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Journal of Natural History

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tnah20>

Austinixa aidae Righi, 1967 and A. hardyi Heard and Manning, 1997 (Decapoda: Brachyura: Pinnotheridae) synonymized, with comments on molecular and morphometric methods in crustacean taxonomy

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Published online: 21 Feb 2007.

To cite this article: J. Scott Harrison & Patrick W. Hanley (2005) *Austinixa aidae* Righi, 1967 and *A. hardyi* Heard and Manning, 1997 (Decapoda: Brachyura: Pinnotheridae) synonymized, with comments on molecular and morphometric methods in crustacean taxonomy, *Journal of Natural History*, 39:42, 3649-3662, DOI: [10.1080/00222930500322862](https://doi.org/10.1080/00222930500322862)

To link to this article: <http://dx.doi.org/10.1080/00222930500322862>

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***Austinixa aidae* Righi, 1967 and *A. hardyi* Heard and Manning, 1997 (Decapoda: Brachyura: Pinnotheridae) synonymized, with comments on molecular and morphometric methods in crustacean taxonomy**

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(Accepted 24 August 2005)

Abstract

While conducting a systematic study of *Austinixa* Heard and Manning, 1997, we encountered difficulties in distinguishing *A. hardyi* Heard and Manning, 1997 from *A. aidae* Righi, 1967, based on characters discussed in the current literature. We re-examined morphological characters previously reported as diagnostic, and assessed their variation within and between these putative species. We expanded the analysis to include three independent types of data: discrete morphological characters, morphometric characters, and mitochondrial DNA sequence data. Partial sequences of the mitochondrial 16S rDNA and cytochrome oxidase I (COI) genes were almost identical between *A. aidae* and *A. hardyi*, raising questions about the validity of *A. hardyi* as a distinct species. Analysis of morphometric characters using multivariate (principal components analysis) and univariate allometric adjustment methods, support the mitochondrial DNA data in suggesting *A. aidae* and *A. hardyi* are a single species. Finally, we provide a brief review of the morphometric and molecular methods used in this study and comment on the usefulness of these methods in crustacean taxonomy.

Keywords: *Austinixa aidae*, *Austinixa hardyi*, mitochondrial DNA, morphometric, Pinnotheridae

Introduction

Crabs of the family Pinnotheridae live as commensals or parasites of other invertebrates, and are found in nearly all of the world's oceans (Schmitt et al. 1973). Taxonomy of this group has been historically difficult because of small size, cryptic habitat, sexual dimorphism, and often complicated life histories. For example, the males, females, and juveniles of *Fabia concharum* Rathbun, 1893 were described originally as three distinct species (Davidson 1968). Many species have been recorded as the most abundant macroinvertebrates in the areas where they are found, yet little is known about them

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Published 23 December 2005.

ISSN 0022-2933 print/ISSN 1464-5262 online © 2005 Taylor & Francis

DOI: 10.1080/00222930500322862

(Manning and Felder 1989; Souza and Gianuca 1995; Alves and Pezzuto 1998). For example, *Austinixa gorei* is a dominant organism of the sandy beaches around Miami, Florida, arguably among the most populated beaches in the USA, yet it was not discovered as a distinct species until 1989 (Manning and Felder 1989).

Renewed interest in the family Pinnotheridae has resulted in numerous taxonomic rearrangements and discoveries in recent years (Pohle and Marques 1998). Evolution within this family of crabs has resulted in pairs and groups of similar species that frequently are difficult to distinguish (Zmarzly 1992).

Recently, Heard and Manning (1997) recognized the genus *Austinixa* for seven species of the *Pinnixa cristata* Rathbun, 1900 complex. Species in this genus are found in sandy beach habitats, inhabiting the upper burrows of thalassinidean shrimps of the *Callichirus major* Say, 1818 complex or that of a few ecologically equivalent thalassinidean species.

A new species, *Austinixa hardyi*, known only from the island of Tobago, was described by Heard and Manning (1997). This species is similar to a northern Caribbean species (*A. gorei* Manning and Felder, 1989) and a northern South American species (*A. aidae* Righi, 1967) in lacking a branchial ridge extending laterally from the orbit. Male and female *A. hardyi* were considered distinct from *A. gorei* in having a bicarinate ventral margin on the propodus of the fourth preopod (P4) and from both *A. aidae* and *A. gorei* in having a posterior ridge on the dactylus of P4. Male specimens were considered distinct from all *Austinixa* species in having dense patches of setae on the carapace (Heard and Manning 1997).

