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Isolation and characterization of partial mitochondrial *CO1* gene from harpacticoid copepod, *Leptocaris canariensis* (Lang, 1965)

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Copepods are gaining attention as superior viable live feed for larviculture and as ecological bioindicators. One of the possible candidates from Malaysia is *Leptocaris canariensis* (Copepoda: Harpacticoida). However, little is known about the molecular aspect of this species. In this study, DNA of individual *L. canariensis* was extracted and the partial mitochondrial *CO1* gene was successfully amplified using universal primers LCO-1490 and HCO-2198. A 582 bp partial mitochondrial *CO1* gene sequence was obtained. Analysis of partial CO1 sequences of *L. canariensis* revealed 100% similarity among all the individual copepods, verifying the purity of samples and the consistency of the optimized extraction and amplification protocols done in this study. BLAST analysis confirmed that the obtained sequences were from CO1 region and of copepod origin (with E-value < e^{-10}). Phylogenetic analysis of *L. canariensis* along with selected outgroups from different taxa level further supports the purity of *L. canariensis* maintained and validates the taxonomy of *L. canariensis* up to the subclass level: Copepoda. This study serves as the first documentation of molecular studies done on harpacticoids from the genus *Leptocaris*. The availability of *L. canariensis* partial CO1 sequence as reference will spearheads many more research in various fields in the near future.

Key words: Harpacticoida, *Leptocaris canariensis*, *CO1* gene, molecular identification.

INTRODUCTION

Copepods are the second largest meiofaunal group in marine sediment environment, after nematodes (Barnes, 1982) and they serve as food for juvenile fish in the marine meiobenthic food web and aquatic pollutant transporters across the food chains (Goetze, 2003; Raisuddin et al., 2007: Lundström et al., 2010; Lauritano et al., 2012). Being an order of copepods, harpacticoids can be found throughout the world in the marine environment and in fresh water (Boxshall and Defaye, 2008). Harpacticoid copepods are gaining attentions as

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Abbreviations: CO1, Cytochrome c oxidase subunit 1; **PCR,** polymerase chain reaction; **BLAST,** basic local alignment search tool.

possible substitutes for calanoid copepods due to their high stocking densities (Støttrup et al., 1986; Cutts, 2003; Støttrup, 2003) and the ability to convert shorter chain n-3 polyunsaturated fatty acids to the essential fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Nanton and Castell, 1998; Cutts, 2003). It was also noted that the nauplii of harpacticoid copepod can be more easily and completely digested compared to rotifers or Artemia (Pederson, 1984; Schipp, 2006).

High numbers of species *Leptocaris* (Harpacticoida) were recorded in mangrove area of west peninsular Malaysia. They were exclusively found on mangrove leave litters (Somerfield et al., 1998). However, there was no known literature on the molecular aspects of harpacticoids from the genus Leptocaris, including those found in Malaysia.

Compared to molecular identification method, morphological identification has some notable shortcomings. Absolute morphological identification of copepod can only be done once they reached late copepodite and adult stage because they showed only limited differences in their morphology during larval stage (Frost, 1971). Apart from that, morphological identification is time consuming, ambiguous and is more prone to misidentification of rare species (Bucklin et al., 2003; Jagadeesan et al., 2009). Molecular analysis has been proven to be important and useful in various fields of studies, especially in the understanding of deep phylogenetic relationships (Blair and Hedges, 2005; Regier et al., 2005), examining intra- and inter-specific relationships and population structure within and between species (Avise et al., 1987; Zhang and Hewitt, 2003; Dippenaar et al., 2010), identification of unknown or immature specimens based on established reference molecular data (Olson, 1991; Bartlett et al., 1992), and in delimiting cryptic species (Goetze, 2003; Hendrixson and Bond, 2005; Thum and Derry, 2008).

