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Verification of cyprid size as a tool in the identification of two European species of *Chthamalus* barnacles using mtDNA-RFLP analysis

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ABSTRACT: Adult barnacles of *Chthamalus stellatus* and *Chthamalus montagui* were collected in August 1998, from Garrettstown Co. Cork and Kilkee Co. Clare, in southwest and west Ireland, respectively. Attached cypris larvae were collected during the settlement season of *C. stellatus* and *C. montagui*, on 2 shores in Co. Cork and 1 shore on Clare Island, Co. Mayo in west Ireland. Cyprid collections were made during either August or September, in 1992, 1994 and 1998 and all cyprids were measured along their carapace length. Size-frequency histograms were produced for each sampling occasion. Total DNA was extracted and the COI–COII mitochondrial genes were PCR amplified, both from selected adults and cyprids of each species collected in 1998. Composite haplotypes, produced by digesting PCR products with the restriction endonucleases *TaqI* and *RsaI*, showed clear identification between the 2 species as adults and larvae. Of the 68 cyprids examined, those measuring 525 µm and under were found to have RFLP profiles corresponding with *C. montagui* adults, while those of 550 µm and larger were found to match *C. stellatus* adults. The findings verify previous length-frequency analysis. Carapace length is an important character in the identification of *C. stellatus* and *C. montagui* cyprids. The PCR primers developed during this study will also amplify DNA from 4 other species of acorn barnacle.

KEY WORDS: *Chthamalus* · Cyprids Identification · Carapace length · PCR-RFLP

INTRODUCTION

Two species of *Chthamalus* barnacles, *C. stellatus* and *C. montagui*, occur together in the intertidal zone on suitable shores from north Africa, along the Atlantic coast, to north Scotland and Ireland as well as in the English Channel and Mediterranean (Crisp et al. 1981). Distributions of the adults of these species may overlap considerably in terms of shore height and wave exposure. *C. montagui* is usually more common in the upper barnacle zone, than it is lower down, where *C. stellatus* may be dominant (Crisp et al. 1981). *C. stellatus* is often abundant on wave-exposed coasts whereas *C. montagui* is more commonly found in embayed situations (Crisp et al. 1981). *C. stellatus* and *C. montagui* are similar in the northern and eastern limits of their geographic distributions and physiological adaptations (Crisp et al. 1981), as well as brooding cycles (Burrows et al. 1992, O'Riordan et al. 1992) and settlement seasons (Power et al. 1999). The processes controlling the distribution patterns of *C. stellatus* and *C. montagui* are as yet unknown. There are some differences between the 2 species in settled cyprid

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density relative to metamorph abundance (Power et al. 1999) and larval development time (Burrows et al. 1999). Studies which compare sympatric larval settlement in the field between these species have been hampered because, although minute structural differences in the cyprid carapace have been reported in *C. stellatus* and *C. montagui* (Jensen et al. 1994), no diagnostic tool for species identification as cyprids has been available.

Carapace length has been suggested as a method of distinguishing between larvae of *Chthamalus stellatus* and *C. montagui* from field samples (Burrows 1988, Burrows et al. 1999, O'Riordan et al. 1999). The present work investigated size as a useful means of identifying *C. stellatus* and *C. montagui* cyprids from field samples collected at different dates in south and west Ireland. The development of a genetic marker allowed the identification of measured cyprids to be verified using mitochondrial DNA-RFLP (Restriction Fragment Length Polymorphism) analysis on identified adults compared with the cyprids of each species. Thus the extent of overlap in size between the cyprids of the 2 species could be determined.

MATERIALS AND METHODS

Sampling sites. Adult barnacles were collected from the middle and upper intertidal areas on 2 shores, in counties Cork (Garrettstown, 51° 38' N, 8° 35' W) and Clare (Kilkee, 52° 41' N, 9° 40' W) Ireland during May 1998. Widely spaced (>150 km) shores were chosen in order to maximise the possibility of sampling genetically different populations of each species, should such differences exist. In the laboratory, adult barnacles of *Chthamalus stellatus* and *C. montagui* were identified and their somatic tissue dissected out, placed in 95% ethanol and stored at –20°C for subsequent analysis.