While conducting a systematic study of the genus *Austinixa*, we encountered difficulties in distinguishing *A. hardyi* from *A. aidae*, based on characters discussed in the current literature. Little to no information has been added to that given in the original description of *A. aidae* by Righi (1967). We re-examined morphological characters and their variation between and within these putative species. Three independent types of data were analysed: discrete morphological characters, morphometric characters, and mitochondrial DNA (mtDNA) sequence data. Using a combination of procedures allowed better assessment of variation within and between the groups in this difficult taxon.

When analyzing morphometric data in an organism where well-defined stages of growth and/or the fixed sizes of adults are unknown, it is important to transform absolute sizes of body parts to some estimate of relative size (shape) of those parts (Reist 1985). This is particularly important when the mean overall body size between samples differs, and it is not known whether the heterogeneity in size is artificially (sampling bias) or biologically caused. Both multivariate and univariate methods have been proposed to deal with this problem. In this study we employed both the multivariate method of principal components analysis (PCA) and a univariate allometric adjustment method (Thorpe 1975).

PCA occasionally has been used to detect morphometric variation at both the population and species level in studies of other decapod crustaceans (Bert et al. 1996; McClure and Wicksten 1997). This multivariate approach is useful in taxonomy because it weights all available variables and provides minimum discrimination between individuals, with no requirement of *a priori* assignment of individuals to groups (Dytham 1999). Scores of the principal components can then be examined to detect any differential clustering of individuals. Inspection of weighting indicates which variables contribute most to these differences, and more importantly, allows one to take allometry into account in morphological comparisons (Rincon 2000).

There has been a huge increase in the use of DNA sequence data in systematic studies over the last 15 years, particularly that of mtDNA. With this increased use, there has been increased discussion on how these markers should be used, and how useful they are in

differentiating species (Moritz 1994; Sites and Crandall 1997; Avise 2000). There are numerous cases where morphologically cryptic species were first detected by large genetic discontinuities. When combined with other types of data (i.e. morphological or nuclear DNA markers), mtDNA markers can be useful in differentiating species. We applied this method for the first time in pinnotherid crabs.

Three main objectives are addressed in this study. First, the taxonomic status of *A. hardyi* with respect to *A. aidae* is assessed through the analysis of discrete morphological, morphometric, and molecular characters. Second, morphometric variation is quantified within and between these putative species. Third, we provide a brief review of the morphometric and molecular methods used in this study and comment on the usefulness of these methods in crustacean taxonomy.

Materials and methods

Samples used

Museum specimens of *A. hardyi* (13 males, 13 females) were obtained from the National Museum of Natural History, Smithsonian Institution (USNM). A paratype male was first photographed for morphometric analysis, and then destroyed for DNA extraction. Museum specimens of *A. aidae* (eight males, one female) were obtained from the Museu de Zoologia, Universidade de São Paulo (MZUSP). In both cases specimens examined included type specimens. Two additional males of *A. aidae* were collected by Alberto Souza south of the type locality of Rio Juqueriquere, Caraguatatuba, São Paulo, Brazil, and preserved in 95% ethanol. One of these specimens was used in morphometric analysis as described, and subsequently destroyed for DNA extraction.

Molecular data collection

DNA extraction was performed with the Qiagen Qiamap Tissue Kit following the procedure outlined by the manufacturer (Qiagen Inc.), with the following exceptions: before addition of proteinase K, whole crabs (≈ 20 mg) were ground in extraction buffer inside 1.5 ml tubes. After digestion with proteinase K, tubes were centrifuged and buffer containing digested tissue and DNA was transferred to a new tube to eliminate remaining exoskeleton. For the museum specimen of *A. hardyi*, the final DNA elution step was performed with a reduced volume of 100 μ l and repeated three times. This increased the final concentration of DNA but reduced overall yield. There was no possibility for cross-contamination between samples as DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing for each sample were performed 6 months apart, with samples never having the possibility of contact.

Two portions of the mitochondrial genome were amplified by PCR: a 554 bp region within the 16s rDNA gene and a 693 bp region within the cytochrome oxidase I (COI) gene. Primer sequences were: 16sa 5'-CGCCTGTTTATCAAAAACAT-3' (Xiong and Kocher 1991) and 16s2 5'-GATATAAGAAGTCTAGCCTG-3'. The latter was designed for this study from species of *Austinixa*. Primer sequences for COI were S1718 5'-GGAGGATTTGGAAATTGATTAGTTC-3' and S2411 5'-GGGATAGCAATYAT TATWGT-3' (Harrison and Crespi 1999).

