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Genetic diversity and polyploidy in the cosmopolitan asexual ostracod *Cypris pubera*

TOM J. LITTLE

MAX-PLANCK INSTITUT FÜR LIMNOLOGIE, AUGUST–THIENEMANN-STRAßE 2, 24306 PLÖN, PLÖN, GERMANY

PRESENT ADDRESS: INSTITUTE FOR EVOLUTIONARY BIOLOGY, UNIVERSITY OF EDINBURGH, KINGS BUILDINGS, WEST MAINS ROAD, EDINBURGH EH9 3JT, SCOTLAND

CORRESPONDING AUTHOR: tom.little@ed.ac.uk

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Freshwater ostracods show extensive breeding system variation, but both the cause of breeding system transitions, and the origins of genotypic diversity in asexuals, have yet to be convincingly explained. The study reports on the genotypic (based on allozyme electrophoresis) characteristics of Northern European populations of the globally distributed ostracod Cypris pubera. No male has ever been described for C. pubera, and none were found in this study. Genotypic profiles indicated deviations from Hardy–Weinberg (HW) equilibrium, confirming that C. pubera reproduces through ameiotic parthenogenesis, that is, the production of female offspring where the formation of ova is through a mitotic process. Cypris pubera seems to be comprised of diploid clones that are brown in colour and a series of polyploid clones that are green. The two morphs often occurred in sympatry, but the green morph was considerably more diverse than the brown. The factors which contribute to asexual diversity or elevated ploidy levels in other taxa, such as hybridization, do not appear to be important for C. pubera. Thus, this study has established a link between polyploidy and genetic diversity in C. pubera, but the origins of both asexuality and polyploidy remain unclear for this ecologically successful ostracod.

INTRODUCTION

The ostracoda are notable for numerous features of broad interest to evolutionary biologists and ecologists (reviewed in Chaplin *et al.*, 1994). Although marine ostracods are exclusively sexually reproducing, freshwater forms show extensive breeding system variation: within species there is among-population variation in reproductive mode, and within genera there are species that reproduce exclusively asexually, while other species reproduce only through sex. Above the species level, genera composed entirely of asexuals are observed, as are genera containing only sexual species. The ostracoda even provide a rare example of a higher taxonomic level (the family Darwinulidae) that is entirely asexual, indicating the possible long-term persistence of asexual forms. Coupled with this breeding system diversity is a tendency for asexuals to show ploidy level elevation. Exclusively sexually reproducing freshwater taxa also show exceptional features, in particular, sexual ostracods consistently display skewed sex ratios. Most of these peculiar features of the ostracoda have yet to be convincingly explained or correlated with ecological features. Thus, freshwater ostracods remain an enigmatic group ripe for studies on a range of key evolutionary issues.

Cypris is a freshwater ostracod genus first described by Sars (Sars, 1928). *Cypris pubera* is a large species (~3 mm in length) with an essentially global distribution. Like many ostracods, *C. pubera* is not strictly part of the plankton, though they are not wholly benthic either. Males have never been described for *C. pubera*, and it is thus assumed to reproduce solely by parthenogenesis. However, no genetic studies have yet been performed on *C. pubera*, and so its asexuality has yet to be confirmed with tests for the genetic disequilibria known to accompany parthenogenesis. Confirming breeding system through genetic approaches is crucial, because the rarity of males in many sexually reproducing

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taxa could lead to false conclusions on the occurrence of asexuality.

In addition, the apparent ecological success of this cosmopolitan species demands explanation. Parthenogenetic organisms are limited in their ability to generate genotypic diversity and thus should have a limited ability to evolve in response to environmental change. This constraint, coupled with the inability of asexuals to arrest the accumulation of deleterious recessive mutations (Muller, 1964), leads to the prediction that parthenogenetic lineages should have limited evolutionary and ecological potential. Most obligately parthenogenetic lineages do indeed appear to be recently derived (Crease *et al.*, 1989; Avise *et al.*, 1992; Chaplin *et al.*, 1994). However, as is the case for *C. pubera*, many parthenogens have broader geographic distributions than related sexual species, indicating that a lack of genetic flexibility does not prevent short-term success (e.g. Lynch, 1984). The evolutionary success of parthenogens may be determined by the amount of genetic variation they possess. Indeed, many parthenogenetic taxa possess substantial genetic variation (Hebert *et al.*, 1988). It is not always clear what processes contribute to asexual variability, but a role for hybridization and/or polyploidy has frequently been suggested (e.g. Little *et al.*, 1997; Turgeon and Hebert, 1994; Little and Hebert, 1997).

