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Morphological analysis of some cryptic species in the *Acanthocyclops vernalis* species complex from North America

Stanley I. Dodson[1], Andrey. K. Grishanin[2], Kevin Gross[1] & Grace A. Wyngaard[2]
[1]*Zoology Department – Birge Hall, 430 Lincoln Drive, University of Wisconsin, Madison, WI 53706-1381, U.S.A. E-mail: sidodson@facstaff.wisc.edu*
[2]*Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow 119991, Russia*
[3]*Department of Biology – MSC 7801, James Madison University, Harrisonburg, VA 22807, U.S.A.*

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Abstract

Patterns of morphological variation and reproductive isolation were examined for several North American populations of copepods in the *Acanthocyclops vernalis* Fischer A., 1853 (Copepoda, Cyclopinae) species complex. The copepods were collected from six sites in Wisconsin, U.S.A. Morphological analysis of 120 adult females revealed that a character used previously to distinguish species in this group was unreliable because of phenotypic plasticity. Most of the morphological variance was due to environment (Laboratory vs. field) and to field site. Relatively little of the variation was due to measurement error or asymmetry. Multivariate ordination analysis produced poorly-defined clusters of individuals, suggesting that different biological species are difficult or impossible to distinguish using a set of easily-measurable morphological characters. In our study, morphological similarity was independent of geographic distance among sites, between 0.05 and 300 km. Isofemale lines within sites showed little or no reproductive isolation, but nearly complete isolation among sites. Reproductive isolation was also independent of morphology. These results suggest that the *Acanthocyclops* population at each site could be considered a distinct cryptic biological species. These copepods expressed morphological stasis – persistence of morphological uniformity despite reproductive isolation. Because of the effect of site and environment on morphology, we recommend using much larger collections (many sites), common garden experiments, and a multi-disciplinary approach (morphological, reproductive, chromosomal, and molecular) as the basis for future taxonomic research on putative copepod species.

Introduction

Cryptic speciation is a common phenomenon in copepods that has been well studied in marine systems. Examples of groups of morphologically similar species include Frost (1989) and McLaren et al. (1989, seven biochemically and genetically distinct species of *Pseudocalanus* in northern oceans), McKinnon et al. (1992, phenotypically plastic but biochemically different sibling species of the inshore copepod *Acartia* in two adjacent Australian bays), Ganz & Burton (1995) and Edmands (1999, outbreeding depression in populations of the intertidal copepod *Tigrious californicus*), Knowlton (2000 excessive lumping of morphologically similar but genetically distinct marine copepods), and Lee (2000) and Lee & Frost (2002, genetic differences between proximate populations of the coastal copepod *Eurytemora affinis*).

North American freshwater copepods in the genus *Acanthocyclops* with 17-segmented antennae have been interpreted as one or more morphological species (Price, 1958; Smith, 1981; Dodson, 1994). Different geographic populations are remarkably similar morphologically, yet show subtle morphological differences that continue to obsess taxonomists. This pattern of difficulty in describing cryptic species is

characteristic of freshwater cyclopoid genera. Enigmatic species swarms have been described for all well-studied and species-rich genera, including, for example, *Acanthocyclops* (Smith, 1981), *Tropocyclops* (Reid, 1991), *Diacyclops* (Reid, 1992), and especially *Cyclops* (Einsle, 1993). Copepodologists are just beginning to use sophisticated genetic and statistical techniques to explore the taxonomic meaning and fitness implications of subtle morphological variation within species complexes (Hoþyñska, 2000; Lajus & Alekseev, 2000).

Dodson (1994) re-described two very similar *Acanthocyclops* species that were distinguished by two characters. *A. vernalis* was distinguished from *A. robustus* by the presence of a patch of spines (P4ANT) on the anterior face of the P4 coxa (see Fig. 1D in this paper and Fig. 4G in Dodson, 1994). A less consistent character was the terminal segment of the P4 endopod, which has two terminal spines (see Fig. 1C in this paper). In *A. vernalis*, the outer spine was described as being often longer than the inner, and the opposite was characteristic of *A. robustus*. Otherwise, no morphological differences were detected between the two species.

Previous work, reported in Dodson (1994), showed that the number of spine-like setae on the P4 endopod terminal segment depended on some environmental factor, probably temperature. Cold water forms were characterized by 5 spines, while warm water forms had only the two terminal spines. This phenotypic plasticity was clearly not useful in distinguishing species, and such variation in one character provided a warning that other morphological characters could be influenced by environmental factors.

As part of a collaborative project, the Dodson (1994) key was used to identify *Acanthocyclops* species used in a series of mating trials between isofemale lines established from adult females collected from 5 of the six sites (Grishanin, unpublished data). As part of the establishment of isofemale lines, it was necessary to identify field-caught specimens as well as individuals in laboratory cultures. In the course of this study, it became clear that the morphological characters being used to separate species were probably unreliable. Specifically, animals from the same site, and even siblings from the same isofemale line expressed presence or absence of P4ANT. While it is possible that two species were present in a single pond, it is unlikely that two species would have the same mother.

