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Genetic confirmation of ragged sea hare *Bursatella leachii* (De Blainville 1817) of Pulicat lake India with Mar Menor (SE Spain) species

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Abstract
This study was to investigate the Genetic characteristic of ragged sea hare *Bursatella leachii* found in Indian water is similar to the species found in Mar Menor (SE Spain) water since pathways/ vector of species introduced into the world wide by the way of transportation activity (shipping activity such as ballast water, sedimentation, anchoring & fouling), aquarium trading (trading of live marine species) and aquaculture activity. The species found in Pulicate Lake, India is genetically matching with the species found in SE Spain water species by 99%. The genetic homogeneity pattern of *Bursatella leachii* found in Mar Menor (SE Spain) and Pulicat Lake in India is the same species available here.

Keywords: Ragged sea hare, *Bursatella leachii*, SE Spain

Introduction
The ragged sea hare *Bursatella leachii* is a marine opisthobranch gastropod molluscs belong to the family Aplysiidae and the order Anaspide. They are distributed worldwide in warm temperate to tropical marine ecosytems [1]. The distribution of this species has generally been reported from the intertidal water zone and down to depth of at least 10m in coastal waters [2, 3]. *Bursatella leachii* is a hermaphrodite with internal cross fertilization; one act as male whiles other act as female. It lays large egg mass strings look like spaghetti noodle. Many gastropod species in the intertidal zone enclose their fertilized eggs within capsular or gelatinous egg masses to provide protection against extreme environment, such as desiccation, temperature, salinity, ultraviolet radiation and water flow [4, 5]. Life span of the *B. leachii* is short; species attend maturity within 2-3 months [6, 7]. Marine ecosystems are considered to be the major source of bioactive compounds [8]. Most of the soft body animals has lack of physical defence so it has evolved highly complex chemical defence which are protect them from the predation, microbial infection etc [9, 10, 8]. Due to sporadic in occurrence at new sites and sudden incursion number of hypothesis one is breeding aggregation [11] second is hydrological conditions in sub-tidal habitat may be a compromise between a preference for low-intensity wave action and their preferences for intertidal algae species [12], third is for aggregation is that it occurs where food is localized [13], population dynamics of *B. leachii* found in association with a cyanobacterial blooms that occur in sea grass beds in tropical conditions [14]. Mediterranean is having hundreds of alien species are considered to have migrated from the Red Sea via the Suez Canal [15]. Similarly to find possible genetic linkages between the species found in Mar Menor (SE Spain) water with Indian water, an attempt was made through gene sequencing.

Material and Methods
Adult *B. leachii* were collected from the intertidal waters where sea grass bed was abundantly available Geographical position $13^0$40. 40" N80^{0}31.57"E (Fig. 1). One sample of *B. leachii* was preserved in 95% ethanol and taken to the Rajiv Gandhi Centre for Aquaculture, MPEDA Sirkali, Nagapattinam, Tamil Nadu for gene sequencing studies.

Fig 1: Collection site of *Bursatella leachii* at Pulicat Lake, Tamil Nadu

Method Used

a. Extraction of Total Genomic DNA

The sample was used for the extraction of total genomic DNA using Phenol Chloroform method standardised by CAGL. Quality of the genomic DNA was assessed using 0.7% agarose gel along with 1kb DNA ladder as size standard**.**

