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Study on the Molecular Variations among Marine Copepods (*Acrocalanus gracilis* and *Euterpina acutifrons)* Isolated from Vellar Estuary-South East Coast of India

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Abstract: Traditional type of classifying copepods does not satisfy the minimum requirement for identifying a particular organism. Analyzing the spatial genetic variation among the species will be more useful for relating with known populations. The present study focused on analyzing variations in the nuclear DNA isolated from *Acrocalanus gracilis and Euterpina acutifrons.* RFLP analysis of the genetic material isolated from two organisms shown that they are entirely different genera. Variation in their genetic content was well displayed by using RFLP analysis.

Key words: Molecular variation · *Acrocalanus gracilis* · *Euterpina acutifrons* · RFLP · DNA isolation · Marine copepods

INTRODUCTION

Biodiversity in marine invertebrates has been proven to be difficult to assess accurately using traditional morphological methods. Major problems include a dearth of characters in many taxa, 'rampant homoplasy [i.e. widespread convergent or parallel evolution], ontogenetic variation and phenotypic plasticity [1-3]. These problems have been complicated by technical limitation on the observation, sampling and culturing of marine organisms and thus the poor understanding of the marine environment [4,5]. Moreover, a dearth of explicit, objective and quantitative morphological analysis has made it difficult to know for sure where, if not in the analyses themselves, the real problems lie [6-8]. The species identification using morphological data is slow and time consuming. Morphological identification of early developmental stages of fish eggs and larvae are difficult due to limited number of morphological characters [9]. To overcome this problem molecular approach is needed for the classification and identification for the copepods. Recently DNA sequencing provide thousands rather than tens [or fewer] of characters, enabling the reconstruction of better- resolved and more robust phylogenetic trees [10].

The molecular analyses have indicated cases of phenotypic plasticity mistaken for species level difference and conversely, morphospecies mistaken for ecophenotypes [11-12]. Molecular studies have shown sometimes-considerable genetic differentiation among population of marine species [13]. The early life stages of marine organisms are not well known, as their morphological identification is very difficult. But, molecular technique is the best technique to identify the marine organisms at early life stages [9]. Traditional methods of copepod classifications were found to be difficult and time consuming one. So it could be easy to test the morphological characters of any species against molecular identification in a blind test of the samples [14]. The study of spatial genetic variation is useful tool for elucidating differences in the populations and which often reveals unexpected patterns of population relatedness [15]. Morphological variability is the potential component for analyzing the geographic differentiation.

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It can be used to determine the existence of cryptic species and understanding how rates of morphological and molecular evolution are related [16].

The present study aimed to distinguish two closed related copepod species using RFLP analysis.

MATERIALS AND METHODS

Collection and Preservation of the Samples: The Zooplankton (Copepods) samples were collected from Vellar estuary (south east coast of India) using horizontal towing of plankton net (0.35 mouth diameter) made up of bolting silk cloth (No.10; 158μm) for 20 to 30 minutes.

The samples were transported to the laboratory immediately after the collection and a portion of the samples were preserved in 5% formaldehyde for taxonomical studies. The copepods were identified on the basis of morphologically by using a light microscope. Copepod [Zooplankton] samples were first screened through 500 μm mesh to remove fish and prawn larvae. The rinsing was made repeatedly to reduce the contamination. After rinsing the adult copepods of *Acrocalanus gracilis* and *Euterpina acutifrons* were picked from the zooplankton samples with the help of fine capillary tube and needles. Then the copepods were washed with double distilled water and preserved in 95% ethanol to facilitate DNA isolation.

It is a monospecific genus as it has only one species. The body is sub-pyriform or arched and the cephalosome is drawn out in front into a greatly prominent rostral projection, which is acute at the tip. Fifth pair of legs formed by two undivided juxtaposed plates.

Urosome 4 or 5 segmented 5 legs symmetrical or dissimiliar on the two sides and shorter terminal segment. The body is parallel side, sausage shaped and nearly 3 times as long as broad. The cephalosome is very evenly rounded. The right and left antennules are similar. The antennae are slightly longer than the body and are provided with two long hairs at their ends. The terminal segments of the exopodites of legs 2, 3 and 4 are generally separated into a proximal and distal portion by the outer marinal spine.