Cyprids of *Chthamalus stellatus* and *C. montagui* were collected from pre-scraped (5 × 5 cm^2) quadrats between 16 August and 2 September 1998 from Garrettstown and Bullens Bay (51° 39' N, 8° 32' W), Co. Cork, and in August 1992 and September 1994 from Portnakilly (53° 47' N, 9° 59' W), Clare Island, Co. Mayo. All attached cyprids were removed using fine forceps and placed in 95% ethanol and stored at –20°C. Cyprids were measured from the anterior to posterior margin of the carapace under a dissecting microscope (40× magnification). Length-frequency histograms of cyprid carapace length were produced using 25 µm class intervals.

Of the cyprids collected at Garrettstown 68 were chosen to be analysed genetically. 14 of the cyprids measured between 400 and 475 µm, and fell within the first modal length group, 23 were within the second modal length group (measuring between 550 and 700 µm) and 31 cyprids were chosen from size classes which were intermediate between the modal length groups (between 500 and 525 µm).

DNA extraction. The DNA extraction was carried out on adult barnacles using the modified CTAB protocol (Doyle & Doyle 1991). Cypris larvae were washed once with sterile distilled water before DNA extraction, which was performed using standard phenol-chloroform procedure (Sambrook et al. 1989).

Genetic marker development. The PCR primers amplifying the Cytochrome Oxidase subunits I and II (COI-COII) region of mitochondrial DNA were developed, based on the COI-COIII DNA sequence of *Balanus improvisus* (Piyapattanakorn unpubl. data) aligned with the COI-COIII sequences already obtained for *Drosophila yakuba*, *Apis mellifera* and *Daphnia pulex* (Clary & Wolstenholme 1985, Crozier & Crozier 1993, Crease & Little 1997, respectively). Highly conserved regions were identified within these sequences and a *B. improvisus* primer (BI-COI) was designed to allow more specific PCR amplification for barnacles. The COII-croz primer developed for *A. mellifera* (Crozier et al. 1989) was retained as the COII primer in this study since the mismatch within the sequences did not affect its specificity in *B. improvisus*. This set of primers produced specific PCR products (~1.3 to 1.6 kb) from various species of acorn barnacles (*Elminius modestus*, *B. improvisus*, *Euraphia depressa*, *Tetraclita squamosa squamosa*, *Chthamalus stellatus* and *C. montagui*) but not from *Semibalanus balanoides*.

A 10 µl PCR reaction routinely used consisted of 5 to 10 ng of extracted DNA, 1× PCR buffer (Advanced biotechnologies), 3.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.1 µM of each primer [BI-COI (GAT ACC CGA GCT TAT TTT AC) and COII-croz (CCA CAA ATT TCT GAA CAT TGA CC)], and 0.75 units of *Taq* polymerase (Advanced biotechnologies). The reaction was carried out as follows: 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2 min with a final extension at 72°C for 7 min, using a Hybaid Omnigene Thermocycler.

RFLP analysis. The 10 µl restriction reaction contained 1× buffer (depending on the enzyme), 1 unit of restriction endonuclease, 300 to 500 ng of PCR product (~4 µl). The reaction was incubated for at least 2 h at 37°C for *RsaI* and at 65°C for *TaqI*.

Electrophoresis. After the PCR product had been digested, the reactions were run on 1.5% (w/v) agarose gel containing 0.2 mg ml^{-1} of Ethidium bromide at 80 volts and visualised on a UV transilluminator. The comparison of RFLP profiles (haplotypes) between gels was facilitated using a 100 bp DNA ladder (GibcoBRL).

RESULTS

Measurements of all cyprids collected in 1992, 1994 and 1998 are shown in Fig. 1. Cyprids ranged in size from 350 to 750 µm over the 3 yr sampled and the data exhibit bimodal size-frequency distributions in each case. The most frequently found size class in the first modal length group was 451 to 475 µm on the 2 southwestern shores in 1998 and 476 to 500 µm on the western shore in 1992 and 1994. The most common cyprid size class in the second modal group was less consistent, ranging between 576 and 650 µm, but with no consistent difference between southern and western shores.