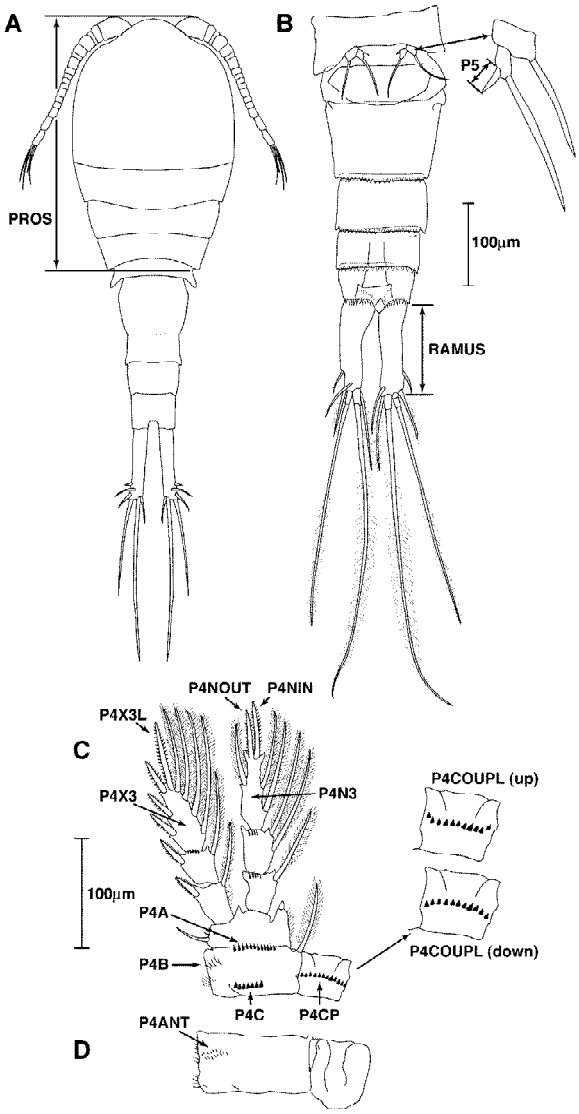

Figure 1. Drawings of the 14 morphological characters used in this analysis. The acronyms are defined in the text. The drawing is representative of a generic member of the *A. vernalis* species complex.

The unreliability of the main distinguishing character produced a desire to know whether it was possible to use morphological characters to describe groups of individuals. Questions that arose included:

- How variable are standard morphological characters used to describe *Acanthocyclops* species?
- To what degree are standard morphological characters correlated? Especially, is there an allometric body-size effect on characters?

- All of the characters show slight variation within a population. How much of this variation is due to effects of environment (field vs. laboratory conditions), site (lake), measurement error, asymmetry, and individual morphological variation?
- Which of the standard characters explain most of the morphological variation?
- Are morphological and geographic distances correlated?
- Is morphological difference correlated with reproductive isolation?

To answer these questions, we measured 120 adult females from the field and laboratory. We were able to take advantage of 48 isofemale lines raised in a common laboratory environment, to address the questions above. Some of these animals were included in mating trials designed to measure reproductive isolation (Grishanin, unpublished data).

Materials and methods

Sites

Copepods were collected in May 2001 from 6 sites in Wisconsin. Three of the sites are in northwest Wisconsin. These are small shallow lakes in Chippewa Co, Lat. 45.2341, Long. 91.1184: 4 miles west and 4 miles north of Holcombe, Wisconsin. Parejko Pond is about 150 m south-southwest of Shorts Pond #1 and Shorts Pond #2, which are only about 50 m apart. Short's #1 is southeast of Short's #2. The remaining three sites are in Dane Co., in south central Wisconsin. Cleveland Road Ditch is a narrow intermittent ditch less than 0.5 m deep when full, at 43.0931° N and 89.6022° W along State Highway 14, near the intersection with Cleveland Road. Trek Pond is a shallow urban retention lake at the Mineral Point exit of Madison's Beltline (Highway 12 & 14) at 43.0606° N, 89.5237° W. Lake Waubesa is a moderate-sized lake in southern Dane Co., at 43.0096° N, 89.6022° W. Copepods were collected in shallow water at the lake's outlet. (Specimens from Lake Waubesa were used in the morphological analysis, but not in the mating trials.) The animals that had developed in the field would have experienced temperatures during their development in the range of 5–10 °C.

Specimens were categorized as:

- Founders – Adult females carrying eggs. These animals were caught in the field, and killed after their young began developing. These animals had been fertilized by one or more males before capture.
- Isofemale Lines: The clutches of Founders were kept isolated and used to start isofemale lines for mating experiments. Male and female offspring were grown from early embryo stage in a common laboratory situation. Lines were maintained by matings among siblings.
- Hybrids – Animals from a few lines that resulted from successful crosses between different lines.

Initially, 48 isofemale lines were maintained at a temperature of about 22 °C, at ambient photoperiod. The animals were fed luxurious amounts of flagellated yellow-brown alga *Cryptomonas ozolini* Skůja from Starr Collection at the University of Texas at Austin (culture number UTEX-LB2194) and newly hatched *Artemia salina* brine shrimp nauplii. Mating occurred readily in the lab and fecundity was high.

After sufficient progeny were produced in the isofemale lines for the purpose of mating trials (see Grishanin, unpublished data), the number of isofemale lines was culled to nine. The present morphological analysis focuses on the founders and their offspring of the nine isofemale lines, along with representatives of 38 isofemale lines not used in mating trials, and 6 specimens from Lake Waubesa.

Adult female specimens were preserved in 70% ETOH. Dissections were done in Hoyer's mounting medium (Dodson and Frey 2001) on microscope slides. The urosome and 4th thoracic segment were separated from the prosome, and positioned so the urosome ventral surface faced up, and the 4th thoracic legs (P4) were flat, undistorted, and with the posterior surface facing up. Dissected specimens were discarded if one or more characters were not visible. A total of 120 individual females were used for data collection.