Cytochrome c oxidase subunit 1 (*CO1*) gene is considered as one of the widely used markers in the studies of population genetics and evolution (Shao et al., 2007) because it is among the most conservative protein-coding genes found in the mitochondrial genomes of animals (Brown, 1985). The phylogenetic relationships of 34 calanoid species belonged to 10 genera and 2 families were successfully determined by Bucklin et al. (2003) with the aid of mitochondrial *CO1* gene of size 693 bp. *CO1* gene has also been proven to be successful in species recognition (Hebert et al., 2003; Waugh, 2007) and specifically efficient in copepods (Bucklin et al., 1999; Hill et al., 2001; Øines and Heuch, 2005). Apart from that, the mitochondrial *CO1* gene variation within a species is far less than the variation that exists between species, making *CO1* gene a good diagnostic molecular marker (Bucklin et al., 1998, 1999).

The aims of this study were (i) to isolate the partial mitochondrial *CO1* gene of *Leptocaris canariensis* (Lang, 1965), and (ii) to characterize the partial CO1 gene of *L. canariensis*. Molecular characteristics of *L. canariensis* were generated through this study. This study serves as the first documentation and characterization of partial mitochondrial *CO1* gene from *L. canariensis* in Malaysia. The result generated from this study can aid in future identification, differentiation and phylogenetic studies of *L. canariensis*.

MATERIALS AND METHODS

Sample collection

Pure cultures of harpacticoid copepods *L. canariensis* were supplied by researchers of Institute of Tropical Aquaculture (AKUATROP), Universiti Malaysia Terengganu (UMT). The pure cultures maintained were initially collected from Merchang (5°1′N; 103°17′E) Terengganu, Malaysia.

DNA extraction, PCR amplification, and sequencing

Individual copepods were extracted and minced in 10 µl of sterile distilled water using fine needle viewed under dissecting microscope. The 10 µl mixture was transferred to 0.2 ml PCR tube. They were then incubated overnight at 4°C after addition of 2.5 µl 10X PCR buffer A (Vivantis Tech., MY) and 6.98 µl of sterile distilled water. The remaining PCR ingredients were added for PCR amplification on the following day. Universal primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) were used to amplify and sequence a 710 bp fragment of partial mitochondrial *CO1* gene from *L. canariensis*. Each 25 µl of PCR reaction contained 16.98 µl of sterile distilled water (with minced copepod), 2.5 µl of 10X Buffer A (Vivantis Tech., MY), 1 µl of $MgCl_2$ (50 mM), 1 µl of dNTPs (10 mM), 1.56 µl of each primers (40 µM) and 0.4 µl of *Taq* polymerase (Vivantis Tech., MY). PCR temperature profile used: initial denaturation at 94°C for 900 s; 40 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 45 s, extension at 72°C for 60 s; final extension at 72°C for 600 s (modified from Vestheim et al., 2005).

The DNA of 30 individual harpacticoid *L. canariensis* were extracted and amplified via this method. Obtained PCR products were visualized on an ethidium-bromide (EtBr) stained 1.5% agarose gel under Ultra-Violet light (UV), and purified using Vivantis GF-1 PCR clean-up kit (Vivantis Tech., MY) before sent for sequencing (First BASE Lab. Sdn. Bhd.).

Data processing and sequence analyses

The obtained sequencing results were viewed and edited using Chromas Ver. 2.33 (Technelysium Pty Ltd) to check for their quality, trimmed off early and end signal losses, ambiguous and unusable base pairs before further analyses. The CO1 gene sequences of 30 samples were aligned with National Center of Biotechnology Information (NCBI) BlastN database by using BLAST (Basic Local Alignment Search Tool) algorithm in the internet for comparison and identification (Altschul et al., 1997). The nucleotide sequences were validated by comparing the amino acid (translated protein) sequences with consensus protein sequences for the same mitochondrial *CO1* gene region.

Phylogenetic analysis using multiple alignment was carried out

Table 1. Pairwise nucleotide distances (Kimura 2-parameter) for partial CO1 gene sequences between *L. canariensis* and outgroups. Two *L. canariensis* CO1 gene sequences (w85 & w71) were chosen to represent all the 30 sequences as they produced identical results. Pairwise nucleotide distances were shown in lower left column while standard error estimates were shown on upper right column.

