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RESEARCH ARTICLE

UNIQUE SEQUENCES IN THE COX1 GENE OF INDIAN MOSQUITO SPECIES

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ABSTRACT

A DNA barcode is a short DNA sequence from a standardized region of the genome used for identifying species. The essential aim of DNA barcoding is to use a large-scale screening of one or more reference genes in order to assign unknown individuals to species, and to enhance discovery of new species (Hebert et al., 2003). Biological taxonomists apply this principle to species classification. The first application of using the DNA sequences in systematic biological taxonomy (also called DNA taxonomy) was conducted by Tautz et al., (2002) and then , Hebert et al., (2003) proposed the concept of DNA Barcoding and suggested its use for a single mtDNA gene, mitochondrial cytochrome c oxidase I (COI), as a common sequence in animal DNA barcoding studies. A unique DNA is a stretch of DNA present in only a single copy in a cell. Genomes contain all the vital genetic solutions discovered by our ancestors in response to all the environmental challenges that they had successfully overcome in the course of evolution. These solutions are maintained by natural selection in the form of unique DNAs. These DNAs give us a chance to successfully overcome similar environmental challenges again and again. The goal of this article is to provide an overview of the rapidly expanding applications of molecular markers (unique sequences within the COX 1 gene) for identifying mosquito species. In the present study, we identify unique sequences in the mosquito COX1 gene using SAS tool.

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INTRODUCTION

Mitochondrial DNA analysis has proven an exceptionally useful tool for population genetics, intraspecific phylogeography, and systematics. In the search for a simple method to identify and compare species, Hebert et al. proposed DNA barcoding, a new system of species identification using the cytochrome *c* oxidase subunit 1 mitochondrial gene (*cox*1 or *COI*) as a standardized single molecular marker for the classification of animal species. One of the requirements of the DNA barcoding approach is that species identification is associated with a voucher belonging to a curated biological collection, enabling follow up and a strategy for corroborating species identification. In addition to the identification of known and new species, barcoding with the *cox*1 gene is suggested as a standard for cryptic taxa discovery, association of different life stages of the same species and wildlife conservation genetics. *Cox*1 appears to have a better phylogenetic signal than the other mitochondrial genes. The presence of unique DNA sequences allows scientists to identify signature sequences that can be later used as probes to detect individual organisms or to detect a particular gene. Changes of even one base pair can be readily detected by most hybridization techniques and by sequencing. Signature sequences are particularly important for diagnosis of viruses, which are the pathogens that lack ribosomal or mitochondrial genes. Their detection and identification is greatly simplified by using these sequences, as traditional methods can take up to a few weeks. The unique DNA sequences can also be used to design primers (short DNA fragments needed to initiate DNA amplification) for polymerase chain reaction (PCR). There is adequate difference between all the genes within one organism, as well as between organisms from different species, to ensure that the selected primers will only amplify the target sequence even if a mixture of different DNA molecules is present.

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MATERIALS AND METHODS

The nucleotide sequences of the COX1 gene was identified using Uniprot database. The UniProt Reference Clusters (UniRef) provide clustered sets of sequences from the UniProt Knowledgebase (including isoforms) and selected UniParc records. This hides redundant sequences and obtains complete coverage of the sequence space at three resolutions:

UniRef100 combines identical sequences and sub-fragments with 11 or more residues from any organism into a single UniRef entry. UniRef90 is built by clustering UniRef100 sequences such that each cluster is composed of sequences that have at least 90% sequence identity to, and 80% overlap with, the longest sequence (a. k. a. seed sequence). UniRef50 is built by clustering UniRef90 seed sequences that have at least 50% sequence identity to, and 80% overlap with, the longest sequence in the cluster.

METHODOLOGY USING SAS

Kumar *et al* sequenced 111 DNA sequences from mitochondrial cytochrome oxidase gene of 6. Here, these sequences have been grouped according to Genus and species to find unique sequences for each species that has been DNA barcoded by Kumar *et al*. Multiple sequences of the same species were present in the data however the sequence with the highest base pair length have been retained for the analysis. The below table (Table 1) gives a list of species with their GenBank accession numbers that was used for Kumar *et al*. The table is grouped by genus and gives a count of the number of sequences that were taken for the analysis. Species were chosen from *Aedes, Anopheles* and *Culex* genera for identifying unique sequences among them. The sequences of these species were cut into short sequences of 50 base pairs (bp) length in the following way – Short sequence 1 was from bp position 1 until 50, Short sequence 2 was from bp position 51 until 100, and so on. The average sequence length of the considered species was 650 and hence the average number of short sequences that resulted from cutting each sequence into 50 bp length was 14. The total number of 50 bp short sequences was 217. Similarly, the sequences were tested for the presence of unique short sequences by cutting into short sequences of 20 bp length. For this, the short sequence 1 was taken from bp position 1 to 20 and the next short sequence was taken from 21 to 40. This yielded around 33 short sequences for each species. The total number of 20 bp short sequences was 540. Each short sequence of a species was then compared to all the short sequences of the rest of the sequences. Exact comparison was done within the short sequence pool. The comparison highlighted the unique and identical sequences between any two species.

