Speciation and the establishment of zonation in an intertidal barnacle: specific settlement vs. selection*

L. APPELBAUM, †‡ Y. ACHITUV † and O. MOKADY ‡
†The Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, 52900, Israel; ‡The Institute for Nature Conservation Research, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Abstract

The tropical barnacle *Tetraclita* forms a belt on hard substrates in the intertidal zone of the Red Sea. Based on morphological data, three distinct species were suggested to exist, occupying different vertical levels — *T. barnesorum*, *T. rufotincta* and *T. achituvi*. In this study we used molecular (12S mitochondrial ribosomal DNA) and ecological data to examine whether this morphological variability reflects genetic differences, or is a result of environmental factors. Adults and spats, collected from settlement plates, were censused and screened genotypically using single strand conformation polymorphism (SSCP) analysis, and settlement dynamics was recorded. We provide evidence for the existence of only two distinct species, and point out both phenotypic plasticity and convergence within and between the proposed species. Cyprids of *T. achituvi* settle specifically at the lower part of the *Tetraclita* belt, and feature one phenotype. In contrast, *T. rufotincta*, occupying the upper and middle portions of the *Tetraclita* belt, settles throughout the range, shows phenotypic plasticity (three variants), and presumably undergoes selection at the lower part. Thus, the vertical zonation of *Tetraclita* is produced by the combination of pre-settlement and post-settlement factors, in *T. achituvi* and *T. rufotincta*, respectively. The examined system may offer a model in which to study the mechanisms underlying sympatric speciation.

Keywords: Cirripedia, convergence, phenotypic plasticity, SSCP, *Tetraclita*

Received 14 January 2002; revision received 9 May 2002; accepted 9 May 2002

Introduction

The distribution pattern of intertidal barnacles is vertically restricted, a pattern known as ‘vertical zonation’ (Stephenson & Stephenson 1972). The lower limit is usually determined by biotic factors such as competition or predation. For example, the distribution of *Chthamalus stellatus* is restricted by competition with *Semibalanus balanoides* (Connell 1961a), whereas the distribution of the latter is, in turn, restricted by the gastropod predator *Thais lapillus* (Connell 1961b; see similar findings by Strathmann & Branscomb 1979). The upper limit is usually set by physical factors, such as heat and desiccation (Connell 1961a). Foster (1971a,b) showed that barnacles occupying higher levels of the substrate are more tolerant to heat and more resistant to desiccation.

Intertidal zonation of rocky shores in the Gulf of Elat, Red Sea, was first studied by Safriel & Lipkin (1964) and Achituv (1972). The most common intertidal barnacle in this area was first described as *Tetraclita squamosa rufotincta* (Pilsbry 1916). Later, Newman & Ross (1976) concluded that this subspecies is a valid species, to be named *T. rufotincta*. Achituv & Borut (1975) described morphological differences between individuals inhabiting different levels within the zone of distribution, and Achituv & Mizrahi (1987) showed differences in allozyme frequencies between these morphotypes. Recently, Ross (1999) has described these morphotypes as distinct species, *T. barnesorum*, *T. rufotincta* and *T. achituvi*.

The reported phenotypic variability may, indeed, reflect genetic differences following speciation processes, but may be the result of environmental induction at the different levels within the range of distribution. Speciation, in turn, may be driven by factors acting at different stages along the life cycle, including the sessile adult and the motile larva. For example, selective larval recruitment may result in a
reproductive barrier and consequent speciation (Templeton 1989; Knowlton 1993). Such a barrier will be especially effective in sessile barnacles, which reproduce by copulation and fertilization of eggs within the mantle cavity, limiting potential mates for a given barnacle to the immediate neighbours. Alternatively, speciation may be associated with post-settlement selection following random larval recruitment.