Morphological character

Morphological characters used in this study are based on those reported in Dodson (1994), with a few additions. Additional characters were added after a careful survey of the appendages, searching for characters that showed promising variation among sites. We favored counts and measurements and avoided shape characters. Morphological characters were measured only on adult females, and were found on the urosome and fifth and fourth thoracic legs. Note that the spine pattern on the basal segment of the fourth thoracic leg is probably related to pre-fertilization mate recognition, and

therefore is of potential taxanomic value (Hołyñska, 2000).

Integumental (sub-cuticular) pore patterns on the body have been successfully used to distinguish cryptic species in other copepod general (Bannister, 1993; Baribwegure, 2001). Our specimens were dissected and mounted before we learned of this morphological character. We were able to see the major sub-cuticular pores on the dorsal surface of the urosome, and concluded that variation in this pore pattern was minimal and not useful.

We recorded data for 14 morphological characters (Fig. 1) on each of 120 individuals. The characters included six length measurements and seven counts and one binary variable. Acronyms are given here to assist in interpreting the results tables.

Six Lengths:

- PROS Length of the prosome axis, from the anterior tip to the wing of the segment carrying the third thoracic leg. (The segment for the fourth leg had been dissected off the thorax.) This measurement is the best surrogate for total body length we had available (Fig. 1A)
- RAMUS Length of the urosome, measured along the outside margin (Fig. 1B).
- P5 Length of the subterminal spine of the fifth thoracic leg (Fig. 1B). þ P4NIN Length of the inner P4 endopod terminal seta (Fig. 1C).
- P4NOUT Length of the outer P4 endopod terminal seta (Fig. 1C).
- P4X3L Length of the (inner) terminal spine of the P4 exopod segment 3 (the distal segment) (Fig. 1C).

Seven Counts:

- P4CP Number of spines in the row across the middle of the coupler of the fourth thoracic legs (Fig 1C).
- P4ANT Number of microspinules in the patch of the P4 basipod, on the anterior face (Fig. 1D).
- P4A Number of microspinules in the distal (marginal, 'A') row of the P4 basipod, posterior face. The spinules are in a line, which is often interrupted in the middle with a space (Fig. 1C).
- P4B Number of microspinules in the distal-lateral patch ('B') of the P4 basipod , posterior face. This count did not include setae along the lateral margin of the segment (Fig. 1C).
- P4C Number of microspinules in the proximal row ('C') of the P4 basipod, posterior face (Fig. 1C).
- P4N3 Number of spine-like setae on the P4 endopod, terminal segment (range was two to five spine-like setae) (Fig. 1C).
- P4X3 Number of spine-like setae on the terminal segment of the P4 exdopod. (range was two to four such setae) (Fig. 1C).

One binary variable.

- P4COUPL This character has two states (in our specimens), and was set = 0 if the spines of the P4 coupler were in a simple downward (proximally-pointing) arc; or = 1 if the outer spine on each side was clearly more distal than the adjacent spine producing an upward pointing arc (both states shown in Fig. 1C, as 'up' and 'down').

Observations and measurements were done with a phase contrast light microscope and an eyepiece micrometer. The characters PROS and RAMUS were measured at a magnification of 63× with a resolution of 11 μm. All other measurements and counts were made at a magnification of 400× and a resolution of 1.7 μm.

Variance partitioning

For each character, we partitioned the total variance into variance related to the field-lab dichotomy, and variance related to individual differences, site, symmetry, and measurement. It was beyond the scope of this study to re-measure all animals on both right and left sides, so we collected data for 21 specimens, measuring characters on both sides, and then re-measuring the same specimens again on the right side, several days after the original measurements. Animals were selected to include at least 2 individuals from each site, and both founders and lab animals were included (except for the Waubesa specimens, which were only founders).

Partitioning of variance is a concept that comes from the world of balanced anova designs for only fixed variables. Because of the nature of our data set, our analysis required a mixed model anova (fixed and random effects) with an unbalanced design. For this reason, the analysis reports first the significance of the only fixed effect (ENVIRONMENT: field vs. laboratory specimens), followed by the relative importance of the variances of the random (stochastic) effects. Variance was partitioned by simultaneous maximum likelihood estimation of the variance components using SAS PROC MIXED (2001). This procedure provides

the machinery to properly account for a mixture of random and fixed effects (Littell et al., 1996).

Once the effect of the fixed variable has been accounted for, this analysis is relatively insensitive to the number of levels (degrees of freedom) for each of the random independent variables, even though the design is unbalanced.

The SAS output does not lend itself directly to statements like 'Variability within individuals accounted for XX% of the total variability in the data', but it will yield conclusions such as 'For PROS, after accounting for differences among environments, the variability among individuals was comparable to the variability among sites (σ^2 = 43 vs σ^2 = 41, respectively), while measurement variability was much less (σ^2 = 4.6).' Finally, the SAS analysis correctly computes the denominator degrees of freedom for the *F*-test of environment (it should be the number of individuals, in this case 21 – this is achieved by nesting INDIVIDUAL in ENVIRONMENT).

The model includes five independent variables:

ENVIRONMENT – was designated as a fixed effect, with two levels: field or laboratory.

INDIVIDUAL – a random effect related to individual variation.

SITE – a random effect with six levels, reflecting the six sites sampled in this study.