Isolation of DNA: The DNA was isolated by the Saline Citrate Solution (SCS) method. 200mg of copepod samples were suspended in 800μl of saline citrate solution (0.14M NaCl and 0.02M Trisodium citrate) and homogenized by mortar and pestle. The homogenate was transferred in to a fresh centrifuge tube and centrifuged at 3000 rpm for 10 min. After centrifugation the supernatant was discarded and the pellet was resuspended in saline citrate solution and centrifuged at 3000 rpm for 5 minutes, The pellet was again resuspended in 400μl of 2M sodium chloride solution and centrifuged at 10,000 rpm for 15 minutes at 4°c. Followed by centrifugation the supernatant was collected in a fresh eppendorf tube. In order to precipitate the DNA double the volume of absolute alcohol was added to the pellet for 6 minutes.

The fibrous DNA was pooled by a clean glass rod and transferred to clean eppendorf tube and processed for RFLP analysis.

Table 1: Taxonomical position of *Euterpina acutifrons*

Phylum	Arthropoda
Class	Maxillipoda (Crustacea)
Order	Harpacticoida
Family	Tachidiidae
Genus	*Euterpina*
Species	*acutifrons*

Table 2: Taxonomical position of *Acrocalanus gracilis*

Phylum	Arthropoda
Class	Maxillipoda (Crustacea)
Order	Calanoida
Family	Paracalanidae
Genus	*Acrocalanus*
Species	*gracilis*

Rflp (Restriction Fragment Length Polymorphism) Analysis: Restriction digestion of the isolated DNA was done using restriction enzymes procured from Genei, Pvt. Ltd., Bangalore. The DNA isolated from copepods was taken in a microfuge tube at the concentration of 5μg /20μl. To the DNA sample 3 μl of assay buffer containing 0.5μl of 5M NaCl and 2 μl of BSA (1mg\ml) were added. 1μl of two different restriction enzymes (Hind III and EcoRI) were added to two different microfuge tubes, which were labeled as *Acrocalanus gracilis* and *Euterpina acutifrons* and incubated for 1 hour at 37°C. The digested products were analyzed using agarose gel electrophoresis prepared with 1.5% agarose. The electrophoresed gel was observed after it was stained with 50 μg\ml of Ethidium bromide. The restriction profiles were photographed by UV Transilluminator fitted with a gel documentation system. (Biotech Pvt. Ltd, Yercard, Salem).

RESULTS

Zooplankton like *Acrocalanus gracilis and Euterpina acutifrons* stored in the alcohol solution was able to release huge amount of DNA sample and the DNA digested with EcoR I and Hind III were found to produce