With the advent of modern molecular technology, one can easily assess whether phenotypic diversity maps onto genotypic diversity, to determine whether the former is accounted for by speciation or plasticity. Using this approach, unjustified lumping as a result of convergence (Mokady & Brickner 2001), as well as unjustified division due to plasticity (Lively 1986a,b; Mokady et al. 2000) have recently been exemplified in coral- and rock-inhabiting barnacles. These studies have shown the sequence polymorphism of specific segments of the mitochondrial DNA (mtDNA) to be adequate for inter- and intraspecific resolution in barnacles. Additionally, the utility of single strand conformational polymorphism (SSCP; Orita et al. 1989a,b), an electrophoresis-based method for visualizing small differences in DNA sequence, was demonstrated (Mokady & Brickner 2001).

Our study was designed to: (i) assess whether the different phenotypes featured by Tetraclita reflect speciation or phenotypic plasticity; and (ii) examine the establishment of zonation in this barnacle genus, with respect to settlement preferences and recruitment dynamics.

Materials and methods

Animal collection and field experiments

Animal collection and field experiments were carried out at the H. Steinitz Marine Biology Laboratory (MBL), Eilat, at the northern tip of the Gulf of Eilat, Red Sea. Adult barnacles were collected from the MBL pier and nearby intertidal rocks.

Settlement was examined on 80 plastic plates (10 × 10 cm), arranged in 8 rows (1-top, 8-bottom) to cover the entire vertical span of the Tetraclita belt. The settlement plates were fixed in place in August 2000, prior to the reproductive season (October to December; Achituv & Barnes 1978). All cyprids (the settling stage of barnacles) and newly settled spats found on the settlement plates were counted at 2-week intervals throughout the recruitment season. At each counting, samples of cyprids and spats were taken from rows 1, 3, 5 and 7, and individually fixed in 100% ethanol for DNA analysis. Between 10 and 30 individuals per row were collected in each sampling, with the exception of the first sampling, in which ≈50 individuals were collected from the bottom row (the only row settled at the time) to enable calibration of the SSCP analytic system.

DNA preparation and polymerase chain reaction amplification

DNA was extracted from individual adult barnacles, and in a few cases from cyprids or spats, as described by Mokady & Brickner (2001). In most cases, following brief evaporation of the ethanol on a piece of plastic film, whole individual cyprids and spats served as template for subsequent DNA amplification, without extraction. Polymerase chain reaction was employed to amplify a fragment of the 12S subunit of the mitochondrial ribosomal DNA (400 bp) using the primer set of Mokady et al. (1994): 5′-GAAACCAGGATTAGATACCC, 5′-TTTCCCGCGAGCGACGGGCG. Amplification was carried out using 1.25 U of Taq DNA polymerase in the presence of 1.5 mM MgCl₂ and 0.1 mM of each dNTP. Amplification consisted of initial denaturation at 94 °C for 2 min, followed by 40 cycles of 0.5 min denaturation at 94 °C, 1 min annealing at 53 °C and 1 min elongation at 72 °C. According to a preliminary analysis of amplified sequences, Tetraclita-specific PCR primers were designed to amplify a 200 bp fragment, revealed to contain most variable positions (Fig. 1): 5′-TATCGCTGTTGTCAGTTAG, 5′-TGTGTACACAAGATAGAGC. These primers improved amplification specificity and intensity, and the resolution of SSCP analysis.
Sequence analysis and phylogenetic reconstruction

Direct sequencing was performed by fluorescent chain termination, on an ABI 377 automated sequencer. Sequences were aligned using clustalx (Version 1.81) software (Thompson et al. 1997), and the aligned sequences were analysed by PAUL Version 4.0d65 (Swofford 1999). Distance matrices were produced using the ‘standard distances – total character difference’ option. Both maximum likelihood and maximum parsimony criteria were used for phylogenetic reconstruction, with transition/transversion (Ti/Tv) ratio estimated from the actual data.

Single strand conformation polymorphism

This method enables large-scale screening, avoiding individual sequencing and distinguishes fragments of equal size but different sequence. The sensitivity (down to single base substitution; Sheffield et al. 1993) is inversely related to fragment length, with best performance at less than 200 bp (Hayashi 1991, 1992).

