Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2016; 4(5): 166-170

Received: 24-07-2016
Accepted: 25-08-2016

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# Mitochondrial *COX1* gene based delineation of putative insect species from Salt Lake of Great Rann of Kutch, India

**Rakshit Ojha, SK Jalali, TM Shivalingaswamy and Abraham Verghese**

## Abstract
Insect biodiversity of Salt Lake of Great Rann of Kutch in western India has not been studied in detail so far. This region is one of its kinds being a seasonal salt marsh representing India and Pakistan. Shore insects are one of the major invertebrates that inhabit this unique habitat and use carbon source for their growth and are considered as greenhouse pests. To identify these less described insect taxa, first specimens were morphologically delineated the species. Thereafter, DNA barcoding based on *COX*1 gene was applied to distinguish specimens collected into 9 different species. The quantification of biodiversity revealed that 80% of specimens belonged to Diptera and 10% each of Hymenoptera and Coleoptera. Based on molecular identification, two specimens that had 99% similarity with GenBank sequences were identified up to species level and were designated as *Australospesis niveipennis* (Diptera: Sepsidae) (insect code as SH6 GenBank accession no. KP227753 and SH7 accession no. KP227754) and one species designated as *Atherigona varia* (Diptera: Muscidae) (insect code SH2 GenBank accession no. KP227750). Three species were in dentified up to genus level, *viz*., *Musca* sp. (Diptera: Muscidae) (insect code SH1 GenBank accession no. KP227749), *Microchironomus* sp. (Diptera: Chironomidae) (insect code SH4 GenBank accession no. 227752) and *Lispe* sp. (Diptera: Muscidae) (insect code SH12 GenBank accession no. KP227758). However, other four insects could be identified up to family level only as it has similarity from 77-91% only in GenBank similarity test. These insects belonged to Chloropidae, Staphylinidae and Evaniidae. A monophyletic tree was observed with 4 clades showing interordinal relationship between 7 species of Order Diptera, 1 species of Coleoptera and 1 species of Hymenoptera. The overall transition/transversion bias R was 3.70 and nucleotide composite distance was 0.232, indicating a strong negative correlation trend, which suggests further sampling of these taxa in the Salt Lake area in different seasons.

**Keywords:** Shore insects; DNA barcoding; Salt Lake; Great Rann of Kutch; *COX1* gene

## Introduction
The Great Rann, in Kutch (24.08°N, 70.64°E), is a seasonal salt marshy lake located in the Thar Desert of Gujarat, India and the Sindh Province of Pakistan, with a total surface area of 7505 square kilometres and is reputed to be the largest salt desert in the world. It is one of the most remarkable and unique landscapes in the entire world [1]. It is a vast desiccated, unbroken bare surface of dark silt, encrusted with salts, which transforms into a spectacular coastal wetland after the rains. The Rann is considered to be a large ecotone, a transitional area between marine and terrestrial ecosystems. During monsoon, the Rann gets inundated for a period of about one month. The vast cover of saline mudflats in the sanctuary has no vegetation, except on the fringes and islets, with growth activity largely xerophytic, triggered by the advent of monsoon rains. The habitat includes 93 species of invertebrates, including 24 insects (http://whc.unesco.org/en/tentativelists/2105/). Except for this report, there is no information on the insect fauna of this unique region. Therefore, a survey was carried out during September 2014, immediately after rains, to collect insects occurring in this vast Salt Lake ecotone. Insects collected during survey were transferred in glass vials and brought to the laboratory for further processing.

## Materials and Methods
The survey was conducted, after the monsoon, in the month of September 2014 in the Great Rann of Kutch, the entire desert was inundated with saline water and there was no sight of any flora in the area.

Surprisingly, we noticed the activity of insects mostly dipterans attracted (Probably Carbon dioxide emission) to the wind shield and windows of our diesel driven car. All the insects that attracted were collected with the help of aspirator and were transferred to plastic vials. The specimens were transported to the laboratory at National Bureau of Agricultural Insect Resources, Bengaluru, and adults were separated by their distinct morphological characteristics. Species composition was dominant with dipterans as many of them breed in salt marshes.