PCR amplification was performed in 50 μ l reactions containing 5 μ l 10 \times PCR buffer, 2.5 mM MgCl₂, 0.2 μ M dNTPs, 2–6 μ l template DNA, 0.5 μ M of each primer, and 1.25

units of Taq polymerase. Reactions were performed with an initial denaturation step at 94°C for 3 min, then 30–40 cycles of 94°C, 30 s/50°C, 90 s/72°C, 120 s, and a final extension step at 72°C for 10 min. After processing with exonuclease I and shrimp alkaline phosphatase, double-stranded PCR products were sequenced in both directions, using an ABI 373/377 automated sequencer and an ABI Big-Dye Ready Reaction Kit, following the standard cycle-sequencing protocol. COI was sequenced in both directions using the same primers used in PCR amplification. 16s was sequenced with internal primers (16saint 5'-TTAATTCAACATCGAGGTCTG-3' and 16sbint 5'-GATATAAGAAGTCTAGCCTG-3') designed for this study. Sequences were aligned using the program Sequencher 3.0 (Gene Codes Corp.) and observed for nucleotide differences.

Discrete morphological characters

The following characters were compared between *A. aidae* (10 males, one female) and *A. hardyi* (13 males and 13 females): patch of setae on dorsal surface of carapace present or absent, ridge on the posterior surface of P4 dactylus present or absent, number of carinae on propodus of P4, movable spine on the basal segment of antennular (A1) flagellum present or absent, and apex of male gonopod sharply curved laterally or straight. These characters were reported as diagnostic in the description of *A. hardyi* (Heard and Manning 1997).

Morphometric data collection

Morphometric data were taken by capturing digital images with a JVC KY-F30 high-resolution video camera attached to an Olympus SZH10 dissecting microscope. Images were changed to grayscale images and saved as TIFF files using Adobe Photoshop 4.0 (Adobe 1997). An image of a scale was taken by the same method at each magnification. Morphometric measurements were then acquired from the digitized images using the program Scion Image (Scion 1998). All images taken at the same magnification and the corresponding scale image were opened simultaneously. The scale was first calibrated and then all images were calibrated simultaneously to this scale. This was repeated for all magnifications used. Character and scale images were saved as calibrated, assuring that necessary re-measurements or calibration of future images are identical in calibration. This method increases both accuracy and precision in taking morphometric measurements.

Characters were measured for males only because only one female *A. aidae* was available. The following measurements were performed on the digitized images: carapace length (CL)=the distance from the tip of the rostrum to the cardiac ridge; carapace width (CW)=the distance of the greatest width, measured between the lateral extremes of the carapace just above the coxae of the pereopods; telson width at the proximal end (TW); width of the sixth abdominal segment at the proximal end (Ab6WP); minimum width of the sixth abdominal segment (Ab6WMN); cheliped length-posterior (ChLP)=the distance along the posterior side from the tip of the fixed finger to the proximal end of the propodus; cheliped length-anterior (ChLA)=the distance along the anterior side from the dactyl/propodus joint to the proximal end; maximum cheliped height (ChHMX); and cheliped height at the proximal end (ChHP). The following measurements were made on pereopods 2–5 (P2, P3, P4, or P5 preceding measurement abbreviations to indicate appropriate pereopod number): anterior merus length (MLA)=distance from the proximal to the distal end of the merus along the anterior side; posterior merus length

(MLP)=distance from the proximal to the distal end of the merus along the posterior side; merus width (MW)=distance at the greatest width of the merus; anterior propodus length (PLA); posterior propodus length (PLP); propodus width (PW); and dactyl length (DL). All cheliped and pereopod measurements were taken along the ventral surface. All measurements were taken to the nearest 0.01 mm.

Morphometric analysis

To compare morphometric characters it is important to use a methodology able to separate ontogenetic and evolutionary allometry (Cock 1996; Klingenberg 1996). In addition, because most measures of shape are in some way related to size, heterogeneity in mean body size between samples must be taken into account before meaningful comparisons can be made (Reist 1985). Because samples of the two nominal species differed in mean body size, a common multivariate technique (PCA), and univariate technique for allometric correction (Thorpe 1975; Reist 1985) were employed.

Reist (1985) suggested that the best approach to use in allometric situations is the univariate allometric adjustment method (ALLOM) of Thorpe (1975). With this method individual characters are adjusted to predictions of what an individual's size for a particular variable would be if that individual were the overall mean standard size. The character adjustments are made by an allometric formula:

$$Y_i = 10^k \quad (1)$$

where Y_i is the predicted character value of the i th specimen and the exponent k is the log of the adjusted measurement (e) derived from the equation:

$$e = \log y_i - b(\log x_i - \log X_{SSM}) \quad (2)$$

Here, y_i is the original unadjusted measurement of the i th specimen, b is the allometric coefficient (the slope of the relationship between the $\log y_i$ and the $\log x_i$), x_i is the standard size measurement of the i th individual, and X_{SSM} is the grand mean of the standard sizes. Log is the base-10 logarithm.