SSCP analysis was performed in a SEA 2000 apparatus (Elchrom Scientific AG, Switzerland), in 30 mM TAE buffer pre-chilled to 9 °C. Samples consisted of 5 µL PCR product, supplemented with 5 µL of loading buffer (81% formamide, 1.25 mM EDTA, 0.025% xylene cyanol, 0.025% Bromophenol Blue). Denatured samples (5 min, 95 °C) were run on horizontal GMA™ gels (Elchrom Scientific) for 20 h, under 72 V (6 V/cm), followed by post-staining with Gel Star® (FMC BioProducts, ME, USA) following manufacturers’ instructions.

Results

Genotypic analysis

The preliminary sequence analysis (Fig. 1) revealed genotypic differences between barnacles collected from the upper or lower part of the Tetrachita belt. Seventeen diagnostic positions clearly separate these groups, which are hereafter referred to as ‘genotype A’ and ‘genotype B’, respectively. The sequences within each group differ slightly from each other (five positions with parsimony-uninformative, taxon-specific character-states). These findings were further substantiated by SSCP genotyping of additional barnacles, using the sequenced products as standards for assigning electrophoretic patterns to genotypes. All 20 adults collected from the upper part were of genotype A. Twenty-two of 23 adults collected from the lower part were of genotype B, and only 1 of genotype A.

To examine the relationships between these genotypes and the three species described by Ross (1999), sequence information was obtained for barnacles identified as T. barnesorum, T. rufotincta and T. achituvi according to Ross’ descriptions. Three individuals of each species were collected from the upper, middle and lower part of the Tetrachita belt, respectively. Surprisingly, these sequences clearly cluster into two groups (Fig. 2), mapping onto the sequences obtained in the preliminary analysis. All T. barnesorum, T. rufotincta and one T. achituvi form one cluster, corresponding to genotype A. The two other T. achituvi form a second cluster, corresponding to genotype B.

The sequence data obtained in this study were deposited in the EMBL databank, under Accession nos AJ426433–AJ426448 and AJ428544–AJ428547.

Settlement dynamics

Figure 3 shows the temporal and spatial pattern of settlement observed in this study. Settlement starts at the lower part of the Tetrachita belt, at the beginning of November, and progresses upward. The settlers reach the upper part of the range at mid-December, which is the peak of the settlement period – highest settlement was observed in the middle of the Tetrachita belt. Later the numbers decrease and the typical Tetrachita belt is formed.

Newly settled spats were screened for genotype by SSCP. Only 6 electrophoretic patterns were found in the 400 individuals thus screened, and representative products featuring each of the patterns were sequenced. Figure 2 shows how the obtained sequences map onto the clusters of ‘genotypes’ A and B. When the magnitude of differences within each group is compared with the electrophoretic patterns visualized by SSCP, two important characteristics of SSCP can be appreciated: (i) even minute sequence differences (1–3 variable positions among ∼200) can result in striking electrophoretic differences; and consequently (ii) it is impossible to reconstruct phylogenetic relationships without calibrating the electrophoretic patterns with sequence data.

The sensitivity of SSCP and its potential utility for molecular ecology studies were anecdotally demonstrated with the identification of a ‘third group’ of barnacles – 29 of the settlers were typified by 1 of 2 electrophoretic patterns differing from the above 6 patterns. These SSCP patterns were calibrated to two closely related sequences, which did not cluster with either ‘genotype’ A or B. Comparing these sequences with available data, we identified the spats as Balanus amphitrite (EMBL Accession no. X78233). It should be noted that B. amphitrite is found in closed lagoons and harbours in the vicinity, and is nearly absent along the shores of the open sea (Achituv 1972). Our results suggest that cyprids of B. amphitrite settle at an overlapping zone and time to Tetrachita and are lost before they can be recognized.

Tetraclitamalus obliteratus, a common intertidal barnacle in Eilat, which inhabits a belt overlapping that of Tetrachita, constituted another potential pitfall with respect to data.