Thus, this study had two goals. First, to subject *C. pubera* to genetic studies that can confirm its mode of reproduction. Second, this study sought to determine levels of genetic diversity and polyplopidy, such that insight in to the ecological success of this asexual taxon might be gained.

METHOD

Ostracods were collected from nine small farm ponds within a 40 km radius of the Max-Planck Institute for Limnology, in Ploen, Northern Germany. Samples were obtained by passing a plankton net (200-μm mesh) through aquatic vegetation at the pond margin. Collections were made from April to August 1997, and there was one collection site per pond, except at the large Lebrade Teich where there were two collection sites at opposite ends of the habitat. Samples were brought to the laboratory where individual ostracods were examined under a microscope and identified to the species level using Bronstein (Bronstein, 1988). Following identifications, individuals were immediately subject to allozyme electrophoresis.

Allozyme phenotypes were discriminated with cellulose acetate electrophoresis (Hebert and Beaton, 1993). Twenty-four individuals from each of four populations were initially screened for variation at 15 enzymes including aldehyde oxidase (AO) (EC 1.2.1), amylase (AMY) arginine phosphokinase (APK) (EC 2.7.2), aspartate aminotransferase (AAT) (EC 2.6.1.1), fumarate hydratase (FUM) (EC 4.2.1.2), glucophosphate isomerase (GPI) (EC 5.1.9), isocitrate dehydrogenase (IDH) (EC 1.1.1.42), lactate dehydrogenase (LDH) (EC 1.1.1.27), malic enzyme (ME) (EC 1.1.1. 40), malic dehydrogenase (MDH) (EC 1.1.1.37), mannose phosphate isomerase (MPI) (EC 5.1.8), peptidase-A (leucyl-glycine) (PEP-A) (EC 3.1.13), PEP-A-2, peptidase-C (phenyl-proline), and phosphoglucomutase (PGM) (EC 2.7.5.1). IDH and MDH did not stain, while AO showed a multi-banded phenotype that proved difficult to interpret. All other but loci resolved clearly but only three were polymorphic (GPI and PGM and MPI) in the initial screening. Individuals from the remaining populations were analysed for variation at only these three loci. Thus, it was assumed that the initial screen, because it includes individual from multiple populations, was sufficient to determine the proportion of polymorphic loci in this geographic location. Individuals from Rixdorf pond, which was dominated by a single clone, were used as internal standards on all gels to ensure consistency in allelic designations.

For each population, allozyme electromorphs were labelled based on their mobility. There was a maximum of four electromorphs per locus, and these were labelled (from slowest to fastest mobility) 's', 'm', 'f' and 'v'. Allozyme bands with unique electrophoretic mobility were assumed to correspond to unique alleles, and distinct multi-locus genotypes were termed clones, but with the following caveat: individuals sharing the same allozyme phenotype may posses amino acid substitutions that do not result in detectable mobility differences or they might differ at loci not assayed. Thus, an allozymic 'clone' likely represents a clonal group whose members happen to share the same allozyme phenotype.

Although heterozygous phenotypes at polymorphic loci were often consistent with the known quaternary structure of the enzymes, aberrant (unbalanced) phenotypes, typical of polyploids, were also detected (Beaton and Hebert, 1988; Hebert and Beaton, 1993; Little and Hebert, 1994). For monomeric enzymes, a phenotype was considered unbalanced if one of the bands of the two-banded heterozygote phenotype stained more intensely than the other. For dimeric enzymes, diploid taxa express a three-banded phenotype with the middle band being the most intense. For polyploids, however, dimeric enzymes may show more intense staining at a band other than middle one (Hebert and Beaton, 1993).