b. PCR Testing- 16srRN

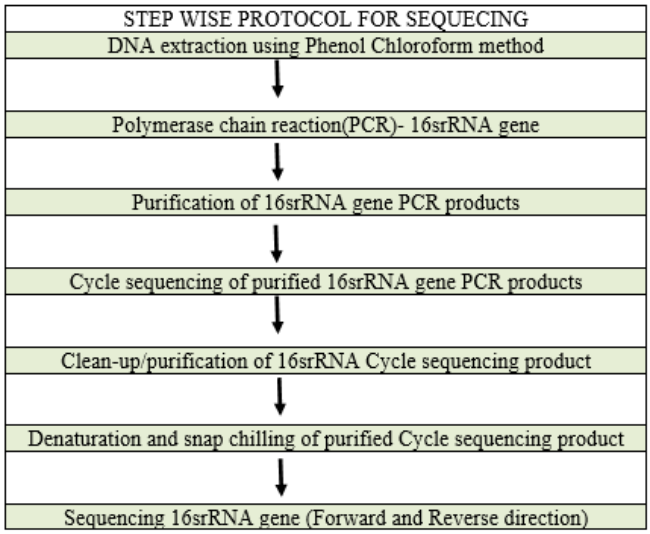
c. A gene amplification

Amplification of 16srRNA gene was carried out using universal 16srRNA forward & reverses primers. Expected band was amplified in the sample. PCR-generated amplicon was confirmed and purified using Gene JET PCR purification kit (Thermo Scientific, EU-Lithuania) to remove the primer dimer and other carryover contaminations. The quality of the product was assessed using 2% agarose gel along with 100bp DNA ladder as size standard and the product was found to be good for sequencing.

Primers used	Sequence (5' to 3')
Universal 16srRNAF (Forward)	CGCCTGTTTAACAAAAACAT
Universal 16srRNA (Reverse)	CCGGTCTGAACTCAGATCATGT

d. Sequencing

Amplified PCR products were purified and prepared for Cycle sequencing using the Big Dye® Terminator 3.1 sequence kit (Applied Biosystems, Foster City, California, USA). After cycle sequencing, the product was purified using Ethanol-EDTA purification protocol to remove the un-incorporated dNTP's, ddNTP's and primer dimer**.** The purified cycle sequencing products were dissolved in 12µl Hi-Di formamide and the samples were subjected for denaturation at 95 ^{0}C for 5mins. Denatured products were subjected for sequencing in forward and reverse direction using Genetic Analyzer 3500 (Life Technologies Corporation, Applied Biosystems®, California 94404, USA) as per manufacture's instruction. Sequences were aligned and edited using Mega software version 6[16] to confirm the species.



Results and Discussion

Bio-invasions are increasing the ecological and economical impacts on different habitats all over the world [17]. The *B. leachii* might have migrated to Mediterranean from the Red Sea via the Suez Canal, since its opening in 1869 [15]. Pulicat lake past history revealing that Arabs are the first landed in the Pulicat Lake followed by Portuguese and Dutch for the trading purpose; the ultimate source to reach to Pulicat lake was the Ships [18]. Due to increase load of ship transportation from one part to other parts of the world, Aquaculture activity, Marine Aquarium trade hundreds of alien species has establish themselves in worldwide [14, 19]. It is important to know the relationship between the species *B. leachii* found in other part of world is genetically similar to the other area or different. Pulicat Lake has rich flora and fauna diversity, which support active commercial fisheries. The Rajiv Gandhi Centre for Aquaculture (RGCA), MPEDA Sirkali, Nagapattinam, Tamilnadu is the institution in India to carry out the research in gene sequencing mapping and creating the data bank for the aquatic species in South India.

The raw data sequences of the samples were subjected for sequence alignment and editing using MEGA software. Sequence similarity search was carried out for all the samples against the sequences submitted in NCBI using NCBI-BLAST. The Species of *B. leachii* found in Pulicat Lake has shown 99% similarity with *B. leachii* specimen found in Mar Menor (SE Spain). The result mentioned above is only based on the BLAST search of aligned sequence of the single sample submitted. It is also advised to sequence minimum three samples with same morphtype to confirm the species.