METHODOLOGY USING SAS

The DNA sequences of the included species were cut into 50 bp length using SAS programs. Programmatically, each nucleotide of a 50 bp sequence of one species was compared with each nucleotide of the second 50 bp sequence that came from a different species. If the sequences do not match each other then the short sequence was considered unique within the genus group. The unique sequences were tabulated and all the unique sequences together made a library of DNA short fragments for that particular genus.

Similarly, the DNA sequences were cut into short sequences of 20 bp length using SAS programs. Programmatically, each nucleotide of a 20 bp sequence of one species was compared with each nucleotide of the second 20 bp sequence that came from a different species. Upon comparison, if the short fragment was found, it was tabulated as a unique sequence in the library of DNA short fragments.

The sequences were cut in the following fashion – the first short sequence was cut from the nucleotide 1 to 50, the second short sequence was cut from nucleotide 51 to 100, the third short sequence was cut from nucleotide 101 to 150 and so on. The same fashion was followed for constructing a pool of 20 bp short fragments. Out of these pools, DNA short fragment libraries were constructed for each genus group.

SAS Methodology for Identification of unique sequences

```
/*Separating the genus and species names*/
data dp22; set dp21;
genus1_indian = compress(scan(species1_indian, 1));
species1_indian = compress(scan(species1_indian, 2));
run;

/*Sorting and grouping*/
%macro dp(genus);

data dp_&genus._indian;
set dp_g2;
where compress(genus1_indian) ="&genus";
genus = "&genus";
run;
proc sort; by genus1_indian ; run;

%mend;
```

```
/*Select genus group with more than 1 species*/
procsql;
create table genus_group as
select * from dp_g2
having count(genus1_indian) >1
; quit;

/*Cutting into 50 bp or 20 bp length*/
data genus_group_20; set genus_group;
array ge(*) frag1--frag35;
do i = 1 to 708 by 20;
ge(i) = substr(sequence,i,20);
end;
run;

data genus_group_50; set genus_group;
array gg(*) frags1--frags15;
do i = 1 to 708 by 50;
gg(i) = substr(sequence,i,50);
end;
run;
/*Comparing the short fragments*/
proccompare data=genus_group_20
compare=genus_group_20 out=ge_20;
var frag1--frag35;
run;
proccompare data=genus_group_50
compare=genus_group_50 out=ge_50;
var frags1--frags15;
run;
procexport data=ge_20 outfile="&path\ge_20. xls"
dbms=excel replace; run;
procexport data=ge_50 outfile="&path\ge_50. xls"
dbms=excel replace; run;
```

RESULTS

Unique Sequence in Aedes speies: The Sequences of all the Aedes species were considered from the 110 Indian Sequences that were analysed by Kumar et al for analysis. The below mentioned sequences occurred only once within the species and also across all the Aedes species considered for analysis.

Unique sequence in Anopheles species: The sequences of all the Anopheles species were considered from the 110 Indian sequences that were analysed by Kumar et al for analysis. The below mentioned sequences occurred only once within the species and also across all anopheles species considered for analysis.

Table 1. List of species with their Gen Bank accession numbers that was used for Kumar et al. The table is grouped by genus and gives a count of the number of sequences that were taken for the analysis