I tested the assumption that populations are comprised of sexual diploids. Congruence (based on a χ^2 test) of genotypic frequencies to Hardy–Weinberg (HW) expectations was examined after pooling balanced and unbalanced heterozygous phenotypes into a single heterozygote class. Individual heterozygosities were measured by direct count and expected heterozygosities (assuming random recombination and outcrossing) were also estimated. From these measures of heterozygosity, Wright's *F*-statistic was calculated. Lastly, clonal diversity in each population was estimated using Simpson's (Simpson, 1949) index of diversity modified for finite populations (Pielou, 1969).

RESULTS

No males were detected in any of the nine populations of *C. pubera* suggesting the predominance of parthenogenetic reproduction. Although specimens were easily keyed out as *C. pubera* using standard morphological features (Bronstein, 1988), there appeared to be two discrete morphological colour variants—a large green morph and a small brown morph.

Allozyme variation in *C. pubera* was detected at three of 12 resolvable loci, thus the proportion of polymorphic loci was 0.25. There were four alleles at GPI, three alleles at PGM and two alleles at MPI (Table I). There were 23 unique multi-locus electrophoretic genotypes and an average of four clones per population (considering the two Lebrade samples as separate). The mean observed heterozygosity, averaged across all loci, was 9.9% (SE = 1.07), which was higher than mean expected heterozygosity of 6.93% (SE = 1.07; paired *t*-test comparing observed and expected heterozygosity, $t = 2.79$, $P = 0.011$), as reflected in the *F* statistic (Table I). The brown morph showed an observed heterozygosity of just 4.9% (SE = 2.03), and a paired *t*-test comparing observed heterozygosity between brown and green clones in those populations that contained both types, indicated a marginally insignificant difference at the 95% level ($t = 1.85$, $P = 0.069$). Simpson's diversity index (Table II) also indicated less polymorphism in the brown morph (paired *t*-test, $t = 2.77$, $P = 0.0503$).

In all but one sample (Lebrade site 2) deviations from HW equilibrium were detected at least one polymorphic allozyme locus. Aberrant heterozygous phenotypes, typical of polyploids, were common (Table I) and were exclusively associated with the green morph. These polyploidy phenotypes never involved the occurrence of more than two different alleles but, rather, showed band intensities suggestive of an unequal number of gene copies (Hebert and Beaton, 1993).

DISCUSSION

Confirming previous taxonomic surveys, no males were detected in populations of *C. pubera*. Accordingly, the electrophoretic analysis revealed patterns of genotypic variation indicating that this ostracod species does not reproduce sexually. Cyclical parthenogenesis or environmentally induced sexuality occurs in other crustaceans, but such reproductive strategies have never been recorded in the ostracoda. Thus, it appears that *C. pubera* reproduces through obligate ameiotic parthenogenesis. This study has also established that many clones of *C. pubera* are polyploid. In particular, 14 of the 20 unique electrophoretic clones possessed unbalanced allozyme phenotypes which are indicative of polyploidy (Beaton and Hebert, 1988; Hebert and Beaton, 1993; Turgeon and Hebert, 1994; Little and Hebert, 1997). *Cypris pubera* also appears to exist in two morphological forms: a green morph and a brown one. All of the polyploids were of the green morph, while the brown morph appeared to be a diploid.

Ignoring for the moment the difficulty of applying species concepts to asexual organisms, it is possible that the green and brown morphs are simply separate species. However, the allozyme results do not support this. In particular, both morphs were identical at the nine monomorphic loci and consistently shared electromorphs at the polymorphic loci. Moreover, in two cases (clones 2a, b and 10a, b; Table I), the brown and green morphs were allozymically identical, while in a third case (clones 19a, b; Table I) the two clones showed banding patterns with identical mobilities, but the green morph had unbalanced heterozygous phenotypes. Thus it appears that *C. pubera* comprises a brown asexual diploid and a green asexual polyploid, with the polyploidy morph showing greater diversity (Tables I and II). Patterns of diversity and breeding system variation linked to colour morph were also reported in a freshwater ostracod from Australia (Chaplin, 1992; Chaplin and Ayre, 1989).

