MedCrave
Step into the World of Research

Journal of Aquaculture & Marine Biology

DNA Barcoding of *Panulirus homarus* from Oman and Yemen

Abstract

Panulirus homarus is one of the economically important lobster species consumed all over the World. Oman is one of the largest producers and exporter of this species. However, due to uncontrolled fishing concerns have been raised about decline population of P. homarus. Despite such ecological threat, information about taxonomic status of this species is lacking. Keeping this in mind in this study we identified *P. Homarus* specimen (n= 25) collected from five different locations using DNA barcoding techniques. Utility of this technique for the identification of *P. Homarus* is shown for the first time from Oman. Our study showed that specimens collected in this study indeed belonged to P. homarus. However, five samples showed high intraspecies (12%) nucleotide divergence and NJ clustering analysis showed that these samplesbranched out to form distinct clade, when compared to the sequenceof other P. homarus. Therefore, these samples indeed represent a cryptic species within *P. Homarus* that needs to be studied further.

Keywords: *Panulirus homarus*; Arabian sea; Yemen; DNA barcoding; Clustering analysis

Research Article

Volume 2 Issue 5 - 2015

Al-Marzouqi A*, Al-Amry w, Al-Hadabi A and Al-Senaidi R

Department of Marine Science and Fisheries, Sultanate of Oman

***Corresponding author:** Al-Marzouqi A, Department of Marine Science and Fisheries, MSFC, Ministry of Agriculture and Fisheries Wealth, Muscat, Sultanate of Oman, Email: aa.almarzouqi@ymail.com

Received: August 20, 2015 | **Published:** September 21, 2015

Abbreviations: NJ: Neighbour Joining; COI: Cytochrome Oxidase I; PCR: Polymerase Chain Reaction; K2P: Kimura 2 Parameter

Introduction

Lobsters are one of the most famous and delicious sea food consumed all over the world. Within lobsters family *Palinuridae, Panulirus homarus* is the most popular lobsters distributed along Indo Pacific region [1]. They are being extensively collected from all over the world. Oman is one of the largest suppliers of the lobsters with annual catch of approximately 2,000 tonnes per year [2]. However due to uncontrolled fishing concerns have been raised by many in the past [3,4]. In fact, in Oman gross revenue from lobster fishery decreased dramatically from 6 million OR to less than 1 million OR in 2011 [2].

Based on the geographic variation and pattern of sculpturing on the abdomen and colouration, four subspecies of P.homarus have been described Lavery et al. [1] namely, the nominotypical green coloured *P.homarus, Panulirush. rubellus, Panulirus h. megasculpta* and P.homarus "Brown" . Among them *Panulirus h. homarus* showed wide distribution and rest of them have restricted distribution. Very recently, based on mtDNA and nDNA genes Lavery et al. [1] validate the taxonomic status of *P.h. rubellus* and *P.h*. "Brown" as genetically distinct species and *P. h. homarus* and *P. h. megasculpta* as indistinguishable. However, despite its economical importance, no attempts have been made to establish taxonomic status of P.homarus found in Oman. In the past Ptacek et al. [5] tried to resolve phylogenetic status of *Panulirus* using 16S and COI gene sequences. In their study they have used only one specimen of *P. Homarus* (AF339457) collected from Oman and suggested further study to confirm the taxonomic status. Till now no attempts have been made to address taxonomic problems of *P. Homarus* from Oman. This necessitates urgent need to clarify the information about the taxonomic status of *Panulirus homarus* found in Oman which has not been compiled so far.

