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Studies on mt DNA Cytochrome c-oxidase subunit 1 (COI) gene locus for identifying species of *Capitella capitata*

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Abstract
This study deals with barcode sequence obtained from *C. capitata* has been submitted to NCBI database and the sequence accession number assigned is AC171908. The results clearly suggest that it seem phylogeography was evident from the phylogram as closely related geographical species was placed side by side in the tree *i.e. C. capitata* of Pichavaram and Vellar, when compared to distance geographic species *i.e*. *C. capitata* of Gulf of mannar or USA waters. It may be expected due to its geographical variations. Hence Cytochrome oxidase could be the potential barcode for *C. capitata.* However, the sequences obtained for this species from USA waters showed more variations. Molecular taxonomic studies clearly indicate that there is COI gene of *C. capitata* carry phylogeographical signals. Information on phylogeography will be a potential tool solving in future conflicts on origin of organisms.

Keywords
Phylogeography, *Capitella capitata,* Cytochrome oxidase, variations.

Highlights
1. *C. capitata* has been submitted to NCBI database and the sequence accession number assigned is AC171908.
2. It may be expected due to its geographical variations.
3. Molecular taxonomic studies clearly indicate that there is COI gene of *C. capitata* carry phylogeographical signals. Information on phylogeography will be a potential tool solving in future conflicts on origin of organisms.

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INTRODUCTION
Taxonomists have described species for centuries without the aid of protein electrophoresis, RAPDs, RFLPs, AFLPs or DNA sequencing (Manwell and Backer, 1963). DNA barcoding is a taxonomic method that uses a short genetic marker in mitochondrial DNA to identify it as belonging to a particular species. Most eukaryotic cells contain mitochondria and mitochondrial DNA (mtDNA) that has a relatively fast mutation rate, which results in significant variance in mtDNA sequences between species and, in principle, a comparatively small variance within species. A648-bp region of the cytochrome-c oxidase subunit-I gene (COI) was initially proposed as a potential 'barcode' (Moulson and Herbert 2003).

It has long been recognized that DNA sequence diversity, whether assessed directly or indirectly through protein analysis, can be used to discriminate species. More than 40 years ago, starch gel electrophoresis of protein was first used to identify species (Manwell and Baker, 1963). Nearly 30 years ago, single gene sequence analysis of ribosomal DNA was used to investigate evolutionary relationships at a high level (Woese and Fox, 1977) and mitochondrial DNA approaches dominated molecular systematic in the late 1970's and 1980's (Avise, 1994). With the increased access to genetic techniques the detection of potentially cryptic species complexes within recognized morphological species is now commonplace (Hateley *et al*., 1992; Sato and Masuda, 1997; Maltagliati *et al*., 2000 and Maltagliati *et al*., 2001). Genetic differentiation between polychaete species has been shown through systems such as horizontal starch gel electrophoresis to determine allele frequencies (Sato and Masuda, 1997); RAPD-PCR fingerprinting analysis (Westheide and Schmidt, 2003); and electrophoretic analysis of allozymes loci (Maltagliati *et al*., 2000).

When analysed together, the differences between RAPD's and allozymes are small and most are not statistically significant Torres *et al*., (2003). Although these methods can show genetic variability between populations and closely related species (Stevens and Hogg, 2003) and they are having been used frequently to study populations of many polychaete species (Abbiati and Maltagliati, 1996) neither are suitable for a genetically based identification system. An accurate genetic ID system would have to be based on a sequence of DNA that is easily attainable and readily analysed. Other molecular techniques have been suggested as possible alternative to morphological identifications, and genetic barcoding may be one viable solution to this problem (Platnick, 2009).