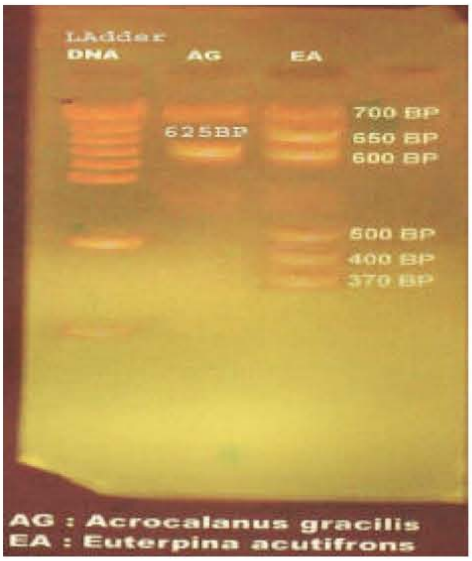

Fig. 1: Restriction fragment frofile using hind

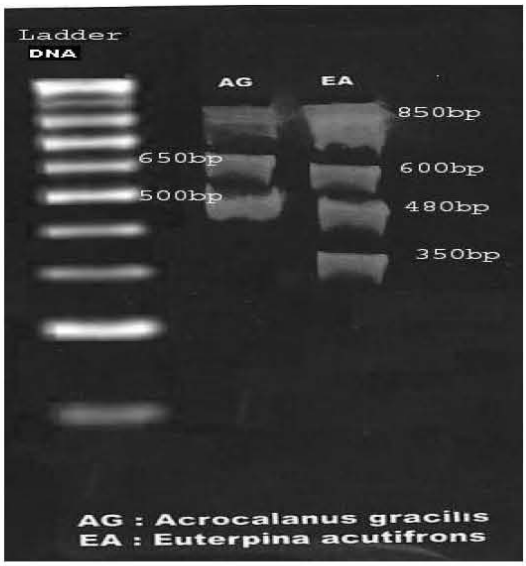

Fig. 2: Restriction fragment frofile using Ecor I

different patterns of DNA fragments. Restriction digested DNA with Hind III and EcoR I was able to produce a characteristic profile for both the copepods; i.e., ***Acrocalanus gracilis*** and ***Euterpina acutifrons***. The EcoR I has 5'G AATTC 3' and 3'CTTAAG 5' recognition sequences and it cleaves at 5' G• AATTC 3' and 3'CTTAA • G 5' positions. Hind III has 5'-AAGCTT- 3' and 3'-TTCGAA-5' recognition sequences and it cleaves at 5'-A•AGCTT3' and 3'-TTCGA•A-5'positions.Restriction fragment profile for Hind III was found to produce two different fragments with the size of: 700 and 625 bp for *Acrocalanus gracilis* and while these bands were different in the case of *Euterpina acutifrons*. It was recorded as six different fragments and each varying in their size. The sizes of each fragments were analyzed as; 700, 650, 600, 500, 400 and 370 bp approximately, (Fig.1). Similarly restriction profile for EcoR I was found to produce three different fragments with the sizes of 850, 650 and 500 bp for *Acrocalanus gracilis* and in the same way it was different in the *Euterpina acutifrons***.** It was recorded as four different fragments and each varying in their size. The sizes of each fragments were analyzed as; 850,600,480 and 350 bp approximately, (Fig. 2).

DISCUSSION

Due to variation in the molecular base pair sequences, species are easily differentiated. Earlier Jegadeesan *et al.* [17] found that the copepod *Paracalanus parvus* was digested with EcoR I and Hind III which produced 5 different fragments with the sizes of 856, 691, 499, 285 and 90 bp respectively. Poongothai [18] found that copepods like *Oithona rigida* and *Pontella danae* digested with EcoRI produced two fragments with the sizes of 1984, 846 bp in *Oithona rigida* and 2752, 1948 bp in *Pontella danae*. In the same way, Hind III also produced two bands with the sizes of 2313, 1708 bp in *Oithona rigida* and 2249, 1437bp in *Pontella danae* respectively. Burton *et al.*[19] reported that variation in eukaryotic rDNA [ribosomalRNA] IGS [intergenic spacer sequence] are used as analyses of molecular mechanism underlying population differentiation and insipient speciation. They found high levels of genetic differentiation in both nuclear and mitochondrial gene in the geographically separated populations of the intertidal copepod *Tigriopus californicus*. From these reports, it is understood that variation in the DNA sequences is a useful tool for differentiation of the species. Another molecular variation work was done in copepod by Bucklin *et al.*[20] and they reported that variation in DNA sequences of two mitochondrial genes 16S rRNA and Cytochrome Oxidase I [COI] showed differentiation within the two sibling species. They concluded that the molecular variation was the only method in discriminating the two sibling species. They also confirmed that taxonomic identification of the copepod species was found to be difficult in identification. Hare *et al.* [21] reported that mitochondrial DNA sequences of a Cytochrome b gene fragment was used in the identification of two morphologically distinct larval wrasses obtained in ichthyoplankton. Similar work

carried by Olson *et al.* [22] reported that 16S r RNA gene was used for the identification of planktonic larvae. These results are similar to our work which shows variation in DNA base pair sequences and we can easily differentiate the variation of two species. RFLP analysis showed the variation of DNA base pair sequences in our work. Likewise many other works in molecular identification of marine species through RFLP was studied. Present work is similar to those studies in identifying the species. Watanabe *et al.* [9] studied that identification of fish eggs and larvae are difficult due to limited number of morphological characters. So they adopted RFLP technique in the quick method of species identification of Japanese eel egg and larvae. Molecular technique such as mtDNA RFLP was applied in distinguishing among closely related taxa [23]. Similar experiment was carried by Silberman *et al.* [24] who have reported that mt DNA RFLP is a genetic marker and is more sensitive in detecting population structure. Hill *et al.* [25] have confirmed that sequence variation in *Calanus* mitochondrial DNA can be a diagnostic and accurate indicator of species identity. Eiane *et al.* [26] have stated that comparison of morphological and molecular identification of calanus species is very effective. Our studies results also found to be consistent with the findings [27] that have also developed the new strategy for species identification of planktonic larvae and insisted that this method will also help in the identification of hybrid larvae. The comparison between the molecular and morphological identification of *Acrocalanus gracilis* and *Euterpina acutifrons* highlights the limitations in morphological identifications methods but the RFLP can clearly demonstrate the potential of molecular techniques to distinguish the two organisms. From this work , it can be concluded that molecular technique such as RFLP showed variation in DNA base pair sequences which help as to differentiate the species when morphological characters are unavailable._ Oright

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