Parameter	1	2	3	4	5	6	7
Leptocaris canariensis (w85)	-	0.000	0.032	0.031	0.035	0.038	0.098
Leptocaris canariensis (w71)	0.000	-	0.032	0.031	0.035	0.038	0.098
Cletopsyllidae sp.	0.362	0.362	-	0.028	0.030	0.035	0.165
Cletocamptus deitersi	0.369	0.369	0.303	-	0.031	0.034	0.141
Coullana sp.	0.422	0.422	0.312	0.331	-	0.032	0.153
Tigriopus californicus	0.450	0.450	0.407	0.391	0.390	-	0.177
Calanus sinicus	1.099	1.099	1.363	1.282	1.327	1.386	-

too using phylogenetic analysis software ClustalX 2.0.12 (Larkin et al., 2007). Uncorrected, pairwise sequence divergences among the obtained mitochondrial *CO1* gene sequence and outgroups were calculated using molecular evolutionary genetics analysis (MEGA), Ver.4.0.2 (Tamura et al., 2007). Similarly, phylogenetic analyses using Neighbour-Joining (Saitou et al., 1987) search with Kimura 2-parameter as model was conducted using MEGA. The tree was bootstrapped using 1000 subreplicates. Outgroups *Cletocamptus deitersi* (AF315012.1), *Cletopsyllidae* sp. (AY327386.1), *Coullana* sp. (AF315015.1), *Tigriopus californicus* (DQ913891.2) and *Calanus sinicus* (EU603284.1) were selected from GenBank database.

RESULTS

Nucleotide identification

BLAST analysis was conducted on all sequencing data of *L. canariensis* by comparing the partial mitochondrial *CO1* gene sequences of *L. canariensis* with the online database of GenBank. All of the BLAST hits showed significant similarity (E-value < e^{-10}) (Chini et al., 2006) with the 30 individual *L. canariensis* partial *CO1* gene sequences. All of the hits retrieved from GenBank database were nucleotide sequences of partial mitochondrial *CO1* gene regardless of species, hence verifying the CO1 origin of *L. canariensis* samples.

The PCR products of 30 *L. canariensis* individuals showed 77% similarity with the partial mitochondrial CO1 gene region of TWO isolates of calanoid copepods, *Boeckella brasiliensis* (GenBank Accession Number: DQ356546.1, DQ356545.1). Five additional hits retrieved with a slightly higher (78-79%) maximum identity percentage values were all from the order Diptera. Nevertheless, the percen-tage of query coverage was highest (87-90%) in calanoid *B. brasiliensis* and showed a considerable difference compared to the other five retrieved hits (78-81%).

Sequence similarity

A perfect matching sequence of 582 bp partial mitochondrial CO1 gene sequence was recovered when multiple alignment analysis was conducted on all obtained partial mitochondrial CO1 gene sequences of *L. canariensis*. The mitochondrial CO1 sequence was submitted to GenBank, NCBI with Accession Number JF707331.

Consistent results were obtained in all of the obtained partial CO1 sequence of *L. canariensis* when compared with selected outgroups in pairwise nucleotide distances analysis (Table 1). Identical results of 0.000 (100% similarity) were seen in the comparisons between individual *L. canariensis* partial mitochondrial CO1 gene sequences (*L. canariensis* w85 & w71) (Table 1).

A range in between 0.30-0.45 were observed for the pairwise nucleotide distances (Kimura 2-parameter) comparison of *L. canariensis* CO1 gene sequences with the CO1 gene sequences of selected harpacticoid copepods outgroups (*Cletopsyllidae* sp., *C. deitersi, Coullana* sp. and *T. californicus*). On the other hand, a huge pairwise nucleotide distances of 1.10 - 1.39 were observed between copepods of the order Harpacticoida (including *L. canariensis*) and the order Calanoida (*C. sinicus*).