Hebert *et al*., (2002) suggested a section of the mitochondrial DNA gene cytochrome-c oxidase subunit-I (COI). Once sequenced, this gene fragment could be used as a 'barcode' to distinguish between species. COI is the best candidate for this taxonomic tool, as it has a high degree of conservation and insertions and deletions are rare (Moritz and Cicero, 2004). It also has many rapidly evolving nucleotide sites, which will allow for differentiation between even recently evolved species (Nylander *et al*., 1999). Compared to the nuclear genome, the mitochondrial genome lacks introns. The mitochondrial genome has restricted exposure to recombination, and has a haploid mode of inheritance (Saccone *et al*., 1999). Hebert *et al*., (2003) demonstrated that the presence of high level of diversity between species sequences allowed for the successful assignment of 98% of samples of cryptic lepidopteron species. The ability to use COI to identify species will enable the identification of cryptic and polymorphic (where a single species may exhibit a range of different morphologies) taxa, and also identify and associate individuals of life stages other than adult to their correct species (Schander and Willassen, 2005).

COI fragments have been successfully taken from Polychaete worms previously to study the variation within phylogenies of Nereidiform Polychaetes, but most studies of Polychaetes using a COI sequence divergence technique are attempting to determine the phylogeny of the studied species, rather than separate between species of morphological similarity. It has not been explored weather DNA Barcoding can be used as an effective identification tool, right down to the species level. High levels of genetic differentiation has been shown between populations of the same polychaete species (Von Soosten *et al*., 1998; Maltagliati *et al*., 2001), including the Nereid *Hediste divericolor* (Hateley *et al*., 1992; Abbiati and Maltagliati, 1996; Rohner *et al*., 1997). It is unknown if such inter-population diversity will affect the use of DNA barcoding to discriminate between species. Considering these lacunae in the present study geographically distinct population of *Capitella capitata* were collected from the three mangrove habitats and subjected to the DNA barcoding study to understand efficiency of the tool to discriminate the populations at species level.

Among 27 species of polychaetes recorded, *Capitella capitata* formed the dominant population in all the three stations and the species is also considered to both the species of important based on its pollution indicater abilities. Hence *Capitella capitata* was taken for the phylogenetic studies.

MATERIALS AND METHODS

Collection of *Capitella capitata*

Samples of *Capitella capitata* (Möller *et al*., 1871), were collected from the Vellar, Pitchavaram and Gulf of Mannar (11°29'24.8"N Lat and 79°45'56.1"E Long, 11°27'18.9"N Lat and 79°47'31.6"E Long and 9º14'39.8"N Lat and 79º10'20.3"E Long) using sieve (0.5mm) Tamilnadu, eastcost of India. Then these polychaetes were preserved in 95% ethanol and stored in freezer until isolation of DNA.

Figure 1. ***Capitella capitata***

DNA isolation

Small pieces of tissue were taken from each individual and the DNA was extracted using the 'salting out method' (Aljanabi and Martinez, 1997).

PCR amplification

Primers (Folmer *et al*., 1994)

LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'

HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

PCR mixtures

Table 1. Showing various PCR components and its composition

S.No.	Component	Quantity
1	Buffer	2.5 µl
2	MgCl2	1.5 µl
3	dNTPs	2 µl
4	LCO 1490 Primer	1 µl
5	HCO 2198 Primer	1 µl
6	Template DNA	1 µl
7	Taq Polymerase	1 µl
8	MilliQ water	15 µl

Electrophoresing the amplicons

PCR amplicons of COI gene was gel checked using 1% China agarose prepared in 1X TAE buffer, bout 3µl Ethidium Bromide was used as staining dye and 5µl of Bromothymol blue was used as tracking dye. 100V DC was maintained between the electrodes of gel apparatus for 20 minutes. Following electrophoresis, the gel was analyzed and pictured in gel doc system (BIOSERVE, Hyderabad).

DNA sequencing

QIAGEN QIAquick™ kit was used for sequencing reaction. The sequencing PCR was done to amplify one strand of barcode gene employing the primer LCO1490 only under standard PCR conditions. The samples were precipitated and suspended in 40µl of loading solution provided with the kit. Sequencing was done with MegaBace sequencer- Bioserve India, Hyderabad.

Phylogram construction

The phylogenetic trees were constructed using MEGA 2.0 (Molecular Evolutionary Genetic Analysis) software. MEGA is an integrated tool for conducting automatic and manual sequence alignment, inferring phylogenetic trees, mining the web base databases, estimating the rates of molecular evolution, and testing evolutionary hypothesis.