Parthenogenetic and polyploidy taxa are often formed through hybridization (Stebbins, 1950; Dufresne and Hebert, 1994; Soltis and Soltis, 1995). However, hybridization results in high heterozygosity (e.g. Dawley and Bogart, 1989; Tinti *et al.*, 1995), and thus interspecific hybridization seems unlikely to be linked to parthenogenesis in *C. pubera*, because this species showed only modest heterozygositites. In ostracods, following the shift to parthenogenesis, polyploidy may arise when ameiotically produced ova are fertilized by the sperm of a closely related male, which is a kind of hybridization (Turgeon and Hebert, 1994). If *C. pubera* has become polyploid in a similar fashion, the sexual

Table I: Summary of allozyme phenotypes, clonal abundances, heterozygosity and F statistics for the ostracod Cypris pubera collected from ponds on in Northern Germany

Clone	Morph	PGI	PGM	MPI	Lebrade (15 May)	Lebrade site 2 (10 June)	Rixdorf (18 May)	Bucholz (22 May)	Flat (30 May)	Lubbersdorf (21 June)	Kirch (17 July)	Hound (23 July)	Krumm (23 July)	Alten Kremp (26 July)
1	G	Mm	Sm*	sm*	27	5	60	30						
2g	G	Mm	Mm	sm	25									
2b	B	Mm	Mm	sm	5						19			
3	G	m*f	Sm*	mm	17									
4	G	Sf	Ss	mm	2	1			1			20		
5	G	Sm*	Sm*	S*m	5	3								
6	G	Ss	Ss	mm	1									
7	G	Sm*	Sm*	mm	1									
8	G	Mm	Sm*	sm	1									
9	G	Mm	Mm	sm*				6						
10g	G	Sm	Ss	mm									22	1
10b	B	Sm	Ss	mm				10						3
11	G	Sm	Sf*	mm					34					
12	B	Ff	Ss	mm					6					
13	G	Mf	Ss	mm		15				1		2		6
14	G	m*v	Mm	mm		1								
15	G	m*f	Sm*	sm*		1								
16	G	m*f	Mm	sm		1								
17	G	s*f	Mm	s*m		2								
18	G	mm	Ss	mm		1								8

(continued)

Table I: Continued

Clone	Morph	PGI	PGM	MPI	Lebrade (15 May)	Lebrade site 2 (10 June)	Rixdorf (18 May)	Bucholz (22 May)	Flat (30 May)	Lubbersdorf (21 June)	Kirch (17 July)	Hound (23 July)	Krumm (23 July)	Alten Kremp (26 July)
19b	B	mm	Ss	mm		10								
19g	G	mf*	S*f	mm						2				
20	G	mm	Mm	ss						35				
21	G	sm	s*f	mm						8				
22	G	sm	s*f	mm						1				
22	G	mf*	Mf	sm						1				
23	G	sf	Mm	mm							3			
Number of clones					9	9	1	3	3	6	2	2	1	4
Sample size					84	40	60	46	41	48	22	22	22	18
Observed heterozygosity					0.111	0.094	0.167	0.137	0.142	0.045	0.083	0.083	0.083	0.046
Expected heterozygosity					0.100	0.088	0.056	0.097	0.092	0.087	0.061	0.045	0.042	0.037
Wrights F					−0.114	−0.064	−1.985	−0.412	−0.537	0.483	−0.354	−0.847	−1.000	−0.255
Hardy–Weinberg					1 of 3	0 of 3	2 of 2	1 of 3	2 of 2	2 of 3	2 of 2	1 of 1	1 of 1	1 of 1

Clones 2 and 10 were comprised of a mixture of green and brown morphs with identical allozyme phenotypes. Clone 19 also comprised both colour morphs but showed different staining intensities of bands with identical mobilities. B, brown morph; G, green morph; MPI, mannose phosphate isomerase; PGI, phosphoglucose isomerase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase. Asterisks designate the more intensely stained band of an unbalanced allozyme phenotype. The row designated Hardy–Weinberg denotes when a significant ($P < 0.05$) Hardy–Weinberg deviation was detected in X of Y polymorphic loci per population.