Based on their morphological characters, the composition of collection was grouped into dipterans (3 Muscidae, 2 Chloropidae, 1 Sepsidae and 1 Chironomidae), Hymenopteran (1 Evaniidae) and a coleopteran (1 Staphylinidae) and were delineated into 9 species and were tentatively determined to their family level (Fig. 1). However, in absence of expert taxonomist for these families in India, particularly insects associated with Salt Lake, molecular technique based on their DNA was adopted for their identification. The specimens that were morphologically designated as tentative species were used for molecular identification based on mitochondrial *COX1* gene in the Molecular Entomology Laboratory at ICAR-NBAIR, Bangalore.

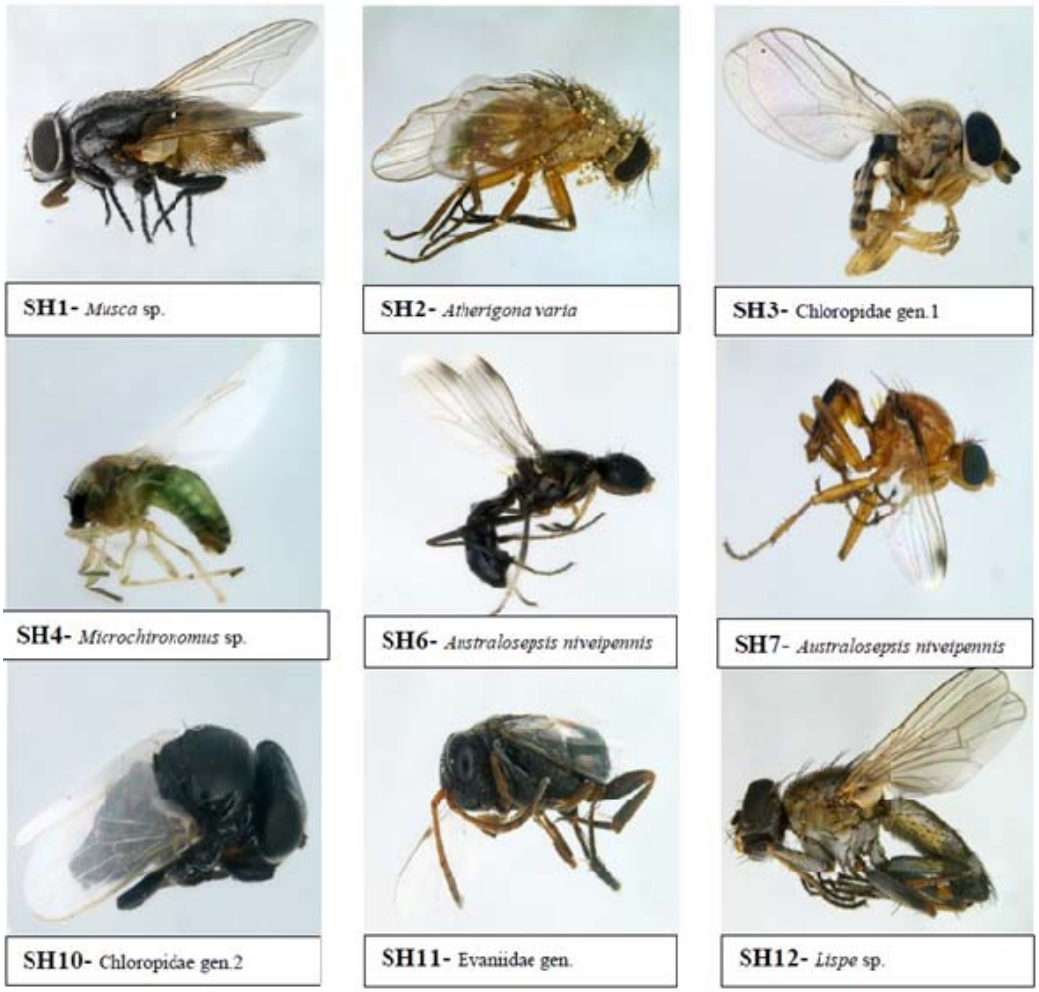


**Fig 1:** Images of shore insects collected from Salt Lake of the Great Rann, Kutch, Gujarat, India

**Amplification of mtDNA *COX1* gene**

DNA extraction was performed on single specimen using Qiagen DNeasy® kit, following the manufacturer's protocols. The remaining individual of same species of each Shore fly kept as voucher specimens at NBAIR, Bangalore, at -70°C. The DNA thus obtained was subjected to PCR amplification of a 658bp region near the 5' terminus of the *COX1* gene following standard protocol [2]. Primers used for amplification of *COX1* gene were: forward primer (LCO 1490 5'-GGTCAACAAATCATAAAGATATTGG-3'), and reverse primer (HCO 2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). Polymerase Chain Reaction were carried out in flat capped 200 µL volume PCR tubes obtained from M/s Tarsons, Kolkata, India. 50 µL reaction volume containing: 5 µL GeNei™ Taq buffer, 1 µL GeNei™ 10mM dNTP mix, 1 µL (20 pmol/µL) forward primer, 1 µL (20 pmol/µL) reverse primer, 1 µL GeNei™ Taq DNA polymerase (1 U/µL), 5 µL DNA (50 ng/µL), and 36 µL sterile water. Thermo cycling consisted of an initial denaturation of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 1 min. PCR was performed using a BioRad C1000™ Thermal Cycler. The amplified products were analysed on 1.5% agarose gel electrophoresis as described [3]. The amplified products were sequenced by M/s Chromous, Bangalore. Each specimen PCR sample was bi-directionally sequenced and checked for homology, insertions and deletions, stop codons, and frame shifts by using NCBI-BLAST and ORF finder.

**Data Analysis**

The sequences of shore flies were aligned using ClustalW with default settings of gap opening penalty 15, and a gap-extension 6.06 in pairwise and 6.06 in multiple alignments and phylogenetic analysis was performed using MEGA 6.0 software. Neighbouring Joining (NJ) tree was constructed using a Kimura-2-parameter ($K_2P$) model with the *cox1* nucleotide sequences of the Indian populations as per [4] and codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated from the dataset. The A, T, G, C, AT and GC content of all 10 sequences was obtained using a computer program designed in the Bioinformatics Lab at ICAR-NBAIR (www.nbair.res.in), Bangalore, India. The AT% at the three codon positions was calculated using the same program. Sequences and other specimen information are available on BOLD Systems (http://www.boldsystems.org).

**Results and Discussion**

Genomic DNA extracted and confirmed on 1% agarose gel from the insects collected from different parts of Salt Lake in Great Rann of Kutch, India, was subjected to *COX1* PCR and 658bp diagnostic fragment of mitochondrial DNA was obtained. All *COX1* nucleotide sequences were checked for homology by using BLAST tool of NCBI. Maximum similar sequence was taken as tentative identification up to order, genera and species levels (Table 1). As no insertions, deletions or stop codons were observed as 2nd frame of DNA, sequences were chosen from ORF finder for submission to GenBank. The quantification of biodiversity revealed that 80% of specimens belonged to Diptera and 10% each to Hymenoptera and Coleoptera. Based on molecular identification, three specimens that had 99% similarity with GenBank sequences were identified up to species level and were designated as *Australospesis niveipennis* (Becker) (Diptera: Sepsidae) (insect code as SH6 GenBank accession no. KP227753 and SH7 accession no. KP227754) and *Atherigona varia* (Diptera: Muscidae) (insect code SH2 GenBank accession no. KP227750). Three species were identified up to genus level, *Musca* sp. (Diptera: Muscidae) insect code SH1 GenBank accession no. KP227749 and *Microchironomus* sp. (? *tener*) (Kieffer) (Diptera: Chironomidae) insect code SH4 GenBank accession no. 227752 and *Lispe* sp. (Diptera: Muscidae) insect code SH12 GenBank accession no. KP227758. Four other insects could be identified only up to family level as it had similarity between 77-91% only. These insects belonged to Chloropidae, Staphylinidae and Evaniidae (Table 1). *Australospesis niveipennis* has been recorded from different states of India [5, 6], however, this is first time it has been recorded from Salt Lake of Rann in Gujarat. *Microchironomus* sp. (? *tener*) has been recorded from China, Japan, Australia [7] and Sweden and usefulness of *COX1* gene to improve species resolution and its potential for implementation in monitoring programmes for Chironomidae collected from Baltic Sea [8] has been demonstrated.

Nucleotide composition of these 10 sequences ranged from 34.2 to 40.1% in T, 14.6-20.8% in C, 11.7-17.3% in G and 28.01- 32.04% in A.% AT (67.34) was higher as compared to% GC (32.66), which was in accordance with invertebrate mitochondrial DNA (Table 2).