The barcode sequences are prepared in FASTA format in notepad files and uploaded into MEGA for multiple alignments. After aligning, the sequences are subjected to phylogram construction based on the standard parameters of the software.

RESULTS

Agaroge gel run representing the amplicons of COI gene

St-1, St-2, and St-3 represents the tissue samples of *C. capitata* collected from Vellar, Pitchavaram and Gulf of Mannar respectively. M represents the marker used.

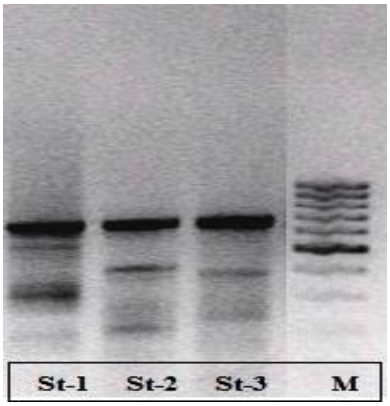


Figure 2. PCR amplification & Gel electrophoresis

Barcode sequences

> *C.capitata* Vellar

GCAAGTTTCATAGAGATTGAGCTTCAGCAAAAAAAGTT
ACAATGTATTACAAAAATTTACAATAATTATTAGGGAC
ACCAGTTTCCGACTGAAAAACATGAAAAATTAGAGAAT
CCGAAACTGAAGATAACCGAAGAGAACTGATATATAAT
ATATTTGATATTCAAGCTACAGTAAGAACAATTAAATTT
GATTCTCCTGGGGTAGAGGTGGATAGGTGGAGGAAGG
GGTGGAGGTGGAGGTGGAGGAGGAAAAGGAGGAGG
CGTGCCATTATCGTTCTCGTCACTGTCAGACCTAGCATC
GGAACTGAAAAAAAGAAAGCATAATGTAATGAATATG
TATGTAAGCTAATAACAATTAAATTTCACTATTAGTATA
AAAATTTCTATAGAATTCATTCGTCCTTTTTACAGATGG
CTTAAGTAGGGGATAGGGGTGAAGGCGGGCCTTTATC
CTCGTCACTAGTAAGGCCAACAGCACACTCAATACATA
CCCATAAATAAACTTGAAGGCCAAGATGCATGTTGATA
GTTCGTGGATGCTCTCTCTTCTTCTGGCGCTTCAGTTTC
GAACTGCACGTCATGTCAAACACCTAAAAA

> *C.capitata Pichavaram*

AAAATTTGTTACAATGTATTACAAAAATTTACAATAATT
ATTAGGGACACCAGTTTCCGACTGAATCACATGAAAAA
TTAGAGAATCCGAAACTGAAGATAACCGAAGAGAACT
GATATATAATATATTTGATATTCAAGCTACAGTAAGAAC
AATTAAATTTGATTCTCCTGGGGTAGAGGTGGATAGGT
GGAGGAAGGGGTGGAGGTGCAGGTGGAGGAGGAAA
AGGAGGAGGCGTGCCATTATCGTTCTCGTCACTGTCAG
ACCTAGCATCGGAACTGAAATTAAGAAAGCATAATGTA
ATGAATATGTATGTAAGCTAATAACAATTAATATTCACT
ATTAGTATAAAAATTTCTATAGAATTCATTCGTCCTAAT
TACAGATGGCTTAAGTAGGGGATAGGGGTGAAGGCG
GGCCTTTATCCTCGTCACTAGTAAGGCCAACAGCACAC
TCAATACATACCCATAAATAAACTTGAAGGCCAAGATG
CATGTTGATAGTTCGTGGATGCTCTCTCTTCTTCTGGCG
CTTCAGTTTCGAACTGCACGTCATGTCAAACACC