© 2002 Blackwell Science Ltd, Molecular Ecology, 11, 1731–1737
Although the adults of *Tetrachthamalus* and *Tetraclita* are easily told apart, it is almost impossible to distinguish between the spats. Sequence data obtained from this species confirmed that no spat of *Tetrachthamalus oblitteratus* was mistakenly collected. This finding suggests that the settlement period of this barnacle does not overlap that of *Tetraclita*.

Figure 4 shows the genotypic composition of *Tetraclita* settlers ('genotypes' A and B; corresponding to *T. rufotincta* and *T. achituvi*, respectively; see below) throughout the recruitment period [data obtained in mid-month November, December and January (not shown) complement the same pattern shown in Fig. 4]. The first barnacles to settle, at the lower part of the *Tetraclita* belt, are mainly of genotype B. These barnacles settle only at the lower part throughout the recruitment period. Later on, as recruitment progresses upward (Fig. 3), genotype A settles in large numbers, throughout the *Tetraclita* belt.
Discussion

Speciation vs. phenotypic plasticity

Genotypic evidence provided by this study, in which adult and juvenile Tetraclita from the Red Sea were screened throughout their vertical range of distribution (a total of 413 individuals), indicates the existence of only two species. This is in contrast to the description of three species of Tetraclita from the very same area on the basis of morphological data (Ross 1999) — T. barnesorum, T. rufotincta and T. achituvi, occupying the upper, middle and lower parts of the range of distribution, respectively. In light of our results, and bearing in mind that T. rufotincta is the original species name prior to any division, we shall hereafter refer to ‘genotypes’ A and B identified in this study as T. rufotincta and T. achituvi, respectively. T. barnesorum does not represent a valid species, but rather a phenotypic variant of T. rufotincta. This assertion confirms the suggestion of Achituv & Mizrahi (1987), following their finding that individuals from the middle and upper parts of the Tetraclita belt feature similar allozymic patterns, which are different than those of individuals from the lower part.

Specimens assigned to T. rufotincta appear in three different phenotypes, answering to all three sets of defining criteria detailed by Ross (1999). The morphological plasticity featured by T. rufotincta may play a role in enabling this species to inhabit the entire Tetraclita belt, including the upper, middle and to a lesser extent the lower parts. This is in line with the relationship between vertical level and shell morphology reported for T. rufotincta by Achituv & Borut (1975). By contrast, T. achituvi expresses only one phenotype and is restricted to the lower part of the belt.

The morphological plasticity demonstrated by T. rufotincta is remarkable, but by no means unique among intertidal barnacles. For example, Barnes & Powell (1950) reported the effect of environmental factors on shell morphology of Balanus crenatus, and Lively (1986a,b) showed a drastic change in shell morphology of Chthamalus stelleri induced by a predator gastropod. The latter case was later confirmed to represent phenotypic induction by direct molecular evidence (Mokady et al. 2000).

At the lower part of the Tetraclita belt the two species are morphologically indistinguishable with respect to shell structure, valve morphology and orifice size, presumably reflecting convergence, and thus fit the definition of sibling species sensu Mayr & Ashlock (1991). They thus add to the list of barnacle sibling species identified in recent years, such as within the genus Chthamalus (Pannacciulli et al. 1997) and Wanella (Mokady & Brickner 2001).

Settlement strategies of the two species

The two species, T. achituvi and T. rufotincta, demonstrate two different settlement strategies. Whereas T. achituvi cyprids settle predominantly at the lower part, coinciding with future adult distribution, those of T. rufotincta settle throughout the Tetraclita belt, including the lower part. Thus, whereas pre-settlement behaviour determines adult distribution in T. achituvi, it is post-settlement selection that plays a major role in shaping the distribution of T. rufotincta. Pre-settlement swimming behaviour of nauplii was previously shown to be responsible for differences in the distribution of two Balanus species (Grosberg 1982). Should similar differences exist between T. achituvi and T. rufotincta (data currently unavailable), they may add to the above in explaining the differences in distribution.