SIDE – a random effect with two levels (left and right) nested in 'INDIVIDUAL' to measure the importance of asymmetry in measurements. These are measurements on the right and left side of a subset of animals. An index of asymmetry was calculated, using standardized data (see below, multivariate section), on an individual basis, as the difference between the value for the left side and the value for the right side. A perfectly symmetrical animal would have an index value of zero. This index was not used for variance partitioning, but was used as an additional test of symmetry – the average index for each character was compared to zero using a simple *t*-test.

MEASURE – a random effect with two levels (first measure and second measurement) nested in 'INDIVIDUAL'. These measurements were done on the same structure, but at least two days apart, to make them as independent as possible. RESIDUAL – a random variable used to account for any left-over variance in the model.

The dependent variable in the model is the SCORE for one of 13 measurements in the data set. We used the raw data score for each variable in the analysis of variance. P4COUPL was not included, because it only showed variability according to site. All Trek and Waubesa animals had one P4COUPL state, and the specimens from the other four sites showed the alternate state. The model used to explore variance in this system is:

SCORE = μ + ENVIRONMENT + INDIVIDUAL + SITE + SIDE (INDIVIDUAL) + MEASURE (INDIVIDUAL) + RESIDUAL

The variance estimates quoted in Table 4 are those that provided the best fit to the data, after the fixed effect (ENVIRONMENT) is accounted for. Sometimes the best-fitting parameters occur on the boundary of the allowable parameter space (i.e., the variance is given as 0), even though we can be certain that the true population-level variance must be greater than zero. When a value of zero variance is reported, this is not a sign that the model-fitting has failed, but is a consequence of the empirical simultaneous best estimate of all the variances. The variables PROS, P4CP, and P4COUPL, which have only one value per individual animal, will of course have zero variance for SIDE.

Multivariate analysis

The multivariate ordination analysis used all 120 specimens, but only 11 of the 14 possible morphological characters – three problematic characters (PROS, P4N3, and P4ANT) were removed at this stage in the analysis. The character PROS was removed because all the other counts and measurements were scaled to a standard body size. The characters P4N3 and P4ANT were removed from the data set, because they are known to be phenotypically plastic. The binary character P4COUPL was included in the ordination analysis. Thus, the morphological space was 11 dimensional (14 total characters minus PROS, P4N3, and P4ANT).

Each of the 11 morphological characters was standardized. Within a character, observations were standardized by subtracting the mean and dividing by the standard deviation for that character. Thus, each standardized character had a mean of zero and a standard deviation of 1.0. In other words, after standardization, the characters were equally weighted for the multivariate analysis. The effect of body size was then removed by regressing each standardized character with PROS. The residuals of this regression were taken as the values to be used in the multivariate analysis.

For multivariate analysis of morphological pattern using the 11 characters, we chose the Non-metric Multidimensional Scaling (NMS) technique (described in the PC-ORD manual, McCune & Mefford, 1999). The NMS technique produced a two-dimensional map, which when compared to the other PC-ORD techniques accounted for the largest amount of the total variation among all 120 individuals. We used a Euclidean distance measure, and tried several seed values to minimize the stress value. (Lower stress value indicates better 'goodness of fit'.)

Morphological and geographic distance

The average morphological position in NMS ordination space of nine isofemale lines (those used in breeding trials) was calculated by averaging the *x*- and *y*-coordinates for 3–5 individuals from each isofemale line. Mophological position in multivariate space was based on the standardized measurements corrected for body length. Pair-wise distances among the nine isofemale lines were calculated using the Euclidean equation and software from PC-ORD (McCune & Mefford, 1999).

Geographic distances were calculated using the spherical Euclidean modified for a spherical surface (the URL is http://jan.ucc.nau.edu/ cvm/latlongdist.php). Isofemale lines from the same site were scored as having zero geographic separation. Morphological and geographic distances were compared using Mantel's test (Mantel, 1967) facilitated by Version 3.0 of the R Package (Legendre & Vaudor, 1991).

Reproductive isolation

Breeding trials were done among 9 isofemale lines. Details of the methods of these crosses are given in Grishanin (unpublished data). An average Index of Reproductive Isolation (IRI) was calculated for each cross and its reciprocal. The IRI ranges from zero (maximum reproduction and survival) to 5 (embryos do not develop).

Results

Three additional taxonomic characters showed unexpected variability (Table 1). The character P4ANT, which was expected to be either present or absent within a species (Dodson, 1994), was observed to be variable among siblings. A few isofemale lines included both animals with and animals without spines. Similarly, the character P4X3 appears to be variable at the individual level, with siblings of the same isofemale line having either 3 or 4 spines, including one instance in which a founding female with 3 spines produced offspring with both 3 and four spines.

A third character, PROS, also varied according to ENVIRONMENT, with founding females significantly larger than their laboratory-raised offspring (Table 1). There are only five lines for which we measured both the field-caught progenitor female and two or more laboratory-raised offspring. In all five lines, the progenitor females averaged larger than their offspring, and only 2 of the 16 offspring were larger than their mother. The weighted average shows that offspring tend to be 0.17 mm shorter (prosomal length) than their mothers, or about 17% shorter.

Each of the 14 variables showed some degree of variation in the total data set (Table 2). In general, the coefficients of variation are in the range of 20–30%. The most variable characters were the smallest and hardest to see (P4ANT and P5) and thus the most subject to measurement error.