Ten individual adults each of *Chthamalus montagui* and *C. stellatus* were initially used to screen with 7 restriction enzymes (*RsaI*, *EcoRI*, *HinfI*, *BfaI*, *VsaI*, *NdeII* and *TaqI*). Six enzymes produced specific RFLP profiles, which can be used to identify *C. montagui* from *C. stellatus* and *EcoRI* showed no difference between the 2 chthamalids. However, only 2 enzymes (*TaqI* and *RsaI*) were selected for use in this study. Approximately 120 individual adults (30 individuals of each species from the Cork and Clare shores) were identified using composite haplotypes produced by *TaqI* and *RsaI*, respectively (Table 1). The common composite haplotype for *C. stellatus* was ED (70%) and for *C. montagui* was AA (94%). Although there were some rare haplotypes occurring with each restriction endonuclease, composite haplotypes between *TaqI* and *RsaI* showed clear identification between *C. montagui* and *C. stellatus* in each case (Table 1 & Fig. 2).

All 68 cyprids were clearly identified by comparing cyprid haplotypes with those of the adults using the above genetic marker. There were 2 *RsaI* haplotypes, C and F, found in cyprid samples which did not occur in adult samples (Table 1). However, the *TaqI* haplotypes of these cyprid samples were common haplotypes, A and E, therefore the species of these cyprids were unambiguously identified (Table 1 & Fig. 2).

Cyprid species, as determined by comparison of adult and larval RFLP profiles (composite haplotypes), and corresponding cyprid size are shown in Fig. 3.

DISCUSSION

This study relies on the analysis of mtDNA from *Chthamalus stellatus* and *C. montagui*, which unlike enzymes, can be obtained from alcohol-preserved material, to identify individuals of each species. The primers developed during the course of this study will also successfully amplify DNA from several other species of acorn barnacles. Previous work on the genetic profiles of *C. stellatus* and *C. montagui* focussed on different enzyme allozyme frequencies as an aid in their separation as adults (Dando et al. 1979) and newly metamorphosed individuals (Burrows 1988). Studies

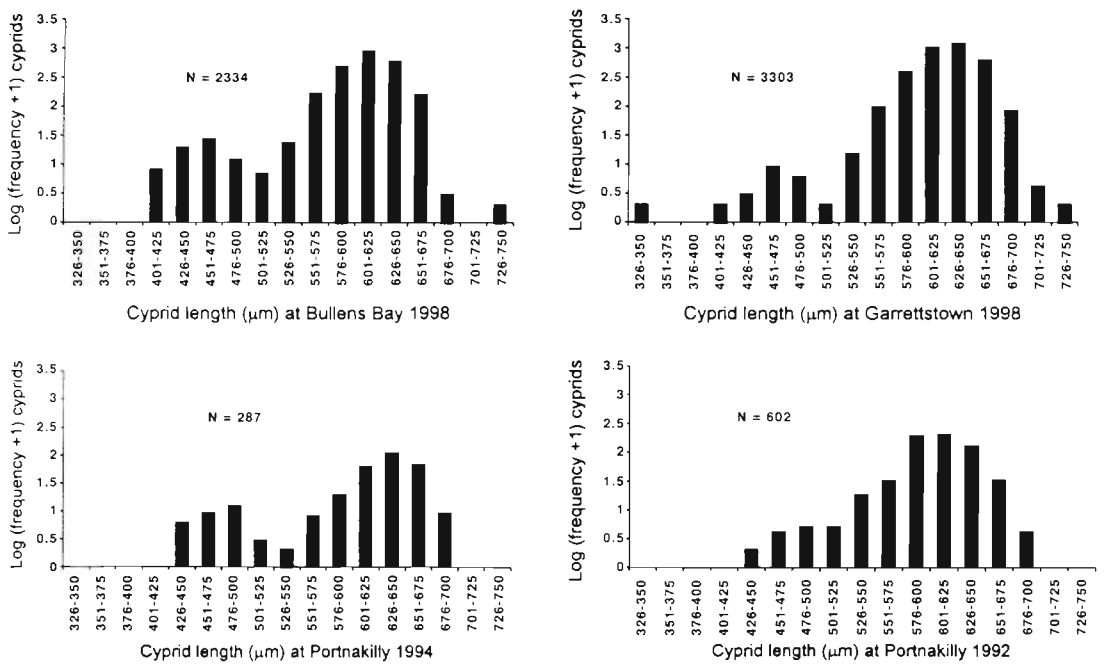

Fig. 1. Size-frequency of cyprids collected in 1998 at Bullens Bay and Garrettstown, southwest Ireland and in 1994 and 1992 at Portnakilly, west Ireland