Phylogenetic analysis

The partial mitochondrial CO1 sequences of *L. canariensis* and selected outgroups were used to create a gene tree using Neighbour-Joining method (Figure 1). The tree branched out into two main clusters according to different orders as expected, with *C. sinicus* (Order: Calanoida) on one cluster while the rest of the copepods from the order Harpacticoida on another main clusters. All the harpacticoid copepod outgroups were resolved with a high bootstrap value of 99%, thus differentiating *L. canariensis* as well.

DISCUSSION

The confirmation of species identity using molecular

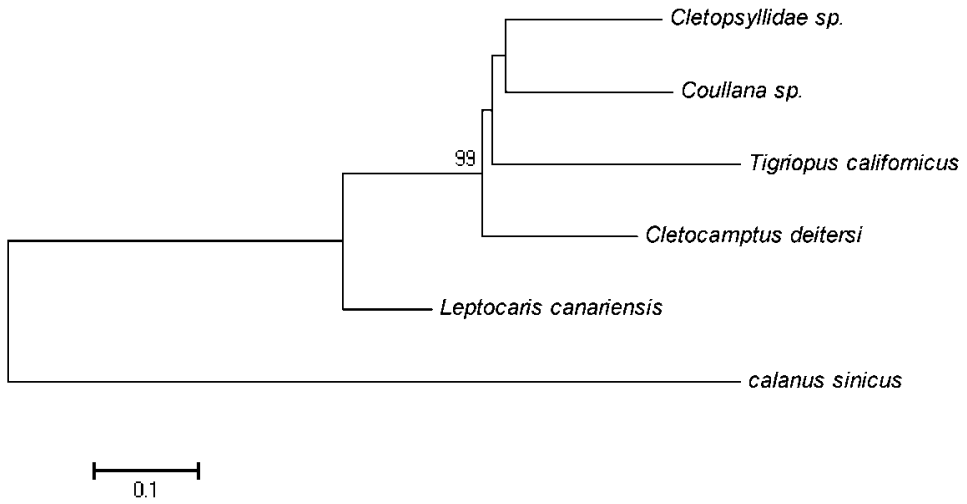

Figure 1. Phenogram of partial mitochondrial CO1 gene region of *L. canariensis* and selected outgroups. Unrooted mitochondrial CO1 gene tree reconstruction was by Neighbour-Joining (Saitou and Nei, 1987) using Kimura 2-parameter; tree was bootstrapped 1000x. Bootstrap value is given at the branch point.

method is crucial in ensuring that the copepod cultures maintained were of single species, especially in organisms as minute and ambiguous as copepods. The recovered 582 bp partial mitochondrial *CO1* gene sequence in this study was in conformation with Laakmaan et al. (2012), where partial *CO1* gene sequences of 525-658 bp were recovered using the same universal primers. The PCR products of *L. canariensis* amplified in this study were confirmed to be the gene sequence of partial CO1 region as all of the hits recovered from BLAST search were *CO1* gene sequences too as expected, with reliable E-value. This confirmation also reflects the ability and effectiveness of universal primers LCO-1490 and HCO-2198 described by Folmer et al. (1994) to target partial CO1 region in most invertebrates, including copepods. The high similarity (77%) observed between a species of calanoid copepod (*B. brasiliensis*) and the harpacticoid studied in this research (*L. canariensis*) further verifies that the partial CO1 gene sequences obtained in this study was of the subclass Copepoda. The appearance of organisms from other subphylum (Hexapoda) appeared in BLAST search was expected as universal primers LCO-1490 and HCO-2198 were used. The closest match was 79% similarity to a *Diptera* sp. CO1 sequence (GenBank Accession Number HM420105.1). Bucklin et al. (1999) also experienced similar situations whereby 88% similarity (closest match) to a *Murex troscheli* (sea snail) CO1 sequence was found when they compare the obtained *CO1* gene sequence of *Calanus finmarchicus* with *CO1* gene sequences in GenBank database.