For this study, the standard size measurement used was carapace width (CW). Use of a univariate adjustment of characters based on CW (i.e. use of CW as a standard size measurement) is justified because CW was correlated strongly with generalized multivariate size in the data set ($\log CW$ and PC1 extracted by a PCA using the covariance matrix $r=0.98$, $P<0.0001$). After allometrically adjusted values for each character were determined, the values were then compared between samples by a one-way ANOVA.

PCA of covariance matrices was used to explore the distribution of individuals in multivariate morphospace and to determine variability within and among samples. Many authors have suggested the use of covariance matrices when data sets are composed of the same kind of measurements (e.g. lengths) (Blackith and Reyment 1971; Wiley 1981). PCA was performed on log-transformed data to equalize standard deviations of measurements across different-sized variables and to help ensure multivariate normality. Because size is usually the variable with highest variance in a data set, the first principal component extracted from a covariance matrix is often a generalized size factor. The remaining components account mostly for shape differences. The PC coefficients of a covariance matrix can therefore be used to account for allometry and size differences (Humphries et al. 1981).

PCA of a correlation matrix does not allow information about allometries to be derived from resulting PC coefficients (Rincon 2000), but it does allow ordination of individuals in

multivariate morphometric space. Additional PCA, using the correlation matrix of allometrically adjusted characters derived using Equation 1, was carried out to provide additional perspective and complementary insight and contrast to PCA of the covariance matrix.

Results

Mitochondrial DNA

No difference was detected between individuals of either species in the 554 bp region of the 16s rDNA gene. In the 693 bp region of COI, individuals of *A. aidae* and *A. hardyi* differed by only two base pairs (0.28%).

Discrete morphological data

No consistent differences in discrete morphological characters were found between specimens identifiable as *A. aidae* and *A. hardyi*. Morphological character data are summarized in Table I for both males and females of each species. Heard and Manning (1997) stated that *A. hardyi* differed from other species of *Austinixa* in possessing dense patches of setae on the carapace anterior to the cardiac crest in males. This bilobed patch of setae was present in all male specimens of *A. aidae* examined here. However, the type specimen of *A. aidae* (a male) had considerably fewer setae in this area than did other male specimens of *A. aidae*. This may be a consequence of setae being partially lost in preservation. It is possible also that the number of setae may decrease in larger specimens, as the type specimen of *A. aidae* is 2.5 mm wider than any other individual observed. Nonetheless, a remnant of the bilobed patch of setae is clearly visible in the type specimen.

Heard and Manning (1997) also stated that a ventral ridge on the posterior surface of the P4 dactylus distinguished *A. hardyi* from *A. aidae*. We found that a dorsal and ventral sulcus was present on the posterior surface of the dactylus, with ridges varying from barely detectable to more pronounced in specimens of both nominal species. Consequently, this character appears not to be diagnostic.

Gonopod tip structure has been found to be extremely useful in the identification of cryptic species in pinnotherid crabs (Manning and Felder 1989). Scanning electron microscope (SEM) images of gonopod tips showed identical structure for specimens of both species (Figure 1).

Table I. Presence or absence of discrete morphological characters considered diagnostic for *Austinixa aidae* and putative *A. hardyi*.

	Male		Female	
	<i>A. aidae</i>	<i>A. hardyi</i>	<i>A. aidae</i>	<i>A. hardyi</i>
1. Patches of setae on dorsal surface of carapace	+	+	—	—
2. Ventral ridge on posterior surface of P4 dactyl	+/-	+/-	+/-	+/-
3. P4 propodus bicarinate	+	+	+	+
4. Movable spine of basal segment of antennular (A1) flagellum	+	+	+	+
5. Apex of male gonopod sharply curved laterally	+	+		