#	Genus	Species	GenBank Accession #	Included in Analysis
111	Grand Count			
10	Aedes Count			Yes
2	Aedes	Aedes (Aedimorphus) vexans	AY917213	
3	Aedes	Aedes (Diceromyia) iyengari	DQ431717	
4	Aedes	Aedes (Fredwardsius) vittatus	AY834246	
5	Aedes	Aedes (Lorrainea) fumidus Coringa	AY729978	
6	Aedes	Aedes (Stegomyia) aegypti	AY729987	
7	Aedes	Aedes (Stegomyia) aegypti	DQ424949	
8	Aedes	Aedes (Stegomyia) albopictus	AY729984	
9	Aedes	Aedes (Stegomyia) albopictus	AY834241	
10	Aedes	Aedes (Stegomyia) albopictus	DQ310142	
11	Aedes	Aedes (Stegomyia) albopictus	DQ424959	
38	Anopheles Count			Yes
12	Anopheles	Anopheles (Anopheles) barbirostris	AY729982	
13	Anopheles	Anopheles (Anopheles) peditaeniatus	DQ149237	
14	Anopheles	Anopheles (Cellia) aitkeni	AY917209	
15	Anopheles	Anopheles (Cellia) annularis	AY917197	
16	Anopheles	Anopheles (Cellia) culicifacies s	AY917198	
17	Anopheles	Anopheles (Cellia) culicifacies s	DQ424962	
18	Anopheles	Anopheles (Cellia) fluviatilis s	AY917202	
19	Anopheles	Anopheles (Cellia) fluviatilis s	DQ154155	
20	Anopheles	Anopheles (Cellia) fluviatilis s	DQ154156	
21	Anopheles	Anopheles (Cellia) fluviatilis s	DQ154158	
22	Anopheles	Anopheles (Cellia) fluviatilis s	DQ310150	
23	Anopheles	Anopheles (Cellia) fluviatilis s	DQ317595	
24	Anopheles	Anopheles (Cellia) fluviatilis s	DQ317596	
25	Anopheles	Anopheles (Cellia) jamesi	AY729972	
26	Anopheles	Anopheles (Cellia) jeyporiensis	DQ154157	
27	Anopheles	Anopheles (Cellia) jeyporiensis	DQ154159	
28	Anopheles	Anopheles (Cellia) jeyporiensis	DQ317591	
29	Anopheles	Anopheles (Cellia) jeyporiensis	DQ317592	
30	Anopheles	Anopheles (Cellia) jeyporiensis	DQ317593	
31	Anopheles	Anopheles (Cellia) maculatus	DQ267690	
32	Anopheles	Anopheles (Cellia) minimus s	AY917196	
33	Anopheles	Anopheles (Cellia) pallidus	AY729974	
34	Anopheles	Anopheles (Cellia) pallidus	AY917212	
35	Anopheles	Anopheles (Cellia) splendidus	AY917207	
36	Anopheles	Anopheles (Cellia) stephensi	AY729980	
37	Anopheles	Anopheles (Cellia) stephensi	DQ154166	
38	Anopheles	Anopheles (Cellia) stephensi	DQ310143	
39	Anopheles	Anopheles (Cellia) stephensi	DQ310148	
40	Anopheles	Anopheles (Cellia) stephensi	DQ317594	
41	Anopheles	Anopheles (Cellia) subpictus s	AY729970	
42	Anopheles	Anopheles (Cellia) subpictus s	AY917203	
43	Anopheles	Anopheles (Cellia) subpictus s	DQ267688	
44	Anopheles	Anopheles (Cellia) subpictus s	DQ310145	
45	Anopheles	Anopheles (Cellia) subpictus A	DQ310146	
46	Anopheles	Anopheles (Cellia) subpictus B	DQ310147	
47	Anopheles	Anopheles (Cellia) subpictus B	DQ310149	
48	Anopheles	Anopheles (Cellia) vagus	AY834247	
49	Anopheles	Anopheles (Cellia) varuna	DQ149241	
34	Culex Count			Yes
53	Culex	Culex (Culex) bitaeniorhynchus	DQ154162	
54	Culex	Culex (Culex) bitaeniorhynchus	DQ267687	
55	Culex	Culex (Culex) fuscocephala	DQ149236	
56	Culex	Culex (Culex) gelidus	AY729965	
57	Culex	Culex (Culex) hutchinsoni	DQ149239	
58	Culex	Culex (Culex) pseudovishnui	AY834248	
59	Culex	Culex (Culex) pseudovishnui	AY917215	
60	Culex	Culex (Culex) quinquefasciatus	AY729977	
61	Culex	Culex (Culex) quinquefasciatus	DQ267689	
62	Culex	Culex (Culex) sitiens	DQ154160	
63	Culex	Culex (Culex) sitiens	DQ154161	
64	Culex	Culex (Culex) sitiens	DQ154163	
65	Culex	Culex (Culex) sitiens	DQ310144	
66	Culex	Culex (Culex) sitiens	DQ317598	
67	Culex	Culex (Culex) tritaeniorhynchus	AY729975	
68	Culex	Culex (Culex) tritaeniorhynchus	AY834249	
69	Culex	Culex (Culex) tritaeniorhynchus	AY917206	
70	Culex	Culex (Culex) tritaeniorhynchus	DQ424952	
71	Culex	Culex (Culex) vishnui	AY729973	
72	Culex	Culex (Culex) vishnui	AY917214	
73	Culex	Culex (Culex) whitmorei	DQ154167	
74	Culex	Culex (Culiciomyia) nigropunctatus	AY729976	
75	Culex	Culex (Culiciomyia) pallidothorax	DQ154154	