The lack of information in the taxonomy of *P. Homarus* is mainly due to morphological variation and characters used in taxonomy and hence in the past many subspecies have been described. Recently developed DNA barcoding technique has been used extensively to resolve such taxonomic ambiguities when species are indistinguishable due to morphological variations [6]. This technique has shown its utility in many studies ranging from insects to fishes. Mitochondrial gene *Cytochrome Oxidase I* (COI) is validly used marker in most of the studies on DNA barcoding. The COI gene sequence is known to show high interspecies nucleotide divergence than intraspecies nucleotide divergence. Generally, COI gene sequences of unknown sample are generated and then it is compared with the BOLD or NCBI databases for similarity search. If query sequence shows 97% or more similarity with the database sequence, then it is said to be identified and if not, it is considered as addition to the database. But if query sequence showed less than 97% similarity under same species in the database then it could represent new species that needs to be examined again for morphological character to confirm its status or a case of misidentification. Both NCBI and BOLD database hosts many sequences for genus *Panulirus*. Thus in this study we aim to check utility of DNA barcoding for species identification of lobsters found along the coast of Oman and to establish the taxonomic and phylogenetic status of *Panulirus homarus* found in Oman using DNA barcoding.

Materials and Methods

Sample collection and morphological data

Whole lobster samples were collected from four different locations in Oman and one location in Yemen. The locations in Oman were Ashkara, Dhalkoot, Duqum, Mirbat. Five representative samples were taken from each site. The samples were caught from

local fishermen/market (Ministry of Agriculture and Fisheries Wealth to confirm and mention it). The samples were transported to the laboratory in Muscat under cool box with ice and stored at -20 °C till further examination. Samples were taken out, photographs were taken and size of each sample was recorded (Table 1). Once the morphological examination was done, each lobster was cut from the leg part. A tissue of size 1cmx1 cm was cut and stored in a tube containing whatman filter paper and silica gel. The sample was dried under room temperature and stored at -20 °C till further analysis was done.

Table 1: Collection details of the samples studied here.

No.	Sample Code	Locality	Collection Date	Length	Weight	Sex
1	A1	Ashkhara	Oct 2013	63.45	-	F
2	A2	Ashkhara	Oct 2013	74.4	-	M
3	A3	Ashkhara	Oct 2013	68.84	-	M
4	A4	Ashkhara	Oct 2013	81.15	-	M
5	A5	Ashkhara	Oct 2013	57.84	-	M
6	D1	Dhalkoot	Oct 2013	82.28	0.609	F
7	D2	Dhalkoot	Oct 2013	70.04	0.303	M
8	D3	Dhalkoot	Oct 2013	74.19	0.290	M
9	D4	Dhalkoot	Oct 2013	68.12	0.272	F
10	D5	Dhalkoot	Oct 2013	63.28	0.244	M
11	X1	Duqum	June 2013	63.78	0.233	M
12	X2	Duqum	June 2013	70.94	0.203	M
13	X3	Duqum	June 2013	58.56	0.184	M
14	X4	Duqum	June 2013	64.39	0.246	M
15	X5	Duqum	June 2013	70.07	0.269	M
16	M1	Mirabat	Oct 2013	82.04	0.545	M
17	M2	Mirabat	Oct 2013	78.52	0.479	F
18	M3	Mirabat	Oct 2013	78.36	0.461	F
19	M4	Mirabat	Oct 2013	77.42	0.476	F
20	M5	Mirabat	Oct 2013	72.14	0.388	F
21	Y1	Yemen	Nov 2013	66.85	0.248	F
22	Y2	Yemen	Nov 2013	50.16	0.119	M
23	Y3	Yemen	Nov 2013	58.88	0.245	F
24	Y4	Yemen	Nov 2013	55.68	0.163	M
25	Y5	Yemen	Nov 2013	55.12	0.198	F

DNA isolation

Twenty five tissue samples from the samples collected were taken out from the storage and thawed at room temperature. The samples were washed with PBS buffer, dried and approximately 25-50 mg of tissue material was homogenized in 200 µl of the buffer. To the homogenized sample, lysis buffer 500 µl was added and preceded for DNA extraction. Further steps were carried out using the phenol: chloroform organic extraction method. The DNA was checked using 0.8% agarose gel running at 60 V for 45 min.