**Table 1:** BLAST similarity of insects collected from Great Rann of Kutch, India

| Insect code | Accession number for submitted samples | Order: Family | Homology % | GenBank maximum similar species |
|---|---|---|---|---|
| SH1 | *Musca* sp. _ KP227749 | Diptera: Muscidae | 94 | *Musca autumnalis*_KF751383.1/Spain |
| SH2 | *Atherigona varia* _ KP227750 | Diptera: Muscidae | 99 | *Atherigona varia*_ KJ510609.1/Singapore |
| SH12 | *Lispe* sp_ KP227758 | Diptera: Muscidae | 90 | *Lispe orientalis* EU627716.1/China |
| SH3 | Chloropidae gen. chlorRann01 sp_ KP227751 | Diptera: Chloropidae | 91 | Tachinidae gen. tachJanzen01 sp _JQ575790.1/Canada |
| SH10 | Chloropidae gen. chlorRann01 sp_ KP227756 | Diptera: Chloropidae | 91 | Tachinidae gen. tachJanzen01 sp._ JQ575790.1/Canada |
| SH4 | *Microchironomus* sp. (? tener) KP227752 | Diptera: Chironomidae | 93 | *Microchironomus tener* _KC750456.1/Australia |
| SH6 | *Australosepsis niveipennis* _ KP227753 | Diptera: Sepsidae | 99 | *Australosepsis niveipennis* & *Sepsis niveipennis*_EU435779.1 & EU435816.1/ Singapore |
| SH7 | *Australosepsis niveipennis* _ KP227754 | Diptera: Sepsidae | 99 | *Australosepsis niveipennis* & *Sepsis niveipennis*_EU435779.1 & EU435816.1/ Singapore |
| SH8 | Staphylinidae gen. staphRann03 sp_ KP227755 | Coleoptera: Staphylinidae | 85 | *Acrolocha pliginskii* _ KJ962995.1/Finland |
| SH11 | Evaniidae gen. evaRann04_ KP227757 | Hymenoptera: Evaniidae | 77 | *Rothevania valdiviana* _ AY800160.1/USA |

**Table 2:** A, T, G, C, AT & GC% for 10 insects collected from Great Rann of Kutch, Gujarat, India

| CODE/Nucleotides | T | C | A | G | AT | GC |
|---|---|---|---|---|---|---|
| SH1 | 38.6 | 16.9 | 28.1 | 16.4 | 66.7 | 33.3 |
| SH2 | 39.4 | 16.1 | 28.7 | 15.8 | 68.1 | 31.9 |
| SH3 | 36.9 | 14.6 | 32.4 | 16.1 | 69.3 | 30.7 |
| SH4 | 40.1 | 18.1 | 24.9 | 16.9 | 65.0 | 35.0 |
| SH6 | 38.7 | 14.8 | 29.4 | 17.1 | 68.1 | 31.9 |
| SH7 | 38.9 | 15.2 | 29.3 | 16.6 | 68.2 | 31.8 |
| SH8 | 34.2 | 18.5 | 29.9 | 17.3 | 64.1 | 35.9 |
| SH10 | 37.1 | 15.7 | 30.9 | 16.4 | 67.9 | 32.1 |
| SH11 | 38.3 | 20.8 | 29.2 | 11.7 | 67.5 | 32.5 |
| SH12 | 38.4 | 16.1 | 30.1 | 15.3 | 68.5 | 31.5 |
| Average | 38.1 | 16.7 | 29.3 | 16.0 | 67.34 | 32.66 |

**Phylogenetic analysis**

A total of 9 designated 'species' were studied, according to the tree generated by applying K2P/NJ model. All these 9 species formed a monophyletic clade (Fig. 2). One major clade consisted of 5 species belonged to order Diptera, which was further distributed into four families, *viz.,* Sepsidae, Chloropidae, Muscidae and one coleopteran species representing family Staphylinidae, whereas 3 sub-clade consisting of 3 species belonged to Muscidae and Chiromonidae. However, one hymenopteran species representing family Evaniidae branched out distantly as an out group showing interordinal relationship. The pattern of nucleotide substitution was inferred from the $K_2P$ model, the overall transition/transversion bias is (R) 3.70 and the overall average between nucleotide composite distance (d) was 0.232 (Table 3).