All the length measurements (RAMUS, P4NOUT, PRNIN, P4X3L, P5) were significantly correlated with PROS, the surrogate for body length (Table 3, the correlation coefficient for $n = 120$, and $\alpha = 0.01$ is about 0.23, Rohlf and Sokal 1981). All the length measurements (with the exception of P5) had an r^2 value of greater than 0.25. The count-variables were less strongly correlated with PROS.

Several of the length measurements (raw data) were also strongly correlated with each other. After the dependent variables had been corrected for the correlation with body length (variables were expressed as the residual of the regression on PROS), length characters were no longer significantly correlated with each other, with the one exception of P4NOUT AND P4NIN.

The total variance for each character (except for PROS) was partitioned among several independent variables (Table 4). ENVIRONMENT (field vs. laboratory) had a significant effect on all the length measurements, and no effect on the counts. After this fixed variable is taken into account, a substantial portion of the remaining variance was due to the factors SITE and to INDIVIDUAL variation. Measurement error and differences between right and left (symmetry) each typically accounted for less than 30% of the variation in the random variables. Exceptions are the trait P4CP, which had over 70% of its variance accounted for by MEASURE, and P4A, which had 56% of

Table 1. Three examples of variable characters: data are given for the first two characters, measured on animals collected in the field (Founders) or from isofemale lines cultured in the laboratory. Prosomal lengths are given for those lines with both the field-collected progenitor and two or more laboratory-raised offspring (n = number of lab animals)

Character	Isofemale line	Founder (field)	Laboratory reared
P4ANT (number of spinules)	021	unknown	0, 9, 10, 11, 11,16, 18
	110	unknown	0, 18
	138	unknown	0, 12
	148	unknown	0, 22
P4X3 (number of setae)	020	3	3,4
	026	3	3,3,4
	361	4	4
	369	4	4
	Trek1	4	4
PROS (mm in length)	020	0.96 mm	0.73 mm (n = 2)
	026	1.04	0.80 mm (n = 5)
	361	1.07	0.86 mm (n = 3)
	369	1.07	0.90 mm (n = 3)
	Trek1	0.85	0.84 mm (n = 3)

Table 2. The average and variance for 13 measurements from 120 animals (the binary character P4COUPL is not included here). For the measured characters, the units are micrometer units. The variances are partitioned in Table 4. CV = Coefficient of variation, the standard deviation divided by the mean, expressed as a percentage. For other symbols, see Text

	PROS	RAMUS	P4N3	P4CP	P4ANT	P4A	P4B	P4C	P4NIN	P4NOUT	P4X3	P4X3	LP5
Average	71.71	77.57	2.18	12.77	11.94	11.60	8.01	8.43	25.11	25.75	3.33	34.68	6.41
Variance	11.59	17.63	0.67	2.3	27.36	2.26	1.68	2.45	7.12	7.48	0.47	8.96	2.77
CV as%	16	23	31	18	62	20	21	29	29	28	14	28	43

its variance accounted for by SIDE. Trait P4A was the character that showed the least variation among sites (and is therefore probably the least likely to be a valuable taxonomic character).

In some cases, a variance component is listed as zero (Table 4), even though we can be certain that the population-level variance must be greater than zero. A zero value indicates either a small value (less than 1%), or it may just be the best-fitting variance decomposition for these particular data.

The specimens naturally showed some variation between the right and left sides (Table 5), but the average index of asymmetry (difference between sides) was always less than one standard deviation from zero. Thus, individual animals can be asymmetrical, but the population as a whole was symmetrical for each of the quantitative characters.

The two-dimensional NMS ordination graph (Fig. 2), using the 11 morphological characters, accounts for about 85% of the total variation among individuals (in two dimensions). The two NMS axes represent two independent linear combinations of the 11 morphological variables; each axis positions each individual according to covariance and association of the variables (McCune & Grace, 2002).

We have drawn boundaries around clusters of individual from the six sites (Fig. 2). Four populations are virtually congruent and therefore morphologically indistinguishable: Parejko, Shorts 1, Shorts 2, and Cleveland Ditch. The remaining two sites (Trek

Table 3. Pair-wise linear correlation coefficients among 14 morphological variables, including PROSOME, a surrogate body length. Correlation coefficients greater the 0.5 are in bold type

		PROS	RAMUS	P4NOUT	P4NIN	P4X3L	P5	P4ANT	P4CP	P4A	P4B	P4C	P4N3	P4X3
Lengths	RAMUS	**0.90**												
	P4NOUT	**0.71**	**0.71**											
	P4NIN	**0.64**	**0.64**	**0.93**										
	P4X3L	0.49	0.47	**0.62**	**0.65**									
	P5	0.37	0.45	0.15	–0.02	0.06								
Counts	P4ANT	0.21	0.20	–0.07	–0.23	–0.08	0.45							
	P4CP	–0.10	–0.18	–0.05	–0.04	–0.04	–0.21	–0.27						
	P4A	0.09	0.01	-0.02	0.00	0.01	–0.03	0.02	0.18					
	P4B	0.24	0.25	0.07	0.08	0.03	0.16	–0.09	0.28	0.31				
	P4C	0.42	0.44	0.10	0.06	0.02	0.28	0.34	–0.12	0.13	0.26			
	P4N3	0.25	0.22	**0.60**	**0.71**	0.47	–0.23	–0.33	0.05	0.04	–0.01	–0.21		
	P4X3	0.29	0.40	0.27	0.34	0.23	–0.03	–0.19	0.10	–0.06	0.22	0.07	0.30	
Binary	P4COUPL	–0.18	–0.24	–0.39	**–0.61**	–0.37	0.38	**0.52**	–0.16	–0.13	–0.17	0.16	**-0.61**	–0.39