PCR amplification and DNA sequencing

Extracted DNA was used for PCR amplification of COI gene using primers: LCO 1490F -GGT CAACAA ATC ATA AAG ATA TTG G and HCO 2198R-AA ACT TCA GGG TGA CCA AAA AAT CA as detailed by DNA barcoding protocol led down by Canadian Centre for DNA barcoding [7]. The PCR reactions were carried out using CCDB protocol. For each reaction of total volume 10.5 µl 10% trehalose was added (6.25µl), 10X PCR buffer (1.25µl), 50 mM $MgCl_2$ (0.625 µl), 10 µM of each primer (0.125 µl each), 10 mMdNTPs (0.0625µl), 5U/µl platinum Taq polymerase (0.06µl) and 2 µl of ddH_2O. To this mixture 2µl of extracted DNA was added. The thermal cycling was carried out according to the standard protocol laid by CBOL, which consisted of an Initial denaturation at 95 °C for 10 minutes followed by first 5 cycles of denaturation, annealing and chain extension at 94 °C (30 secs), 45-50 °C (30 secs), 72 °C (60 secs) respectively. The second stage consisted of 30 cycles of denaturation, annealing and chain extension at 94 °C (30 secs), 51-54°C (30 secs), 72 °C (60 secs) respectively The final chain extension step was for 10 minutes at 72 °C and a final hold at 4 °C. The PCR products were checked on 2% Agarose gel followed purification using Exo-SAP. DNA sequencing was carried out with forward as well as reverse primers of the universal

primer. Sequencing was performed according to standard protocol provided for Big Dye Terminator kit® V 3.1 (Applied Bio systems) using ABI automated DNA sequence (ABI 310 Genetic Analyzer). 3 µl of cleaned purified PCR product was used for each 10 µl reaction.

Table 2: BLAST identification of samples studied here.

No.	Samples	BLAST Identification	Accession No.	Similarty (%)
1	Ashkara_4	*Panulirus homarushomarus*	JQ229926	99
2	Ashkara_5	*Panulirus homarushomarus*	AF339457	96
		Panulirusornatushomarus	AF339467	88
		Panulirus homarushomarus	JQ229924	87
3	Ashkara_3	*Panulirus homarushomarus*	JQ229926	99
4	Dhalkoot_4	*Panulirus homarushomarus*	AF339457	97
		Panulirusornatushomarus	AF339467	89
		Panulirus homarushomarus	JQ229924	88
5	Dhalkoot_3	*Panulirus homarushomarus*	JQ229926	99
6	Dhalkoot_2	*Panulirus homarushomarus*	JQ229926	99
7	Dhalkoot_5	*Panulirus homarushomarus*	JQ229918	99
8	Duqum_3	*Panulirus homarushomarus*	JQ229920	99
9	Duqum_5	*Panulirus homarushomarus*	AF339457	97
		Panulirus homarushomarus	JQ229924	88
10	Duqum_4	*Panulirus homarushomarus*	JQ229926	99
11	Duqum_1	*Panulirus homarushomarus*	JQ229926	100
12	Mirbat_3	*Panulirus homarushomarus*	JQ229926	99
13	Mirbat_5	*Panulirus homarushomarus*	JQ229918	99
14	Mirbat_2	*Panulirus homarushomarus*	JQ229925	99
15	Mirbat_4	*Panulirus homarushomarus*	JQ229918	99
16	Mirabat_1	*Panulirus homarushomarus*	AF339457	96
		Panulirus homarushomarus	KF715552	88
		Panulirus homarushomarus	KC959889	88
17	Yemen_5	*Panulirus homarushomarus*	JQ229918	99
18	Yemen_2	*Panulirus homarushomarus*	AF339457	97
		Panulirus homarushomarus	JQ229884	88
19	Yemen_1	*Panulirus homarushomarus*	AF339457	97
		Panulirusornatushomarus	AF339467	88
20	Yemen_4	*Panulirus homarushomarus*	JQ229918	99
21	Yemen_3	*Panulirus homarushomarus*	JQ229918	99