The FASTA sequence provided below (after alignment and editing) of Mitochondrial 16srRNA gene:

>16S SEAHARE
CGCCTGTTTATCAAAAACATAGCCTAGAGAAAATTA
TTCTAGGTGAAGCCTGCCCAGTGAAAATTTTTAACG
GCCGCGGTACTTTGACCGTGCTAAGGTAGCGTAATC
AGTTGACTTTTAAATGAAGTCTTGTATGAATGGATCT
ATGGGATTAAGCTGTCTCTTATCTACTATACTGAAAT
TACTAATTAGGTGAAAAGGCCTAAAAATGAAAAAG
GACGAGAAGACCCTTAGAGTTTTGATTATAAATTTTT
GTTGGGGCGACGGGAAGACATTATAACTCTTCTATA
ATAAGACTTACTTGCCGGTTTTTTCAAGTATGGATAA
ACTACCTGAGGGATAACAGCATAATCCATTAATGGG
TTTGTGACCTCGATGTTGGACTAGGAAACTTATATTC
TAGCAGAATATATAGTTAGGTTCT

Sequence without alignment and editing

>16S SEAHARE F (Forward sequence)
GTCAGGAAAGGCCCGGKTTTYAAAACCGGAWCTGA
GTTYAAACCGGGGACTTTGACCGTGCTAAGGTAGCG
TAACAGTTGACTTTTAAATGAAGTCTTGTATGAATGG
ATCTATGGGATTAAGCTGTCTCTTATCTACTATACTG
AAATTACTAATTAGGTGAAAAGGCCTAAAAATGAAA
AAGGACGAGAAGACCCTTAGAGTTTTGATTATAAAT
TTTTGTTGGGGCGACGGGAAGACATTATAACTCTTCT
ATAATAAGACTTACTTGCCGGTTTTTTCAAGTATGGA
TAAACTACCTGAGGGATAACAGCATAATCCATTAAT
GGGTTTGTGACC

>16S SEAHARE R (Reverse sequence)
CSRRCGGGTCTGKATCGGASATGTTTTTGTTAACAGG
SGRGKCCRKTTCRAGGTCACAAACCCATTTTGGATTA
TGCTGTTATCCCTCAGGTAGTTTATCCATACTTGAAA
AAACCGGCAAGTAAGTCTTATTATAGAAGAGTTATA
ATGTCTTCCCGTCGCCCCAACAWWAATTTATAATCA
AAACTCTAAGGGTCTTCTCGTCCTTTTTCATTTTTAG
GCCTTTTCACCTAATTAGTAATTTCAGTATAGTAGAT
AAGAGACAGCTTAATCCCATAGATCCATTCATACAA
GACTTCATTTAAAAGTCAACTGATTACGCTACCTTAG
CACGGTCAAAGTACCGCGGCCGTTAAAAATTTTCAC
TGGGCAGGCTTCACCTAGAATAATTTTCTCTAGGCTA
TGTTTTTGTTAWWTTTTTTTT

>16S SEAHARE R RC (Reverse complementary of the reverse sequence)
AAAAAAAAWWTAACAAAAACATAGCCTAGAGAAA
ATTATTCTAGGTGAAGCCTGCCCAGTGAAAATTTTTA
ACGGCCGCGGTACTTTGACCGTGCTAAGGTAGCGTA
ATCAGTTGACTTTTAAATGAAGTCTTGTATGAATGGA
TCTATGGGATTAAGCTGTCTCTTATCTACTATACTGA
AATTACTAATTAGGTGAAAAGGCCTAAAAATGAAAA
AGGACGAGAAGACCCTTAGAGTTTTGATTATAAATT
WWTGTTGGGGCGACGGGAAGACATTATAACTCTTCT
ATAATAAGACTTACTTGCCGGTTTTTTCAAGTATGGA
TAAACTACCTGAGGGATAACAGCATAATCCAAAATG
GGTTTGTGACCTYGAAMYGGMCYCSCCTGTTAACAA
AAACATSTCCGATMCAGACCCGYYSG