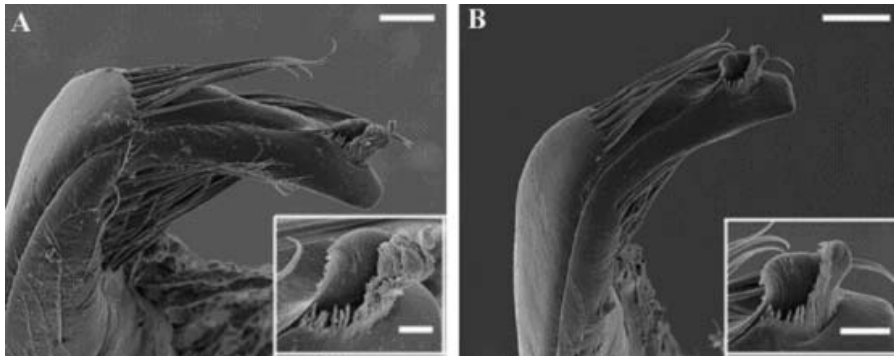


Figure 1. Gonopod tips of *Austinixa aidae* and *A. hardyi*. (A) *A. aidae* (holotype MZUSP 2232) male gonopod tip magnified $160\times$, inserted image is the opening of the gonopod magnified $750\times$; (B) *A. hardyi* (paratype USNM 284177) male gonopod tip magnified $200\times$, inserted image is the opening of the gonopod magnified $1000\times$. Scale bars: $100\mu\text{m}$ (main images); $30\mu\text{m}$ (inserted images).

Morphometric variation

Results from PCA of the covariance matrix indicated the first, second, and third principal components accounted for 93.7%, 2.2%, and 1.2% of the total variance, respectively. The coefficients of the first principal component (PC1) were all of the same sign and had weights of similar magnitude (Table II). The subsequent components were of mixed sign and variable weight. This suggests that the variation along PC1 is size related, thus not informative with respect to shape; whereas PC2 and PC3 can be treated as shape vectors (Blackith and Reyment 1971; Wiley 1981).

Projections of individual scores along the first and second principal components showed divergence, with slight overlap along both PC1 and PC2 (Figure 2). Projection of individuals along second and third principal components showed near-complete overlap along PC3 and divergence, with some overlap along PC2 (Figure 3). Separation of specimens of each nominal species along PC2 was due largely to propodal width of third pereiopod (P3PW) and anterior cheliped length (ChLA). Other characters weighting heavily are summarized in Table II.

A second PCA, using the correlation matrix of allometrically adjusted characters, indicated that the first, second, and third principal components accounted for 33.5%, 17.7%, and 14.1% of the total variance, respectively (Table III). Here, the effect of size had been previously removed. PC1 is now a component that reflects mostly the contribution of the dactyl length of P5 (P5DL), carapace length (CL), and the propodal length of P3 (P3PLA). Additional characters that appeared to weight heavily are summarized in Table III, but were generally measures of the P3 propodus and P5.

Scatterplots of PC1 versus PC2 of the correlation PCA showed divergence with some overlap, along PC1, with complete overlap along PC2 (Figure 4). Projections of all other principal components showed complete overlap, providing no additional discrimination between *A. aidae* and putative *A. hardyi*.

The allometric model generally revealed a good fit to the data. Carapace width (CW) explained a high proportion of variation in morphometric characters (81.3–96.5%), and allometric coefficients differed significantly from zero. Results of an ANOVA of allometrically adjusted characters are summarized in Table III. The majority of the morphometric characters showing significant differences between *A. aidae* and putative

Table II. Weights for the first three principal components computed from the covariance matrix from log-transformed data of *Austinixa aidae* and putative *A. hardyi*.

Character	PC1 (93.3%)	PC2 (2.2%)	PC3 (1.2%)
CL	-0.144	-0.152	0.072
P2MLA	-0.157	0.087	-0.182
P2MLP	-0.162	0.097	-0.180
P2MW	-0.148	-0.062	-0.393
P2PLA	-0.159	-0.065	-0.175
P2PLP	-0.147	0.004	-0.225
P2PW	-0.161	-0.139	-0.208
P3MLA	-0.169	0.078	0.107
P3MLP	-0.158	0.066	0.144
P3MW	-0.170	-0.159	-0.046
P3PLA	-0.152	-0.090	0.218
P3PLP	-0.158	-0.045	0.207
P3PW	-0.180	-0.348	0.069
P3DL	-0.157	-0.196	0.204
P4MLA	-0.182	0.233	0.004
P4MLP	-0.188	0.250	-0.005
P4MW	-0.192	0.003	-0.263
P4PLA	-0.175	0.146	0.020
P4PLP	-0.175	0.191	0.035
P4PW	-0.168	-0.075	-0.276
P4DL	-0.171	-0.025	-0.078
P5MLA	-0.169	-0.017	-0.05
P5MLP	-0.174	0.068	-0.052
P5MW	-0.176	-0.174	-0.208
P5PLA	-0.151	-0.213	0.337
P5PLP	-0.156	-0.297	0.188
P5PW	-0.153	-0.190	-0.129
P5DL	-0.145	-0.289	0.149
ChLP	-0.199	0.269	0.213
ChLA	-0.204	0.309	0.140
ChHMX	-0.214	0.282	0.066
ChHP	-0.216	0.100	0.127
TW	-0.148	-0.040	0.047
Ab6WMN	-0.148	-0.003	0.036
Ab6WP	-0.153	-0.011	0.031

A. hardyi were associated with P3 and P5. These included all morphometric variables of the propodus, merus width, and dactyl length. Propodal width of the second pereopod and carapace length also were found to differ significantly.