Table 4. Partitioning of Variance for each of 13 characters, each character was measured on the same 21 animals. The binary character P4COUPL is not included in this analysis. Numbers are the percent of the variance due to the random variables, after variance due to ENVIRONMENT (field vs. laboratory-reared) has been accounted for. '0' = less than 0.001; '—' = variance for SIDE not available because the character does not occur on right and left sides

	Lengths						**Counts**						
Characters	PROS	RAMUS	P4NIN	P4NOUT	P4X3L	P5	P4N3	P4CP	P4ANT	P4A	P4B	P4C	P4X3
Fixed variable													
ENVIRONMENT *p*	<0.0001	<0.0001	<0.0001	<0.0001	<.0001	0.0032	0.952	0.251	0.911	.5917	0.0696	0.0750	0.081
Random variables													
Individual	50	10	33	96	98	8	17	28	27	31	16	57	70
Site	47	86	65	0	0	85	75	0	41	10	59	21	10
Measure (Indiv)	3	2	0	0	0	1	0	72	0	2	19	1	20
Side (Indiv)	–	0	2	4	2	5	0	–	29	56	6	20	0
Residual	0	3	1	0	0	1	8	0	3	1	0	1	0

Table 5. Symmetry index (= Left – Right, using standardized data) for each of 11 characters, based on measurements of 21 animals. None of the averages is significantly different from zero. The total data set is 14 morphological characters, but the characters PROS, P4CP and P4COUPL for which there is only one value per individual (no symmetry) could not be included here

	RAMUS	P4N3	P4ANT	P4A	P4B	P4C	P4NIN	P4NOUT	P4X3	P4X3L	P5
Average Index Value	–1.52	0	0.10	0.19	–0.10	0.10	0.95	–0.19	0	0.05	0.14
Standard Deviation	0.73	0	1.84	0.44	0.24	0.24	0.31	0.29	0	0.33	0.22

and Waubesa) are distinct from the first 4 sites, but probably not distinct from one another.

The two strongest correlations between NMS axes and non-plastic characters were for P4COUPL (first axis, r= 0.80; second axis r = –0.54) and P4NIN (–0.526 and 0.78). These are highly significant correlations (n = 74, $p << 0.001$). The pattern of positive and negative correlations with the two axes means

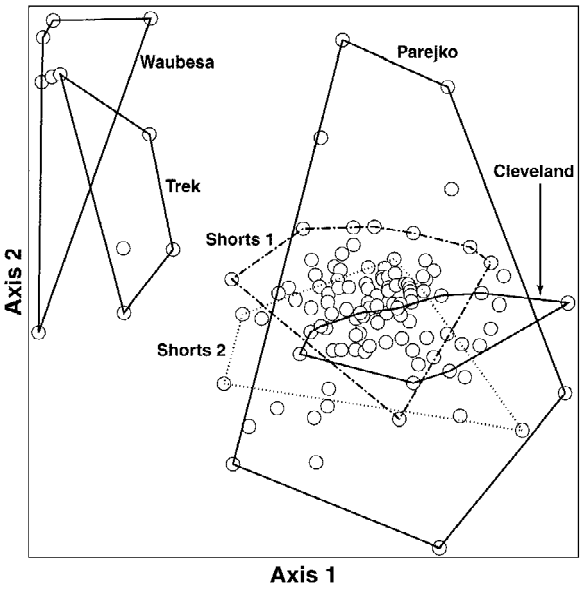

Figure 2. The 2-dimensional graph of the Non-metric Multidimensional Scaling (NMS) ordination. The ordination uses 11 morphological characters, from 120 specimens. Circles represent individuals. Boundaries are drawn around collections of individuals from each of the six sites. Circles in regions of overlapping boundaries indicate individuals that derive from one or the other of the two sites. All individuals were raised under 'common garden' conditions, except for the Lake Waubesa specimens.

Table 6. Linear correlation coefficients (Pearson's 'r') of each of 13 morphological variables with the 1st and 2nd axes of the NMS multivariate analysis. N = 120 individuals. Note that the variables are standardized according to mean, variance, and body length. The critical value for the correlation coefficient (á = 0.1, 119 df, uncorrected for multiple comparisons) is 0.179

Character	Axis 1	Axis 2
RAMUS	–0.001	0.016
P4NIN	–0.526	0.776
P4NOUT	–0.319	0.696
P4X3L	–0.283	0.613
P5	0.620	–0.482
P4CP	–0.503	–0.052
P4N3	–0.574	0.714
P4ANT	0.722	–0.490
P4A	–0.218	–0.321
P4B	–0.350	–0.428
P4C	0.277	–0.484
P4X3	–0.506	0.188
P4CPL	0.796	–0.544

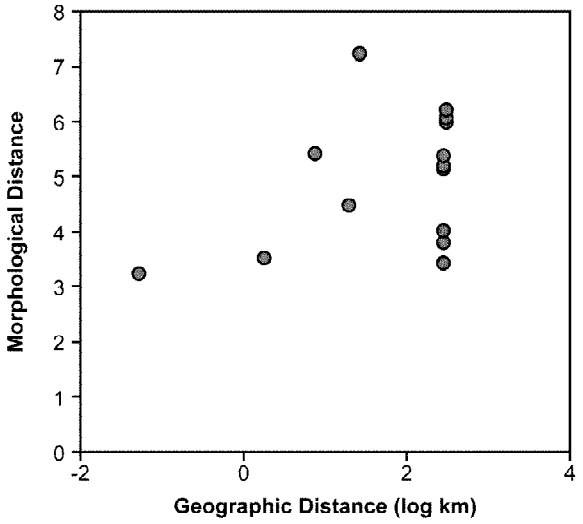

Figure 3. The relationship between geographic distance and morphological distance.

that specimens with the 'up' condition of P4COUPL tended to have short terminal spines (P4NIN) and specimens with the 'down' condition of P4COUPL tended to have long spines (P4NIN). The r^2 values for these two characters are 64–28%, suggesting that there is still a lot of unexplained morphological variance. This is the situation mentioned in the Introduction: great morphological similarity with some tantalizing but slight differences.