Bioinformatics Analysis

Obtained sequences were edited to remove ambiguous base calls. The forward and the reverse sequences were assembled using Chromas Pro (http://www.technelysium.com.au/chromas_lite.html). FASTA format of these sequences were used for species identification using BLAST search at NCBI (http://blast.ncbi.nlm.nih.gov/) and species identification tool at BOLD (http://www.barcodinglife.com). The samples is said to be indentified if it showed sequence match of 97% or more than that with other *P. Homarus* sequences submitted in the databases.And if not then it is considered to show high intraspecies nucleotide divergence. We have also downloaded conspecific sequences of *P. Homarus* from the Gene Bank for clustering analysis. All the obtained sequences were aligned using Bio Edit Hall [8]. The phylogenetic tree was constructed using MEGA6 software [9]. The phylogenetic analysis was done using NJ method in MEGA6. The Kimura 2 parameter (K2P) model of base substitution was used to calculate pair wise genetic distances in MEGA 6 software using *Panulirius ornatusas* an out group. The distance matrix was also constructed to calculate intraspecies and interspecies nucleotide divergences.

Results

In this study, we have sequenced 25 samples of *P. Homarus* collected from five different locations along the coast of Oman

using COI. Expected length of sequences (650bp) was obtained for all samples. Species identification of studied *P. Homarus* based on both BOLD and NCBI database is shown in Table 1. Out of the 25 samples 19 samples could be identified till species level as they showed sequence match of more than or equal to 97% with *P. Homarus* sequences submitted elsewhere from the World. Sequences of the rest of the samples (Yemen (1,2), Duqum 5, Dhalkoot 4, Ashkara 5 and Mirabat 1) showed sequence match of less that 97% suggesting high intraspecies.However, these sequences showed 96% similarity with *P. Homarus homarus* (AF339457) submitted by Ptacek et al. [5]. Otherwise these five sequences also showed sequence match of 88% with the sequence of *P. Homarus* (JQ229924) submitted from India. Intraspecies nucleotide divergence for samples studied here was in the range of 0% to 14%.

To further confirm the taxonomic status of the samples studied here, 120 COI sequences of *P. Homarus* were downloaded from the NCBI database. Clustering analysis was performed using these sequences along with the sequences generated in this study using Neighbor Joining (NJ) method in MEGA 6 software [9]. Clustering analysis showed five distinct clades. Two separate clades belonged to *P. homarushomarus*, third cluster belonged to recently confirmed subspecies, P. h. *rubellus* and fourth, separate clade of *P. Homarus* "Brown" was also seen. The clustering analysis clearly showed fifth distinct cluster diverged from the remaining *P. Homarus* sequences (Yemen (1,2), Duqum 5, Dhalkoot 4, Ashkara 5 and Mirabat 1) representing putative new species. Sequences of all other samples were distributed in the clade formed by sequences of *P. Homarus* downloaded from the databases (Figure 1).