The data shown in Fig. 4 may suggest that T. rufotincta commences settlement a little later than T. achituvi (number of recruited cyprids an order of magnitude lower at the end of October). Temporal differences in recruitment may also reflect differences in the onset of reproduction or in developmental dynamics. We tested the former possibility
Environmental conditions and zonation

Lower vertical levels are presumed to provide favourable abiotic conditions for Tetraclita (Barnes 1959; Achituv 1972; Hunt & Alexander 1991). The longer immersion time offers more feeding time, and less exposure to desiccation. Owing to these factors, lower parts of the intertidal substrate are competed for by sessile barnacles (e.g., Stanley & Newman 1980; Achituv 1981; Paine 1981). Cyprids and spats are particularly prone to desiccation because of greater surface area to volume ratios (Foster 1971a). A possible explanation for the gradual settlement from the lower part upwards (results reconfirmed at the 2001 settlement period), involves the sequence in which rock temperature is relieved – the length of immersion is longer at lower levels, and thus upper levels reach higher temperatures, and retain them for longer periods of the day. Thus, at the beginning of the recruitment period, the conditions at higher levels do not permit recruitment. Only later toward winter, with the gradual decline in ambient temperature, maximal temperatures reached at the upper part of the Tetraclita belt are low enough to support settlement. The adult barnacle will survive the temperatures of the next summers owing to its thick shell and ability for self-isolation by shutting the opercular valves.

In contrast, lower levels offer less favourable biotic conditions, in the form of competitors and predators. Macroalgae, which flourish at lower levels, bloom in February–May (Genin et al. 1995), the time of initial growth of Tetraclita. Algae were observed to cover the barnacles in some cases, possibly reducing the flow of water and food or interfering directly with food filtration (Connell 1961a). The initial stages of algal blooms may even compete with cyprids for available substrate. Predation by the gastropod Thais is another potential risk to barnacles at lower levels (Connell 1961b).

T. achituvi has a thin shell, with a relatively large orifice (shell aperture). Achituv & Borut (1975) argued that these characteristics are adaptive at the lower part of the range, but will not allow survival of desiccation at the upper part. We tested this assertion by relocating settlement plates from the lower part of the range to the upper part and vice versa, in mid-February (~4 weeks after the end of the recruitment period). The plates were examined again 6 weeks later, revealing that all T. achituvi spats (ageing up to 10 week) died following relocation upwards (results not shown). By contrast, most but not all of T. rufotincta died when relocated downwards. With respect to the phenotypic plasticity of T. rufotincta, this may reflect temporal (ontogenetic) limits to the ability to change the developmental response to the environment.

Conclusion

Our research constitutes another item in the ever-growing list of modern studies, in which phenotypic plasticity and convergence were only revealed with the aid of molecular data. This was clearly exemplified in our case, where it was crucial to screen early stages prior to selection of spats – it is virtually impossible to tell the two Tetraclita species apart at early post-recruitment stages (cyprids and spats).

The physical proximity and partial overlap in distribution at the lower part of the Tetraclita belt, coupled with the pattern of similarities and dissimilarities revealed in this study, suggest that T. achituvi and T. rufotincta may offer a model system in which to study sympatric speciation. Our results, together with additional research aimed at identifying potential and actual reproductive barriers between these species may offer insights into mechanisms underlying sympatric speciation.

Acknowledgements

The authors are grateful to the Tobias Landau Foundation and the Bar-Ilan University Research Authority, for generous support of this research. Thanks are also due to the H. Steinitz Marine Biology Laboratory, at the Interuniversity Institute of Eilat, for use of diving facilities and hospitality of its staff.

References


Foster BA (1971a) Desiccation as a factor in the intertidal zonation of barnacles. Marine Biology, 8, 12–29.


This study was part of the M.Sc. research of Liav Appelbaum, supervised jointly by Ofer Mokady and Yair Achituv. Yair Achituv has been studying the biology and ecology of intertidal barnacles, along the Mediterranean and Red Sea coasts of Israel for over 25 years. Ofer Mokady’s laboratory uses molecular markers to address questions at levels varying from the individual to the species, in a number of model systems.