Because Figure 2 represents the large majority (85%) of the total morphological variation using just two dimensions, it makes a good visualization of the average morphological distances of individuals from different sites. A comparison of 2 dimensional morphological and geographic distances using Mantel's test and the data in Table 6 showed no significant correlation ($n = 36$, $p = 0.26$).

The Index of Reproductive Isolation (IRI) values tend to be zero (no reproductive isolation) for different isofemale lines within a site, and for crosses within an isofemale line (Table 6). For crosses between lines from different sites, there was not a significant correlation between IRI and geographic distance (re-

gression analysis, $p = 0.09$, $n = 16$) or for IRI and morphological distance ($p = 0.78$).

Discussion

This study revealed cryptic speciation in the *A. vernalis* species group, with little correlation between morphology and reproductive isolation. Characters traditionally used for systematics in this species group are subject to environmental plasticity. In addition, we found no correlation between morphological and geographic distances.

Body length is not a particularly useful taxonomic character, because it depends on environmental factors such as temperature and nutrition. On the other hand, correlated variables that are independent of body length and environment could be taxonomically useful. Our results failed to produce groups of correlated characters, once the effect of body size was removed.

The desired result of ordination analysis is to identify characters that vary in concert, producing clusters of tight and distinct points in multivariate space. What we see in Figure 2 is only a moderate separation of individuals and a small amount of clustering. The two diffuse clusters can be interpreted as evidence for the existence of two morphological forms. These two clusters appear to be separated according to site.

Our results show that both environment (field vs. laboratory) and site account for substantial portions of the variance in morphological characters. The results emphasize the importance of using specimens reared under common garden conditions for systematic studies.

Also, axonomists are encouraged to look at a large number of sites. Six is clearly too few sites to give a general idea of the pattern of morphological variation within this cyclopoid species complex, if such a real pattern exists. For example, in our ordination, are the gaps between clusters of species a general pattern, or are the gaps only due to the small sample size? An ordination based on ten times as many sites might be much more informative, and might be sufficient to allow a taxonomist to draw conclusions about the number of morphological species.

Our data suggest that it is reasonable to continue identifying individuals in the *A. vernalis* complex, without taking into account either measurement error or symmetry. Both these sources of variance tended to be minor, compared to environment and site. That is, it is reasonable to measure a specimen once, and to use data from either the right or left side.

The results of this morphological analysis, especially of the NMS ordination, suggest that it is premature to decide which characters best separate species in this complex. It is clear that body size (PROS), P4ANT, and P4X3L are not reliable, because they vary within isofemale lines.

Three characters are most likely to be taxonomically valuable. P4COUPL and P4NIN are strong candidates, because of their correlation with the two NMS ordination axes. (P4NIN was used in Dodson [1994] as a character that separated *A. vernalis* and *A. robustus*.) P4COUPL is a new character for the *A. vernalis* group, first observed in this study. It is present in all the individuals from Trek and Waubesa, and absent from all specimens of the other four sites. Thus, P4COUPL may be a valuable character, but our sample size is just too small to evaluate how this character varies over the landscape, and how it varies with other characters.

Our sample size of sites is clearly too small to support a general conclusion about the relationship between geographic separation and morphological similarity. We see that sites separated at the scale of m to km tend to be morphologically similar. However, sites separated by 300 km can be just as similar. In our data set, the morphologically most distinct sites are also the furthest apart, but this may well be a result of small sample size.

With further study, we might find that the Trek and Waubesa sites represent populations of a biological species that could be called *A. robustus*, and the remaining four sites represent sites of biological species in the *A. vernalis* species complex. Individuals from these four sites are similar, with overlapping areas of morphological variation (Fig. 2).

Morphological stasis is the phenomenon of the persistence of extreme morphological similarity over time, despite reproductive isolation (Wake et al., 1983). Our copepods appear to be expressing morphological stasis. Other studies have reported a similar situation. Smith (1981) came to similar conclusions for 20 populations representing the *Acanthocyclops* vernalis species group in southeastern Wisconsin. He identified at least 6 reproductive isolates most of which were not morphologically distinct. Smith (1981) reported ambiguous results for reproductive isolation among 7 populations of *Diacyclops bicuspidatus*, but Monchenko (2000) reported three reproductive isolates from a study of four Kiev and Crimea sites. Price (1958) reported 7 reproductive isolates (biological

Table 7. Average pair-wise distances among 9 isofemale lines. The morphological distances are calculated using laboratory animals only. Geographic distances are straight line distance in km. Index of Reproductive Isolation (IRI, from Grishanin unpublished data) for 20 crosses among some of the nine laboratory isofemale lines