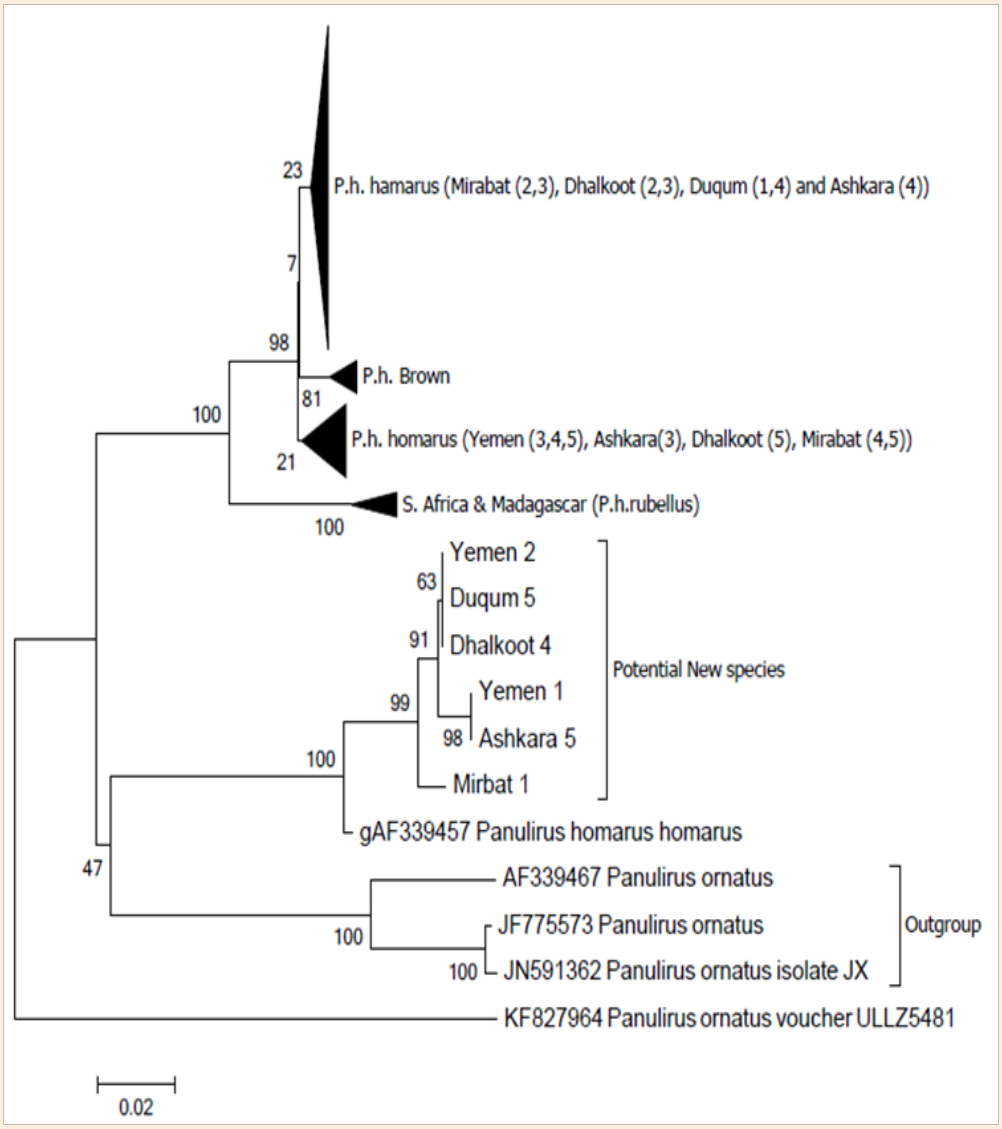

Figure 1: Clustering analysis of samples studied here using MEGA 6 software with *P. ornatus* as an out-group.

Discussion

Taxonomy of *P. Homarus* has always been confusing due to morphologic variation found in this species complex. Despite being so important in commercial point of view, till now no study was done on DNA barcoding of *P. Homarus* from Oman. This is the first study reporting the utility of DNA barcoding for species identification of P. homarus. Our study showed that samples collected in this study indeed belonged to P. homarus. Moreover, our study also revealed the putative new species of *P. Homarus* from Oman that needs to be studied further.

Based on similarity search we could identify 19 out of 25 samples till species level as they showed sequence match of 97% to 100% with conspecific sequences available in the database (Table 2). No case was observed where our query sequences showed similarity match with the sequences under different species name. Thus confirming that samples studied here indeed belong to *P. Homarus* and also assuring the utility of similarity search for species identification.The intraspecies nucleotide divergence for these samples was in the range of 0% to 3% hence, based on threshold intraspecies nucleotide divergence, it is clear that these samples are indeed conspecific. However, five samples (namely, Yemen (1&2), Duqum 5, Dhalkoot 4, Ashkara 5 and Mirabat 1) showed sequence similarity match in the range of 88% to 96% thus showing high intraspecies nucleotide divergence. Interestingly these five samples matches with COI sequence of *P. Homarus* (AF339457) collected from Oman by Ptacek et al. [5].