Table 1. Composite haplotypes of adults and larvae of *Chthamalus montagui* and *C. stellatus*. These are made up of haplotypes produced by *RsaI* (first letter) and *TaqI* (second letter). *RsaI* (A to F) and *TaqI* (A to G) haplotypes may be visualised in Fig. 2

Species	Composite haplotypes	Adult frequency		Cyprid frequency	Total frequency
		Garrettstown	Kilkee	Garrettstown	
Chthamalus stellatus	DE	21	21	20	62
	FE	0	0	1	1
	DD	4	4	0	8
	DF	2	1	1	4
	DC	2	2	1	5
	DG	1	0	0	1
	EC	0	1	0	1
	EG	0	1	0	1
Chthamalus montagui	AA	26	28	44	98
	BA	2	0	0	2
	CA	0	0	1	1
	AB	1	0	0	1

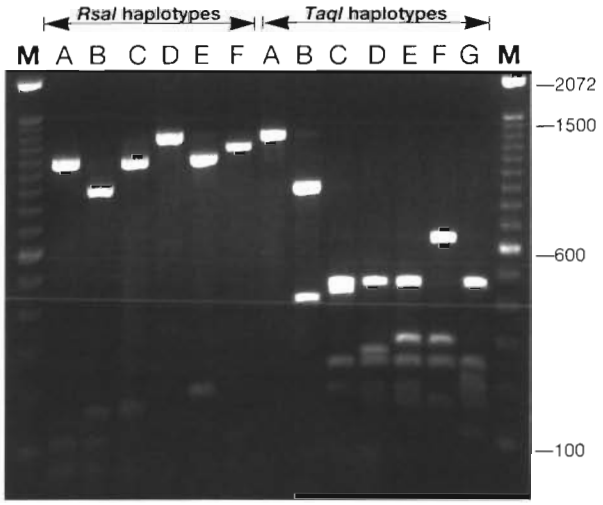

Fig. 2. Photograph of a 1.5% (w/v) agarose gel showing the RFLP profiles of the COI-COII PCR product, after digestion with endonucleases *RsaI* and *TaqI* [A,B,C,D,E,F & G: different haplotypes produced with each restriction endonuclease, M: 100 bp DNA ladder (GibcoBRL)]

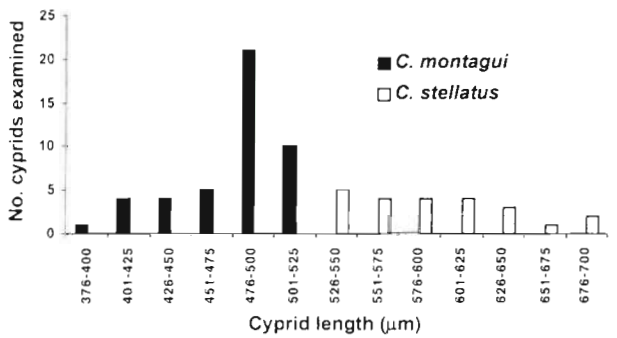

Fig. 3. Sizes of genetically-identified cyprids collected at Garrettstown, southwest Ireland

on the genetic variation within *C. montagui* (Dando & Southward 1980, 1981, Pannacciulli et al. 1997) and *C. stellatus* populations (Pannacciulli et al. 1997) have also used allozymes.