Morphological distances		**S026**	**S102**	**S115**	**S130**	**S142**	**S360**	**S361**	**S369**	**Trk1**
	S026	0	1.61	1.43	1.24	1.42	1.68	1.46	1.05	1.62
	S102	1.61	0	0.53	0.47	0.48	0.77	0.45	0.19	1.61
	S115	1.43	0.53	0	0.21	0.07	1.07	0.75	0.72	1.34
	S130	1.24	0.47	0.21	0	0.23	1.14	0.8	0.66	1.21
	S142	1.42	0.48	0.07	0.23	0	1	0.68	0.66	1.39
	S360	1.68	0.77	1.07	1.14	1	0	0.34	0.67	2.34
	S361	1.46	0.45	0.75	0.8	0.68	0.34	0	0.41	2.01
	S369	1.05	0.19	0.72	0.66	0.66	0.67	0.41	0	1.77
	Trk1	1.62	1.61	1.34	1.39	1.39	2.34	2.01	1.77	0
Geographic distances		**S026**	**S102**	**S115**	**S130**	**S142**	**S360**	**S361**	**S369**	**Trk1**
	S026	0	1.8	1.8	1.8	1.8	274.2	274.2	274.2	280.3
	S102	1.8	0	0	0	0	275.4	275.4	275.4	281.6
	S115	1.8	0	0	0	0	275.4	275.4	275.4	281.6
	S130	1.8	0	0	0	0	275.4	275.4	275.4	281.6
	S142	1.8	0	0	0	0	275.4	275.4	275.4	281.6
	S360	274.2	275.4	275.4	275.4	275.4	0	0	0	7.3
	S361	274.2	275.4	275.4	275.4	275.4	0	0	0	7.3
	S369	274.2	275.4	275.4	275.4	275.4	0	0	0	7.3
	Trk1	280.3	281.6	281.6	281.6	281.6	7.3	7.3	7.3	0
INDEX OF REPRODUCTIVE ISOLATION		**MALES ACROSS**								
		S026	**S102**	**S115**	**S130**	**S142**	**S360**	**S361**	**S369Trk1**	
FEMALES VERTICAL	**S026**	0			3.8				4.8	
	S102		0	2.5	3.9			3.6		
	S115		3.9	2.4					3.9	
	S130	4.8	5		0	0		3.9		3.5
	S142				0.8	0.8	3.4			
	S360					4	0			
	S361		4		5			0		
	S369	4.8		3.7				0	0	
	Trk1				4.8					0

species) among 30 populations of *Acanthocyclops vernalis* from the Toronto, Ontario region. He claimed some of the isolates were morphologically distinguishable, but the differences were based on what our study has shown to be morphological plasticity. Lee & Frost (2002) found that different populations of the same morphological species (*Eurytemora affinis*) were morphologically similar, even when they were separated by thousands of km. However, these populations were reproductive isolated. Thus, there appears to be considerable evidence for morphological stasis as a common phenomenon in copepods.

The breeding trials are preliminary in the sense that they are not between enough isofemale lines from a wide range of geographic and morphological separation. Generalization is premature, but our IRI results suggest that there is one biological species per site. This is because the IRI values for crosses between strains from the same site, or for the control crosses within isofemale lines were mostly near zero (no isolation). However, all crosses between lines from different sites had high IRI values, independent of geographic separation or morphological similarity. These

preliminary results invite more crosses, over a range of geographic and morphological distances.

Observations of Lajus & Alekseev (2000) suggest that three morphologically distinct populations of *Acanthocyclops signifer* live at different points along the shore of a single lake, albeit a very large lake, Lake Baikal. Our results lead us to predict that the morphological differences shown by *A. signifer* are either the expression of phenotypic plasticity resulting from environmental differences, or that they represent reproductively-isolated cryptic species.

Marine examples also suggest a high level of morphological stasis. Knowlton (2000) concluded that in many marine groups, a morphological approach has lead to excessive lumping of cryptic biological or molecular-criterion species. She found that morphologically similar species were often distinct using molecular markers, suggesting the prevalence of morphological stasis. Ganz & Burton (1995) found two reproductive isolates in five Pacific coast intertidal sites. These five populations were all molecularly distinct and morphologically very similar.

It is clear that extensive field and laboratory studies are needed to evaluate the usefulness of morphological characters to the taxonomy of cyclopoid copepods. To make progress in understanding biodiversity in cyclopoid species complexes, we recommend using large sample sizes of individuals per site, scores of sites, and common garden experiments to separate environmental from genetic effects. Results of common garden experiments can also be used to provide data for quantitative genetics analyses, to measure the heritability of different characters, and thus to be able to avoid using plastic characters. Breeding trials are also necessary to understand the relationship between morphological similarity and reproductive isolation.

Our results suggest the following conclusions concerning the *Acanthocyclops vernalis* species group:

- Morphological characters used previously to distinguish species are unreliable because they show phenotypic plasticity between field and laboratory members of isofemale lines.
- These organisms are symmetrical.
- Measurement error is only a small part of the total morphological variation.
- Morphology is conservative among sites and does not reflect reproductive isolation
- Morphological similarity is independent of geographic among sites, between 0.05 and 300 km.
- Reproductive isolation is minimal between animals from within sites, but nearly complete when two sites are compared.
- Reproductive isolation is independent of morphological difference or geographic separation.
- An extreme but consistent interpretation of our data is that the *Acanthocyclops* populations at each site are distinct biological species, with indistinguishable morphologies.
- Because of the effect of site and environment on morphology, we recommend using much larger collections as a basis for future taxonomic research.

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