The current study uses the cytochrome c oxidase subunit-1 *COX1* to delineate putative insect species as advocated that DNA barcodes based on studies conducted [2]. Shore insects associated with the Great Rann of Kutch ecosystem are less studied invertebrates, since the insects of this part are not common to other region. During our survey most of the insects were attracted to the diesel driven car showed their probable attraction to $CO_2$ emission. The specimens collected were molecularly characterized, in the absence of prior work done in this field apart from in Great Salt Lake in northern Utah, United States [9], Use of *COX1* gene has been advocated for recognizing putative species determination, which accelerates taxonomic workflows [10, 11]. Bootstrap consensus monophyletic tree was observed, interordinal relationship showed 3 orders, *viz.,* Diptera, Coleoptera and Hymenoptera, where hymenopteran species forming an out-group. However, one coleopteran species showed 56% relation with a dipteran species, which could be due to low interspecies ratio calculated by software, but longer branch length of coleopteran species shows separation from dipteran species. Nucleotide composition indicated AT rich DNA, which is a well-known characteristic of insect mitochondrial DNA was found to be 67.34% than 32.66% of GC content. The overall transition/transversion bias (R) was 3.70 whereas overall average between nucleotide composite distance was 0.232 indicating towards strong negative correlation of trend. In an earlier study, DNA barcoding using *COX1* gene was used to bring out the evolutionary relatedness between various *Epinephelus* species (fishes of family Serranidae) collected from Andaman coastal region in India [12]. The study suggested that further sampling of these taxa in the Salt Lake area in different seasons.

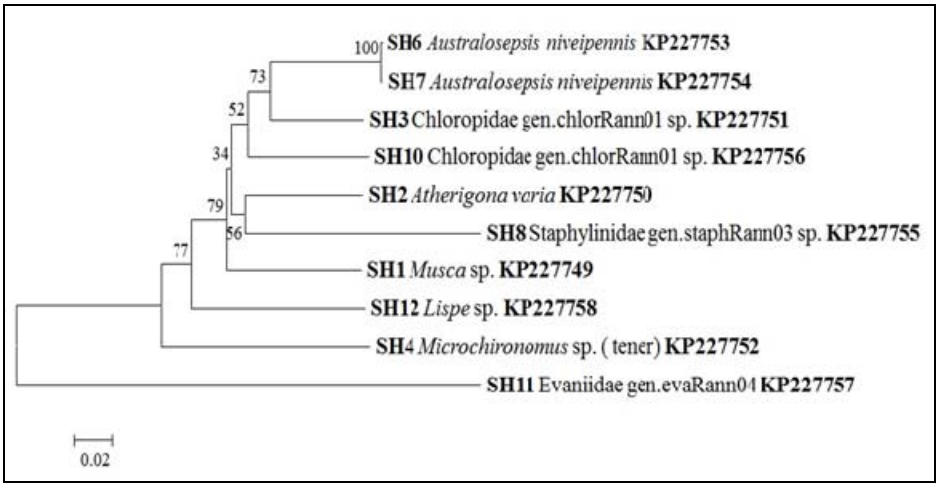


**Fig 2:** Neighbour-Joining (NJ) tree with Kimura-2-Parameter model and bootstrap support (1000 replicates) showing clustering of insects for cox1 sequences. Two major clades can be seen, with sufficient bootstrap support. Among 10 shore insects SH11 is separating from the upper clade as out group.

**Table 3:** Maximum composite likelihood estimate of the pattern of nucleotide substitution for 10 insects' mitochondrial *cox1* gene

| Nucleotides | A | T | C | G |
|---|---|---|---|---|
| A | - | 2.92 | 1.26 | 2.6 |
| T | 2.25 | - | 23.3 | 1.26 |
| C | 2.25 | 54.11 | - | 1.26 |
| G | 4.64 | 2.92 | 1.26 | - |

The overall transition/transversion bias is $R$ = 3.70 # The Nucleotide composition distance overall average is 0.232

**Conclusion**

As many groups of arthropods lack expert taxonomists, an integrated approach is a current need to identify unknown species of unexplored places in India and the world. Large scale sampling of unknown taxa and availability of DNA tools will increase the species identification as this paper has demonstrated interestingly. Even with limited collections, we could identify shore insects and establish interordinal relationship between orders representing a part of the invertebrate biodiversity of Great Rann of Kutch, Gujarat, India.

**Acknowledgement**

This work is part of Ph. D. work of the first author of Jain University, Bengaluru.

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