…… Continue

76	Culex	Culex (Eumelanomyia) brevipalpis	AY834238
77	Culex	Culex (Eumelanomyia) brevipalpis	DQ424960
78	Culex	Culex (Eumelanomyia) brevipalpis	DQ424961
79	Culex	Culex (Eumelanomyia) malayi	DQ149238
80	Culex	Culex (Eumelanomyia) pluvialis	DQ317597
81	Culex	Culex (Lophoceraomyia) infantulus	AY729966
82	Culex	Culex (Lophoceraomyia) infantulus	DQ267691
83	Culex	Culex (Lophoceraomyia) minor	AY917211
84	Culex	Culex (Lophoceraomyia) minutissimus	DQ149240
85	Culex	Culex (Lophoceraomyia) rubithoracis	AY729981
86	Culex	Culex (Lutzia) fuscanus	AY729985

Table 2. Unique sequences of Aedes species of Indian subcontinent

Species	Length of full sequence	Highlighting unique sequence	Length of unique sequence	Remark
Aedes fumidus	518	TCATCAGGAACTGCCCATGCGGGAGCTTCTGTA	33	UNIQUE
Aedes albopictus	688	GGAGCTTCAGTAGATTTAGCTATTTTTTCT	30	UNIQUE
Aedes aegypti	635	ATAATTGGAGGATTTGGAAATTGATTAGTT	30	UNIQUE
Aedes vittatus	513	TCCTCTTTCATCAGGAGTAG	20	UNIQUE
Aedes vexans	519	AATTTTCTCTCTTCATTTAGCCGG	24	UNIQUE
Aedes iyengari	496	TCTCATCAGGTACTGCTCATGCTGGGGCCTCAG	33	UNIQUE

Table 3. Unique sequences of Anopheles species of Indian subcontinent

Species	Length of full sequence	Highlighting unique sequence	Length of unique sequence	Remark
Anopheles annularis	732	AACTGTTTACCCCCCTCTTTCTTCTGGGATTGCTCATGCAGGAGCTTCAG	50	UNIQUE
Anopheles barbirostris	665	ATATCCACCTTTATCTTCTGGAATTGCACATGCAGGAGCTTCTGTTGATT	50	UNIQUE
Anopheles culicifacies	681	GCTATTTTTTCTTTACATTTAGCAGGGATTTCTTCAATTTTAGGAGCAGT	50	UNIQUE
Anopheles fluviatilis	710	TGCACATGCTGGGGCTTCAGTAGACTTAGCTATTTTCTCTTTACATTTAG	50	UNIQUE
Anopheles jamesii	520	CCTCCTCTTTCTTCAGGAATTGCTCACGCGGGGGCTTCAGTAGATTTAGC	50	UNIQUE
Anopheles jeyporiensis	702	GCTTCAGTTGATTTAGCTATTTTCTCTTTACATTTGGCAGGAATTTCTTC	50	UNIQUE
Anopheles minimus	710	TGGAGCTTCAGTAGATTTAGCTATTTTTTCACTACATTTAGCTGGAATTT	50	UNIQUE
Anopheles pallidus	522	CATCAGGAATTGCTCATGCAGGAGCTTCAG	30	UNIQUE
Anopheles peditaeniatus	677	ACTACAGTAATTAATATACGATCACCAGGGATTACATTAGACCGAATACC	50	UNIQUE
Anopheles splendidus	499	TTAATATTAGGAGCACCAGATATAGCATTTCCTCG	35	UNIQUE
Anopheles stephensi	651	GATTGCACATGCAGGAGCATCAGTTGATTTAGCAATTTTTTCTCTACATT	50	UNIQUE
Anopheles subpictus	499	AATTGATTAGTTCCATTAATACTAG	25	UNIQUE
Anopheles vagus	701	CTTCAATTTTAGGAGCAGTAAATTTTATTACTACAGTTATTAATATGCGA	50	UNIQUE
Anopheles aitkenii	507	TTTCTTCTGGTATTGCTCATGCAGGAGCTTCTGTAGATTTAGCAATTTTC	50	UNIQUE
Anopheles barbirostris	665	ATTACTACTGTTATTAATATACGTTCACCAGGAATTACTTTAGATCGAATA CCATTATTTGTCTGATC	68	UNIQUE