Table II: Simpson's diversity index to account for clonal diversity in populations of the freshwater ostracod Cypris pubera

	Lebrade 1	Lebrade 2	Rixdorf	Bucholz	Flat	Lubbersdorf	Kirch	Hound	Krumm	Alten Kremp	Total (all populations combined)
Brown and green morphs combined	0.768	0.741	0.000	0.522	0.298	0.447	0.247	0.173	0.000	0.699	0.885
Green morph only	0.741	0.726	0.000	0.286	0.057	0.447	NA	0.173	0.000	0.590	0.838
Brown morph only	0.000	0.000	NA	0.000	0.000	NA	0.000	NA	NA	0.000	0.700

There were two distinct colour morphs in these populations. NA indicates populations where only a single colour morph was present.

populations involved in this process were not detected in Northern Germany. However, it does not seem likely that polyploidy in *C. pubera* has arisen through matings with a sexual taxon, as these cases often show the presence of three or more distinct alleles at a locus (e.g. Turgeon and Hebert, 1994). *Cypris pubera* never appeared to possess more than two alleles at a single locus. Elevated ploidy in *C. pubera* may have arisen through internal genomic duplications, as is common in plants (e.g. Stebbins, 1950; Dufresne and Hebert, 1994; Soltis and Soltis, 1995). Higher resolution techniques such as microsatellites would help to gain a fuller impression of polymorphism in this species and, in particular, might help to gain a precise measurement of ploidy level. This in turn would aid in heterozygosity estimates.

Another source of genotypic diversity in asexuals occurs when a sexual group makes multiple transitions to asexuality. In this case, the levels of genotypic diversity present in the original sexual group may be mirrored in the newly formed asexuals. Such a process has been observed for the crustacean *Daphnia* (Crease *et al.*, 1989). In any taxon, verification of this process requires the detection of the source sexual populations. Although *C. pubera* has historically been sampled widely, and males have not been found (Bronstein, 1988), the occurrence of rare sexual populations would not be surprising. For example, the ostracod *Cyprinotus incongruens* is cosmopolitan in the northern hemisphere, but sexual populations have only rarely been detected (Rossi *et al.*, 1993). Similarly, *Cytherissa lacustris* is sexual in Lake Baikal, but males are absent throughout the remainder of its broad Eurasian range (Bronstein, 1988).

It remains conceivable that that some genetic diversity has arisen from mutations following the loss of sexuality. As mutational contributions to genotypic variability are expected to increase slowly, this substantial levels of genotypic variability in *C. pubera* raises the possibility that it is a relatively ancient asexual (Schon *et al.*, 2003). Alternatively, Song *et al.* (Song *et al.*, 1995) have shown that the genomes of newly formed polyploids may diversify markedly over just a few generations of reproduction by selfing (Soltis and Soltis, 1995). However, *C. pubera* did not show the high levels of homozygosity typical of selfers. Thus, the origin(s) of asexuality and genotypic diversity in *C. pubera* remain obscure, as is the case for other ostracod taxa. Determining these origins may help explain the widespread occurrence of asexuality in the ostracods, or the apparent ecological success of some taxa.

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REFERENCES

Avise, J. C., Quattro, J. M. and Vrijenhoek, R. C. (1992) Molecular clones within organismal clones: mitochondrial DNA phylogenies and the evolutionary histories of unisexual vertebrates. *Evol. Biol.*, **26**, 225–246.

Beaton, M. J. and Hebert, P. D. N. (1988) Geographical parthenogenesis and polyploidy in *Daphnia pulex*. *Am. Nat.*, **132**, 837–845.

Brostein, Z. S. (1988) *Fresh-Water Ostracoda*. A.A. Balkema, Rotterdam.

Chaplin, J. A. (1992) Variation in the mode of reproduction among individuals of the ostracod *Candonocypris novaezelandiae*. *Heredity*, **68**, 411–424.