Discussion

Taxonomy and distribution

Although morphometric variation was found when comparing types and syntypes of *A. aidae* and *A. hardyi*, no consistent diagnostic differences in either morphology or mtDNA sequences were found. The data do not support the existence of two species, and we conclude that *A. hardyi* should be considered a junior synonym of *A. aidae*.

The known distribution of *A. aidae* is expanded from its previously only known location, Rio Juqueriquere, Caragatatuba, São Paulo, Brazil to the island of Tobago. Rio

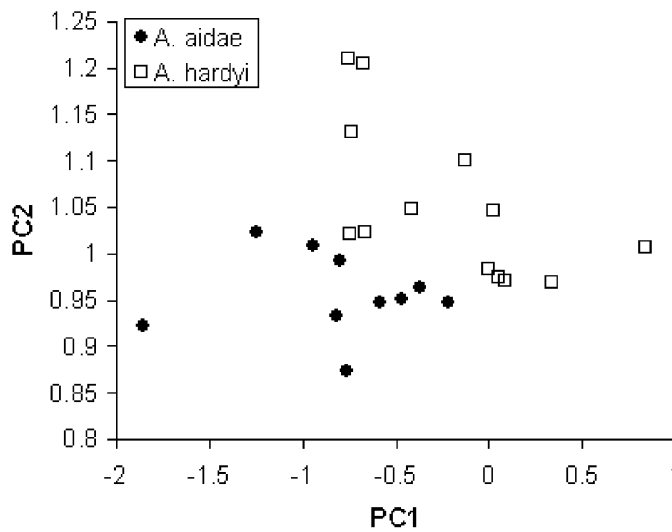


Figure 2. Scores plotted on the first and second principal components computed from the covariance matrix from the log-transformed data of *Austinixa aidae* and putative *A. hardyi*.

Juqueriquere appears to be the southern limit of this species. It is replaced to the south by a congener, *A. patagoniensis* Rathbun, 1918. Based on Heard and Manning (1997), *A. aidae* appears to be more common in Tobago than in the southern part of its range. The host of *A. aidae* has not been recorded in Brazil, but like most other species of *Austinixa*, specimens from Tobago prefer a thalassinidean shrimp of the *Callichirus major* complex as a host (Heard and Manning 1997). Further collections are needed to determine if *A. aidae* lives north of Tobago in the Caribbean Sea or along the Caribbean coast of Central America, where no species of *Austinixa* have been reported to date.

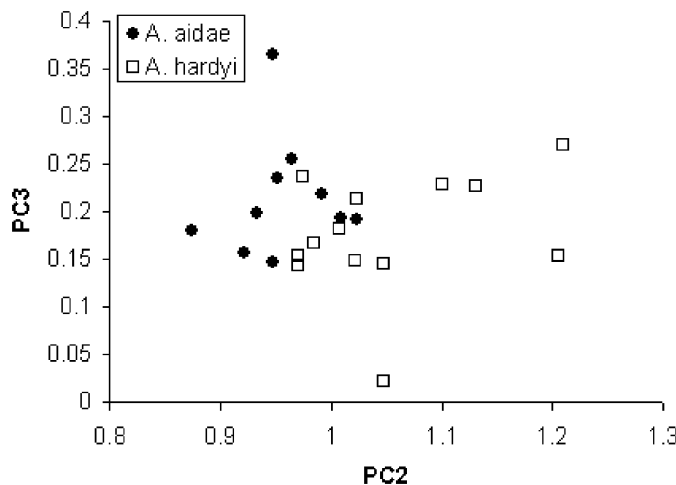


Figure 3. Scores plotted on the second and third principal components computed from the covariance matrix from the log-transformed data of *Austinixa aidae* and putative *A. hardyi*.

Table III. Weights for the first three principal components computed from the correlation matrix from the allometrically adjusted data; associated *P* values for an ANOVA of allometrically adjusted characters (ALLOM) comparing *Austinixa aidae* and putative *A. hardyi*.