Absolute (100%) similarities (pairwise nucleotide value = 0.000) were observed among all the 30 individual *L. canariensis* partial *CO1* gene sequences while huge differences of pairwise nucleotide distances values were observed when compared to other outgroups (Table 1). The absence of pairwise nucleotide distance found in all 30 *L. canariensis* samples indicated that all the samples were of the same species. This is expected as Bucklin et al. (1999) reported that the *CO1* gene region differed between species by approximately 30% of nucleotides, but exhibited little or no variation within species. Eberl et al. (2007) also showed that based on the uncorrected divergence (*p*-distances), CO1 sequences of harpacticoid *Macrosetella gracilis* sampled at Atlantic and Pacific have a genetic distance of less than 2% (Kimura 2-parameter distances). The degree of relatedness between *L. canariensis* and the other outgroups is reflected in this study and indirectly supports the taxonomy of *L. canariensis*. *L. canariensis* is expected to be more similar (smaller genetic distances) to other harpacticoid copepods compared to calanoid copepod, as shown in this study (Table 1).

Multiple alignment analysis of all the obtained partial CO1 sequences of *L. canariensis* on the other hand enabled the recovery of a matching sequence with the length of 582 bp partial *CO1* gene sequence. This recovery was expected as Folmer et al. (1994) reported in their study that universal primers LCO-1490 and HCO-2198 were able to amplify partial mitochondrial *CO1* gene sequence of 710 bp and readable sequences of approximately 651 bp were obtainable in more than 80 species ranging from 11 different phyla. This perfectly matched 582 bp CO1 sequence implied that all the *L.*

canariensis samples tested were of pure culture and no divergence or contamination of other morphologically similar species exists in the cultured stock population. This obtained *CO1* gene sequence can serve as a reference partial *CO1* gene sequences of *L. canariensis* for any future studies such as identification, population studies, intraspecific and interspecific discrimination of *L. canariensis*.

Phylogenetic analysis on the partial mitochondrial *CO1* gene sequences of *L. canariensis* and selected outgroups using Neighbour-Joining method confirmed the taxonomic hierarchy of *L. canariensis* up to order level (Copepoda) (Figure 1). Based on this phenogram, it is deducible that all the samples were equally identical in terms of genetic distance and as harpacticoids, *L. canariensis* is more closely related to other harpacticoid copepods compared to the calanoid copepod (*C. sinicus*). This confirmation is important because studies done on harpacticoid *Tigriopus californicus* (Burton and Lee, 1994; Ganz and Burton, 1995) found that mitochondrial DNA (especially *CO1* gene) revealed extreme genetic divergence even over short geographical distances. Studies have also shown that some marine invertebrates (including copepods) will undergo cryptic speciation, diverging at molecular level but remains morphologically similar (Knowlton, 1993; Lee, 2000; Goetze, 2003).

The present study showed the ability of partial *CO1* gene in differentiating between species (Figure 1). However, the 0.000 pairwise divergence between same species (*L. canariensis*) indicates that this biomarker is not suitable to be used in resolving relationships within similar species. The limited usefulness of mitochondrial *CO1* gene region, with a resolving power for some groups at genus level and above, may be due to the rapid evolving rate within this region (Miyata et al., 1982; Machida et al., 2006). The successful confirmation of *L. canariensis* up to the order level (Harpacticoida) was consistent with the conclusion made by Costa et al. (2007), stating that the mitochondrial CO1 region has a 95% success rate in classifying species to the correct order.

Conclusion

The accuracy of using molecular methods to identify and validate harpacticoid copepods have been shown in this study. The purity of the *L. canariensis* population is confirmed with 100% similarity among the partial mitochondrial *CO1* gene sequences obtained. The ability of using mitochondrial CO1 gene as DNA barcoding marker was proven when amplification of *CO1* gene was feasible in all of the samples tested.

However, it is suggested to include another gene from nuclear genome to make the data more robust. Apart from that, partial mitochondrial *CO1* gene may serve as a good biomarker for distinguishing between species, but were poor in resolving differences within species, especially in *L. canariensis*. The presented information here will spear-head more future research on creating a copepod DNA bank and ease their monitoring as possible ecological bioindicators and as viable live feed candidates.

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