Table 4. Unique sequences of Culex species of the Indian subcontinent

Species	Length of full sequence	Highlighting unique sequence	Length of unique sequence	Remark
Culex hutchinsoni	606	TATTTGTTTGATCAGTAGTAATTACTGCTGTTTTATTACTTCTTTCTTTA	50	UNIQUE
Culex whitmorei	681	TACTACAGTAATTAATATACGATCTTCAGGAATTACACTT	40	UNIQUE
Culex nigropunctatus	522	TTCTTCTGGGACTGCTCATGCAGGAGCTTCAGTTGATTTAGCTATTTTTT	50	UNIQUE
Culex pallidothorax	674	CTTTATTTGTATGATCTGTAATTATTACTGC	31	UNIQUE
Culex brevipalpis	674	TATTTGTATGATCTGTTATTATTACTGCTAT	31	UNIQUE
Culex malayi	691	TTCTATTTTAGGAGCA	16	UNIQUE
Culex pluvialis	498	CCTCTTTCTTCTGGAACAGCTCATGCTGGGGCTTCTGTTGATTTAGCAAT	50	UNIQUE
Culex minutissimus	685	TTAGCTGGAGCTATTACTATATTATTAACAGATCGAAATT	40	UNIQUE
Culex rubithoracis	519	TCATCTGGAACTGCTCACGCGGGGGCTTCAGTTGATTTAGCTATTTTTTC	50	UNIQUE
Culex fuscanus	495	CTGTTTACCCCCCTCTTTCATCTGGAACTGCTCATGCAGGTGCATCAGTT	50	UNIQUE
Culex gelidus	490	CTATTTTTTCTTTACATTTAGCTGGGATTTCATCAATTTTAGGAGCAGTA	50	UNIQUE
Culex infantulus	490	TGTTGATTTAGCTATTTTTTCTTTACATTTAGCAGGAATTTCTTCTATTT	50	UNIQUE
Culex vishnui	514	AACAGTTTATCCTCCTTTATCATCTGGAACAGCTCATGCTGGGGCCTCAG	50	UNIQUE
Culex tritaeniorhynchus	502	CTATCATCTGGAACAGCACATGCTGGAGCTTCAGTTGATTTAGCTATTTT	50	UNIQUE
Culex pipiens	521	AATGGGGCTGGGACTGGATGAACAGTGTATCCCCCTCTTT	50	UNIQUE
Culex pseudovishnui	499	GGAGCTTCAGTTGATTTAGCTATTTTTTCTTTACATTTAGCAGGTATTTC	50	UNIQUE
Culex minor	487	TCTCTCCATTTAGCTGGAATTTCTTCTATTTTAGGAGCTGTAAATTTTAT	50	UNIQUE
Culex fuscocephala	705	TTTAGCTGGGATTTCATCAATTTTAGGTGCTGTAAATTTTATTACAACAG	50	UNIQUE

DISCUSSION

Any unique DNA sequence which can be used in DNA hybridization, PCR or restriction mapping experiments to identify that sequence can be used as a DNA marker. A gene or DNA sequence having a known location on a chromosome and associated with a particular gene or trait refers to DNA marker.

Markers should exhibit high level of polymorphism. In other words, there should be variability in the markers. It should demonstrate measurable differences in expression between trait types and/or gene of interest. DNA markers are useful in the assessment of genetic diversity. Molecular markers detect the genetic diversity of organisms based on the mutation of nuclear acids and related amino acid translation in proteins.

These markers have become a powerful and comparatively efficient tool in genetic studies when morphological, cytological and biochemical markers do not allow insect discrimination at both inter- and intra-specific levels, allowing researchers to explore the track of speciation and evolution in many insect species. The mt-COX1 gene sequence is suitable for this role because its mutation rate is often fast enough to distinguish closely related species and also because its sequence is conserved among conspecifics. Contrary to the primary objection raised by skeptics that mt-COX1 sequence differences are too small to be detected between closely related species, more than 2% sequence divergence is typically detected between closely related animal species, suggesting that the barcode is effective for animals. In the present study, the identification of unique sequences within the barcode gene can be used to identify species as well as to locate cryptic species.

Conflict of interests: The author declares no conflict of interests.

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