Character	PC1 (33.5%)	PC2 (17.7%)	PC3 (10.1%)	ALLOM
CL	-0.243	-0.070	0.087	***
P2MLA	-0.125	0.152	-0.300	
P2MLP	-0.049	0.125	-0.323	
P2MW	-0.161	-0.082	-0.225	
P2PLA	-0.227	-0.036	-0.179	
P2PLP	-0.153	0.013	-0.179	
P2PW	-0.191	-0.130	-0.112	**
P3MLA	-0.151	0.202	0.173	
P3MLP	-0.120	0.212	0.132	
P3MW	-0.183	-0.203	0.058	**
P3PLA	-0.237	0.035	0.196	***
P3PLP	-0.212	0.044	0.235	***
P3PW	-0.205	-0.145	0.042	**
P3DL	-0.193	-0.101	0.169	***
P4MLA	-0.049	0.358	-0.091	
P4MLP	-0.042	0.357	-0.105	
P4MW	-0.152	0.017	-0.272	
P4PLA	-0.139	0.262	-0.066	
P4PLP	-0.109	0.329	-0.061	
P4PW	-0.171	-0.111	-0.279	
P4DL	-0.150	0.072	-0.195	
P5MLA	-0.208	-0.011	-0.100	
P5MLP	-0.059	0.080	-0.112	
P5MW	-0.205	-0.187	-0.177	**
P5PLA	-0.223	0.013	0.148	***
P5PLP	-0.203	-0.094	0.059	**
P5PW	-0.197	-0.091	-0.152	*
P5DL	-0.255	-0.074	0.067	***
ChLP	-0.052	0.290	0.182	
ChLA	-0.051	0.262	0.117	
ChHMX	-0.080	0.260	0.050	
ChHP	-0.166	0.148	0.101	
TW	-0.180	-0.076	0.175	
Ab6WMN	-0.195	-0.036	0.194	
Ab6WP	-0.177	-0.080	0.209	

P*=0.05, *P*=0.01, ****P*=0.001.

Morphometric variation

The data presented here indicate morphometric differences between specimens from Tobago and those from Brazil. These included width of the P3 and P5 propodus, and P5 dactylus length. The ALLOM method indicated that specimens from Brazil had a significantly longer carapace than those from Tobago, an observation also made by Heard and Manning (1997).

Morphometric variation has been observed in other species of pinnotherid crabs. Individuals of *Pinnixa franciscana* Rathbun, 1918 found in protected coastal habitats differ from those found in open-coast habitats in extent of lateral elongation of the carapace, length of the P4 propodus, and degree of pronouncement of a cardiac ridge. This intraspecific variation was hypothesized to stem from ecological differences (Rathbun

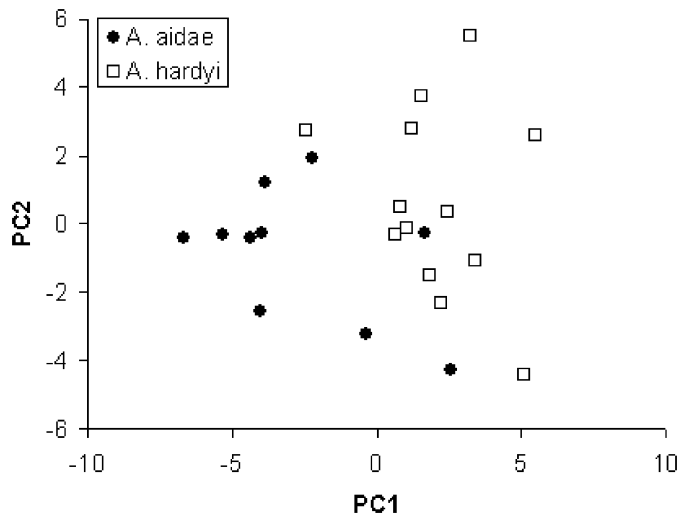


Figure 4. Scores for the first and second principal components computed from the correlation matrix from the allometrically adjusted data of *Austinixa aidae* and putative *A. hardyi*.

1918; Zmarzly 1992). Alves and Pezzuto (1998) noted different growth rates between bi-yearly recruitment classes of *A. patagoniensis*, possibly due to lower water temperatures during one of the recruitment periods. A similar pattern was observed in *Pinnotheres ostreum* Say, 1817 (Christensen and McDermott 1958). Differences in growth rates have the potential to affect growth patterns and resulting shapes of morphological characters. Morphometric variation observed in *A. aidae* may be due to ecological adaptations to factors such as host or substrate differences between locations. Studies of development and a fine-scale geographic assessment of morphology are needed to better understand patterns of variation in *A. aidae*.