> *C.capitata Gulf of Mannar*

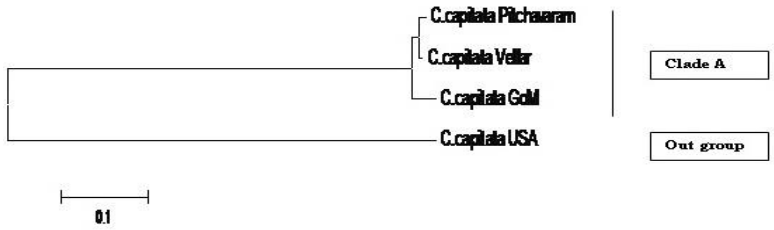
ACAAAAATTTACAATAATTATTAGGGACACCAGTTTCC
GACTGAAAAACATGAAAAATTAGAGAATCCGAAACTG
AAGATAACCGAAGAGAACTGATATATAATATATTTGAT
ATTCAAGCTGCAGTAAGAACAATTAAATTTGATTCTCCT
GGGGTAGAGGTGGATAGGTGGAGGAAGGGGTGGAG
GTGGAGGTGGAGGAGGATTAGGAGGAGGCGTGCCAT
TATCGTTCTCGTCACTGTCAGACCTAGCATCGGAACTG
AAATAAAGAAAGCATAATGTAATGAATATGTATGTAAG
CTAATAACAATTAAATTTCACTATTAGTATAAGCGTTTC
TATAGAATTCATTCGTCCTTTTTACAGTTGGCTTAAGTA
GGGGATAGGGGTGAAGGCGGGCCTTTATCCTCGTCAC
TAGTAAGGCCTTACAGCACACTCAATACATACCCATAA
ATAAACTTGAATTTTAAGATGCATGTTGATAGTTCGTG
GATGCTCTCTCTTCTTCTGGCGCTTGTCGTGA

>*C.capitata [Acc. No. AC171908]*

AAAATCCATGATCTTTGAAGTTCTGCTGATTCATCAGTA
CACAAACCCAGACTTGAAACAGGTTCAGTAATTTCGTT
CCCCACACATGTAATCTGGTCCTGCAGTTAGTTAGTTAT
GACTATTATTTATTCGACCAATCAGTGCTCAGGGCTTCG
GATTAGAATTCAAACAGTAGATCCTTCAACCACATGCC
ATCCCTGATGTTTTCTGATGGTACAGTATCACCAAATAC
GTTACATAATCCTTGGTACAGTCCCTACCCCCTAGAAAA
AAAAAATAAATAAAAGGGAGGAGGAGCCGATCCCTGA
TGGCAACTATTCCCATTTGGATCTTTTTCTCTCATTACCA
ACTTCTGTCACCTGATATTCGCAATATTTTGTATACATG
TAGCAAGAAGTCCATTCATTTGTGCTCTCGTCATTAAT

Table represents the barcoded sequences of *C. capitata* collected from different geographies. *C. capitata* from USA waters was extracted via NCBI (Acc. no.AC171908) will act as an out-group sequence.

Phylogram



Phylogenetic tree constructed via NJ distance methods using Kimura 2 Parameters. *Capitella capitata* COI sequence extracted from NCBI (Acc. No.AC171908) was used as an out-group sequence.

DISCUSSION AND CONCLUSION

The salting out protocol yielded good quantity of DNA for further analysis. Though background amplification of genes was visualized in agarose gels, they interfered less during sequencing process. About 599 bps are recovered from the COI gene of *C. capitata* collected from Vellar estuary, whereas the 565 bps and 522 bps are recovered from COI gene of *C. capitata* collected from Pichavaram and Gulf of Mannar respectively. The sequences were submitted to NCBI and awaited for accession numbers (Von Soosten *et al*., 1998; Maltagliati *et al*., 2001).