A previous suggestion that size could be used as a tool to distinguish these species, was based on bimodally distributed carapace length data from August 1996 (O'Riordan et al. 1999) and comparisons of these lengths with laboratory-reared *Chthamalus stellatus* (Al-Yahya 1991) and *C. montagui* cyprids (Burrows 1988, Stone 1989, Whillis et al. 1990, Al-Yahya 1991, O'Riordan 1992, Jensen et al. 1994, Moyse et al. 1995). Cyprids measuring 483 µm and smaller were suggested to be *C. montagui* and those of 563 µm and larger to be *C. stellatus* (O'Riordan et al. 1999). Another indication that this might be a useful technique was reported by Burrows (1988), who extrapolated cyprid sizes of each species from their wild and laboratory-reared naupliar sizes at Plymouth, England (see also Burrows et al. 1999).

In the present study, the use of carapace length to differentiate between *Chthamalus stellatus* and *C. montagui* was verified using mtDNA-RFLP analysis on material collected at Garrettstown, southwest Ireland. The cypris larvae collected at Garrettstown and at Bullens Bay, Co. Cork in August/September 1998 demonstrated a bimodal size distribution similar to that reported by O'Riordan et al. (1999) from 6 shores (including Garrettstown and Bullens Bay) in Co. Cork during August 1996. Although the most frequent cyprid size class in the first modal group was slightly larger (by 25 µm) than that of O'Riordan et al. (1999), that of the second modal group had shifted to the right by at least the same amount, hence a ca 150 µm separation was maintained between the size modes in both studies. In the present study, of the cyprids examined genetically (over the observed bimodal size-frequency

range), all 14 cyprids of 475 µm or less in carapace length were *C. montagui*, while all 23 cyprids measuring 550 µm or more were *C. stellatus*. Cyprids of intermediate length between the 2 modal groups (infrequent lengths i.e. those between 476 and 525 µm) were of particular interest, because according to their size, they could have been either unusually small *C. stellatus* or unusually large *C. montagui*. Of the 31 cyprids analysed within this size range, all were *C. montagui*. Thus, from the material examined here, a very small but consistent size difference of <25 µm seems to separate the ranges in cyprid size of the 2 species in samples taken from southwest Ireland.

The length-frequency data of cyprids collected from Portnakilly in the west Ireland in 1992 and 1994 are also bimodal, although the modal length groups seem to be less distinct than on southwestern shores. Cyprid size (Pyefinch 1948, Crisp 1962) and embryo size (Crisp 1959, Barnes & Barnes 1965) in field-collected *Semibalanus balanoides*, have been reported to vary with location and with latitude in particular, which has been linked to the rate at which the embryo develops (Barnes & Barnes 1976). However, 'considerable variation' reported in cyprid length of *S. balanoides* at Millport in Scotland (Barnes 1953) is in fact less, at 10% of the modal length, than is reported here for *Chthamalus* cyprids (which vary between 21 and 38% of the modal length on the different sampling dates). *Chthamalus stellatus* nauplii from the north coast of France were larger than those at the Spanish coast and embryos developed faster at higher culture temperatures (Patel & Crisp 1960), but this work was carried out before *C. montagui* was separately identified by Southward (1976) and could have been a mixture of nauplii of both species. Cyprid size was the only character to distinguish between the larvae of *Chthamalus fissus* and *Chthamalus dalli* from California, the mean length of the former being slightly (~45 µm) larger (Miller et al. 1989). This is a much smaller separation than that observed between size modes of *C. stellatus* and *C. montagui* (minimum 100 µm) in the present study. In addition, no intraspecific differences were found in the naupliar sizes of laboratory-reared *C. fissus* and *C. dalli* when grown at low (13°C) and high (18°C) temperatures (Miller et al. 1989).

Further genetic confirmation is needed, as to the extent of overlap in cyprid size of *Chthamalus stellatus* and *C. montagui* at other latitudes, prior to using length as a general identification tool where the species occur sympatrically. Spatial and temporal variation in the sizes of field-collected cyprids of *C. stellatus* and *C. montagui* are currently being investigated from a number of European sites (O'Riordan 1999). Species of *Chthamalus* are frequently sympatric over part of their range (Southward 1976, Dando et al. 1979, Dando & Southward 1980, 1981) but studies on the settlement phase of sympatric *C. stellatus* and *C. montagui* populations are dependent upon a diagnostic tool such as carapace length to reliably and quickly identify large numbers of cyprids from field samples along the species ranges.

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