in taxonomy have led to misdirected conservation efforts (see O'Brien & Mayr, 1991; Avise & Hamrick, 1996 for summaries; also see Daugherty et al., 1990; Bowen et al., 1991). The current thrust of governmental policy in Western Australia is to protect and conserve species and ecological communities (Humphreys & Armstrong, unpublished report). In response, mining and other companies planning to develop an area are required to conduct intensive Environmental Impact Assessments, which can be logistically difficult and expensive in remote areas of the state. Hence, what the companies require is certainty of procedure. This is further complicated by a potentially major and contentious issue: how are species defined and recognised for the purpose of meeting the legal requirements? Mayden (1997) observes that there are at least 22 concepts of species currently in use. Two sets of information often lead to congruent results, but how should decisions be made when the two data sets are incongruent, as in the present case? The most reasonable biological resolution, namely to conduct properly designed cross–breeding experiments at least to the F_2 stage will, in most if not all situations involving stygofauna, be practically impossible to execute. Consequently, we suggest that a number of actions be taken, to establish a framework from which science and industry can develop suitable protocols. First, and most importantly, comprehensive systematic studies of the stygobitic fauna of the Pilbara are urgently needed. The real issue facing managers is how to manage something about which very little is known. Secondly, there needs to be informed and substantive debate directed towards developing an acceptable definition of a legal "operational species concept" which has sound biological basis. There have been recent arguments for a strictly molecular approach to taxonomy —i.e., a phenetic concept based on genetic distance or phylogeny (Tautz et al., 2003). While there are reasonable arguments against this extreme approach to the problem (see commentaries by Lipscomb et al., 2003; Seberg et al., 2003), the utility of molecular data as additional characters in making taxonomic decisions is not refuted. Finally, a regional approach needs to be adopted to place local findings in a broader context. This study demonstrates the complexity of issues facing government, industry and the scientific community, and makes clear the need for action in order to establish the framework within which to make well–informed decisions on issues of environmental concern.

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