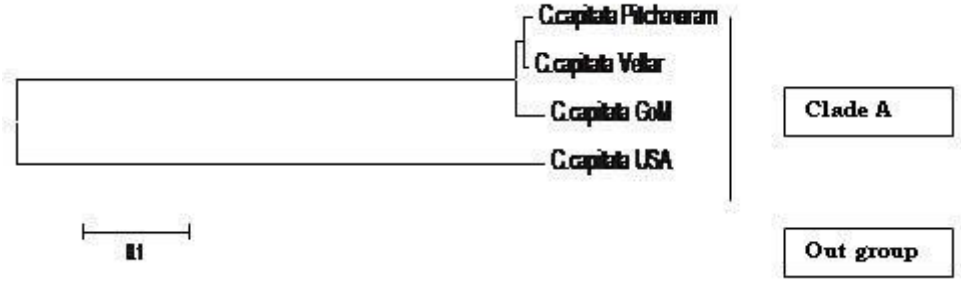
The phylogram constructed via NJ method using K2P distance showed two clear branching patterns, where the out group was clearly placed outside of the cluster. The clade A consists of all *C. capitata* collected from different geographies. The *C. capitata* collected from Vellar and Pitchavaram shows closer relatedness and places separately *C. capitata* collected from Gulf of Mannar. It seems that the species from nearby geographies possess higher degree of relatedness when compare to the far way species (Gulf of Mannar). This can be further evidenced from the reference code compared (USA No: AC171908) which is separated and further from the sampler collected from India. So, it is quite evident that within the same species barcode gene possesses variations. Also, it seems phylogeography was evident from the phylogram as closely related geographical species were placed side by side in the tree *i.e. C. capitata* of Pichavaram and Vellar, when compared to distance geographic species of Gulf of Mannar or USA waters.

To prove the COI gene of *C. capitata* to carry phylogeographical signals large sampling should be done that might facilitate more interesting results but as per the objectives of this work. *C. capitata* collected from the study area was barcoded and their phyologeographical signals in the COI gene was studied and clearly established. Among the three stations, *Capitella capitata* was successfully barcoded to 599 bps. The sequence is given below.

> *C.capitata* Vellar

GCAAGTTTCATAGAGATTGAGCTTCAGCAAAAAAAGTT
ACAATGTATTACAAAAATTTACAATAATTATTAGGGAC
ACCAGTTTCCGACTGAAAAACATGAAAAATTAGAGAAT
CCGAAACTGAAGATAACCGAAGAGAACTGATATATAAT
ATATTTGATATTCAAGCTACAGTAAGAACAATTAAATTT
GATTCTCCTGGGGTAGAGGTGGATAGGTGGAGGAAGG
GGTGGAGGTGGAGGTGGAGGAGGAAAAGGAGGAGG
CGTGCCATTATCGTTCTCGTCACTGTCAGACCTAGCATC
GGAACTGAAAAAAAGAAAGCATAATGTAATGAATATG
TATGTAAGCTAATAACAATTAAATTTCACTATTAGTATA
AAAATTTCTATAGAATTCATTCGTCCTTTTTACAGATGG
CTTAAGTAGGGGATAGGGGTGAAGGCGGGCCTTTATC
CTCGTCACTAGTAAGGCCAACAGCACACTCAATACATA
CCCATAAATAAACTTGAAGGCCAAGATGCATGTTGATA
GTTCGTGGATGCTCTCTCTTCTTCTGGCGCTTCAGTTTC
GAACTGCACGTCATGTCAAACACCTAAAAA

The barcode sequence obtained in the present study for *C. capitata* has been submitted to NCBI database and the sequence accession number assigned is AC171908. The results clearly suggested that phylogeography was evident from the phylogram as closely related geographical species was placed side by side in the tree *i.e. C. capitata* of Pichavaram and Vellar, when compared to distance geographic species *i.e. C. capitata* of Gulf of Mannar or USA waters level (Woese and Fox, 1977).



This was expected due to its geographical variations. Hence Cytochrome oxidase could be the potential barcode for *C. capitata.* However, the sequences obtained for this species from USA waters showed more variations than the Indian waters (Abbiati and Maltagliati, 1996).

To prove the COI gene of *C. capitata* to carry phylogeographical signals, large sampling should be done that might facilitate more interesting results. But as per the objectives of this work *C. capitata* collected from the study area was barcoded and their phyologeographical signal in the COI gene was studied.

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