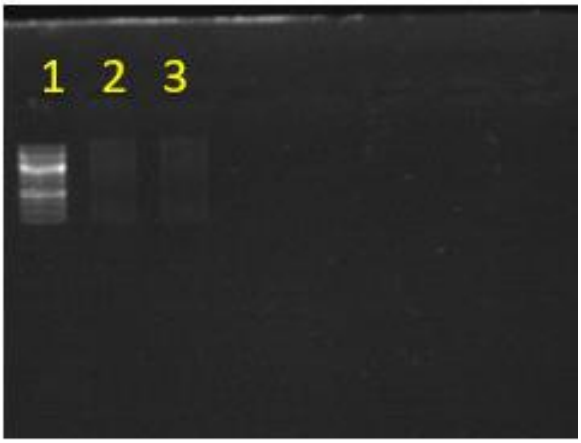


Fig 2: Gel image of Genomic DNA and 16S rRNA gene of *Bursatella leachii*. L1-1kb ladder, L2- Sample 1, L3-Sample 1a

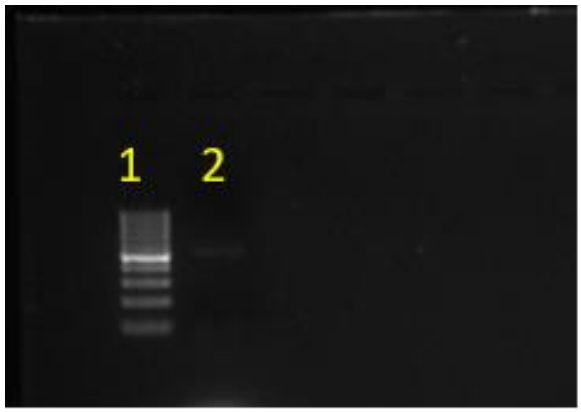


Fig 3: Gel image of PCR amplified 16S rRNA gene (~550bp) L1-100bp ladder, L2- Sample 1

Single sample analysis of *B. leachii* from Pulicat Lake with COI mitochondrial gene sequence of 550-bp in length and 16S gene Fig 2 & 3 shows the gel images of Genomic DNA and 16S rRNA gene. The Black morphotype of *B. leachii* recorded in Mar Menor and Mediterranean location with light morphotype found exclusively inside Mar Menor; the genetic homogeneity found in both the samples of *B. leachii* (Black & light morphotype) due to absence of variation in the mitochondrial DNA loci and individual sharing the same mitochondrial lineage [19]. Similar type of results has been observed for *E. timida* another Opisthobranch inhabiting the Mar Menor coastal lagoon and the Mediterranean Sea [20]. In fact, most pantropical species of sea slug have not survived the advent of molecular systematics [21] with the sole execption of the pelagic aeolid nudibranch *Glaucus atlanticus* and *Bursatella leachii* which could explain their unique range, is that both species occur in cold and temperate waters in South Africa, and this region could serve as a gateway that allows the maintenance of gene fow between the South Atlantic and Indian Ocean [22]. Although Pacific and Atlantic populations of *B.leachii* are genetically distinct, the divergence between population from these two geographic region is relatively small, suggesting B.leachii is a truly pantropical species which displays geographic structure among major basins. More importantly sequenced Mediterranean and Atlantic animal share similar or identical haplotypes [23].

Findings reveals that the species of *B. leachii* found in Mar Menor South East Spain and the species found in Pulicat Lake India shows genetic homogeneity at mitochondrial genes.

Moreover, the difference in morphotype of *B. leachii* may differ place to place due to feeding preferences and availability of food and seasonal variation or locality, but no variation in gene sequence.

Conclusions

Molecular studies is getting importance to determine the origin of the species population worldwide. In the present study we used mtDNA sequence analysis data to infer the population structure of *B.leachii* to determine the genetic affinities of the Mediterraean species population with the Pulicat lake population. It proves that *B.leachii* is a true pantropical species, migration of these species considered both man made & natural dispersal.

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