Review of morphometric methods

Results of the PCA of the covariance matrix provided questionable results that were not consistent with results obtained from allometric character adjustment methods. PC2 was the main axis indicating variation between *A. aidae* and *A. hardyi*. Three of the four cheliped measurements weighted quite heavily along PC2, and ChLA had the second highest weight on this axis. Other methods of comparisons employed not only contradicted this, but strongly indicated no statistical differences in these characters with *P* values ranging from 0.75 to 0.98. Simple observation of log-log bivariate plots also gave no indication of systematic bias in these characters.

Some authors have suggested that PCA may produce spurious results when PC1 accounts for extremely high levels of the total variation. In this situation, the remaining PCs, which are assumed to contain the variation of interest (shape variation), account for such a small amount of the total variation that each PC may not have a unique meaning, PC1 may contain shape information, and the remaining PCs may also contain size information (Blackith and Reyment 1971; Humphries et al. 1981; Reist 1985; Rincon 2000). Although there is no consensus on how much variation is too much in PC1 and too little in the remaining PCs, with 93.7% of the variation accounted for by PC1, and only 6.3% by PC2–PC13, PCA may produce misleading results with this data set.

Numerous univariate methods have been applied to taxonomic studies using morphometric data sets to deal with the problem of separating size and shape information. These methods include logarithmic transformations, ratios, logarithmic transformation of ratios, and allometric character adjustment methods (Reist 1985). Debate continues as to which method best separates shape information and size influences due to either heterogeneity in size between samples or different allometric patterns. A host of potential problems are known to exist when using ratios. However, ratios continue to be the most commonly used method. Potential problems with using ratios include compounding error terms, creating or increasing correlation between numerator variables, and most importantly not removing the effects of size if allometric relationships exist between variables (Atchley et al. 1976; Thorpe 1976; Atchley and Anderson 1978; Reist 1985).

The allometric adjustment method of Thorpe (1975) has yet to be used to analyze morphometric characters in systematic studies of crustaceans and most other invertebrate taxa. Reist (1985) compared numerous univariate and multivariate methods of morphometric analysis and determined this allometric adjustment method to be superior in generating shape variates that are independent of size and in having minimal adverse effects on the data. Future systematic studies of crustacean taxa, using morphometric data, would benefit from using this method in place of the common practice of using ratios.

Mitochondrial DNA sequence variation

The mitochondrial gene sequences compared in this study have been shown to be variable between species of *Austinixa*, with interspecies sequence divergence ranging from 3 to 9% for 16s and 13 to 22% for COI (Harrison 2004). The sibling species *A. behreae* Manning and Felder, 1989 and *A. chacei* Wass, 1955 in the northern Gulf of Mexico are almost indistinguishable morphologically, and show sequence divergences of 3 and 13% for 16s and COI, respectively (Harrison 2004). The amount of variation seen between individuals from Brazil and Tobago are characteristic of that between individuals of an interbreeding population. It is important to emphasize that only one individual was sampled from each location, which is significantly below the sample size needed to accurately estimate genetic diversity within and among populations (Crandall and Templeton 1993). Nonetheless, the observed lack of variation between individuals contributes important information to the question being addressed, and adds support to the morphological data in rejecting the hypothesis of the existence of two species.

Mitochondrial DNA data and taxonomy

Data from mtDNA have provided scientists with a wealth of information about natural variation within and among species and some comments on its use in studies of species discrimination are in order. mtDNA is haploid, non-recombining, and maternally inherited in most organisms. Therefore, the effective population size is one-quarter that of a single-copy nuclear locus. This has important repercussions in that a mitochondrial locus will sort to fixation approximately four times faster than nuclear loci. This results in fixed diagnosable haplotypes being common between structured populations within a species (Sites and Crandall 1997). In addition, in the event of hybridization, the mtDNA haplotype of one species can rapidly introgress into the other, making the species indistinguishable with respect to mitochondrial markers (Sites and Crandall 1997; Slowinski and Page 1999). For these reasons, mtDNA markers should not be used exclusively in species

definition, but can contribute important insight when used in combination with other types of data, including morphological data and nuclear DNA markers (Moritz 1994; Sites and Crandall 1997; Avise 2000).

Summary

This is the first study to combine morphology, morphometric, and genetic methods in a taxonomic study of pinnotherid crabs. Although the univariate allometric adjustment method employed in this study is commonly used in vertebrate taxa, this is the first study to use it for taxonomic comparison in decapod crustaceans. The use of a combination of three independent types of data provided strong support for the existence of only a single species while allowing quantification of morphometric variation that exists between extreme locations in the distribution.

Acknowledgments

We are greatly indebted to A. Souza for collection of specimens. Thoughtful critiques of the manuscript were provided by M. Wicksten, J. Manhart, J. Woolley, and J. Gold.

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