Chaplin, J. A. and Ayre, D. J. (1989) Genetic evidence in the contribution of sexual and asexual reproduction to populations of the freshwater ostracod *Candonocypris novaezelandiae*. *Freshw. Biol.*, **22**, 275–284.

Chaplin, J. A., Havel, J. E. and Hebert, P. D. N. (1994) Sex and Ostracods. *Trends Ecol. Evol.*, **9**, 435–439.

Crease, T. J., Stanton, D. J. and Hebert, P. D. N. (1989) Polyphyletic origins of asexuality in *Daphnia pulex*.2. Mitochondrial-DNA variation. *Evolution*, **43**, 1016–1026.

Dawley, R. M. and Bogart, J. P. (1989) *Evolution and Ecology of Unisexual Vertebrates*. New York State Museum, Albany.

Dufresne, F. and Hebert, P. D. N. (1994) Hybridization and the origins of polyploidy. *Proc. R. Soc. Lond., B*, **258**, 141–146.

Hebert, P. D. N. and Beaton, M. J. (1993) *Methodologies for Allozyme Analysis Using Cellulose Acetate Electrophoresis*. Helena Laboratories, Beaumont, Texas.

Hebert, P. D. N., Ward, R. D. and Weider, L. J. (1988) Clonal-diversity patterns and breeding-system variation in *Daphnia pulex*, an asexual-sexual complex. *Evolution*, **42**, 147–159.

Little, T. J., Demelo, R., Taylor, D. J. *et al.* (1997) Genetic characterization of an arctic zooplankter: insights into geographic polyploidy. *Proc. R. Soc. Lond., B*, **264**, 1363–1370.

Little, T. J. and Hebert, P. D. N. (1994) Abundant asexuality in tropical freshwater ostracodes. *Heredity*, **73**, 549–555.

Little, T. J. and Hebert, P. D. N. (1997) Clonal diversity in high arctic ostracodes. *J. Evol. Biol.*, **10**, 233–252.

Lynch, M. (1984) Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Q. Rev. Biol.*, **59**, 257–290.

Muller, H. J. (1964) The relation of recombination to mutational advance. *Mutation Res.*, **1**, 2–9.

Pielou, E. C. (1969) The measurement of diversity in different types of biological collections. *J Theor. Biol.*, **13**, 131–144.

Rossi, V., Giordano, P. and Menozzi, P. (1993) Genetic variability in parthenogenetic populations of *Heterocypris incongruens* (Crustacea, Ostracoda). In K. G. Mckenzie and P. J. Jones (eds), *Ostracoda in the Earth and Life Sciences*. A.A. Balkema, Rotterdam, pp. 369–383.

Sars, G. O. (1928) *An Account of the Crustacea of Norway*. Bergen Museum, Oslo.

Schon, I., Martens, K., Van Doninck, K. *et al.* (2003) Evolution in the slow lane: molecular rates of evolution in sexual and asexual ostracods (Crustacea: Ostracoda). *Biol. J. Linn. Soc.*, **79**, 93–100.

Simpson, E. H. (1949) Measurement of diversity. *Nature*, **163**, 668.

Soltis, D. E. and Soltis, P. S. (1995) The dynamic nature of polyploid genomes. *Proc. Natl. Acad. Sci. U. S. A.*, **92**, 8089–8091.

Song, K., Ping, L., Keliang, T. *et al.* (1995) Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. *Proc. Natl. Acad. Sci. U. S. A.*, **92**, 7719–7723.

Stebbins, G. L. (1950) *Variation and Evolution in Plants*. Columbia University Press, New York.

Tinti, F., Mantovani, B. and Scali, V. (1995) Reproductive features of homospecific hybridogenetically-derived stick insects suggest how unisexuals can evolve. *J. Evol. Biol.*, **8**, 81–92.

Turgeon, J. and Hebert, P. D. N. (1994) Evolutionary interactions between sexual and all-female taxa of *Cyprinotus* (Ostracoda: Cyprididae). *Evolution*, **48**, 1855–1865.