NJ clustering analysis was performed by downloading 120 sequences of *P. Homarus* from the databases. Based on reciprocal monophyly, all samples studied here were found in the clade of *P. Homarus* thus suggesting samples studied here indeed belonged to P. homarus. NJ clustering analysis based on these samples showed all samples in one monophyleticclade of P. homarus, no case of paraphyly or polyphyly was observed. Clustering analysis further showed five distinct sub clades. The two distinct clades of P.h. hamarus (I & II), third clade belonged to P.hamarus "Brown" and fourth clade included *P.h. rubellus*. Interestingly, fifth clade was branched out to form distinct clade that includes five samples. Nevertheless, all the samples studied here (except samples in the fifth clade) studied here were distributed in the two clades of P.homarus (I & II). The intraspecies divergence between these samples was in the range of 1% to 3%. Thus similarity search and NJ clustering analysis done in this study showed that samples studied here (except clade V) indeed belonged to P. homarus.

Thus in this study utility of DNA barcoding for identification of *P. Homarus* was shown for the first time from Oman. Previous studies Lavery et al. [1] based on morphological characters have suggested the presence of subspecies *P. Homarus megasculpta* in Oman water. However, Lavery et al. [1] failed to sequence samples from Oman. In this study, we have collected samples from various localities of Oman and our survey identified that all the samples indeed belonged to *P. Homarus* and no high genetic divergence (except clade V) was seen P. homarus. Thus our study is in congruency with the observation made by Lavery et al. [1] that *P. Homarus megasculpta* could be just a morphological variation, however, details studies involving more samples are needed to further support this observation.

NJ clustering analysis showed clade V that includes samples namely, Yemen (1 & 2), Duqum 5, Dhalkoot 4, Ashkara 5 and Mirabat 1 (Figure 1). In this clade, one samples studied by Ptacek et al. [5] was also included. The intraspecies divergence between these samples is in the range of 1% to 3%, however when compared with other samples in this study and sequences downloaded from the databases the intraspecies divergence was 12%. We assume that this clade represent a putative new species. Based on the nucleotide divergences of 16S, COI and ITS genes and sympatric distribution, Lavery et al. [1] confirmed the subspecies status of *P.h. rubellus*. In their study, nucleotide divergence between P. h. *rubellus* and other neighbouring species was 8.5% to 9.3% for COI gene. Using this assumption, nucleotide divergence obtained in our study was much higher (12% to 16%) and thus fifth clade shown here indeed belonged to putative new species. Similarly, Tourinho et al. [10] based on COI gene observed interspecies divergence of 14.4% to 17.8% in another commercially important species *Panulirisargus.* Based on such high intraspecies divergencetheir study concluded that the Caribbean and Southwest Atlantic lobster population indeed belonged to different species. Intraspecies divergences obtained in this study are in the same range to that of nucleotide divergence of congeneric species of *Panulirus*. Further study by Ptacek et al. [5] based on molecular divergences, suggested species radiation in this genus. Given the high intraspecies divergences obtained in this study and sympatric distribution of the samplesstudied here, we believe that the samples in the clade V indeed belonged to different species that needs to study further.

Acknowledgement

The authors: Wahida Alamry, Abdulaziz Almarzooqi, Ahmed Alhadabi, wish to thank the data collectors Rashid alsinady during collecting samples from the fields and Sultana Aljardani how participated in genetic analysis training. This project been funded and completed by Ministry of Agriculture and Fisheries Wealth.

References

1. Lavery SD, Farhadi A, Farahmand H, Chan TY, Azhdehakoshpour A, et al. (2014) Ecolutionary divergence of geographic subspecies within scalloped spiny lobster *Panulirus homarus* (Linnaeus 1758). PloS One 9(6): e97247.
2. Mehanna S, Al-Shijibi S, Al-jafary J, Al- Senaidi R (2012) Population dynamics and management of scalloped spiny lobster *Panulirus homarus* in Oman coastal waters. Journal of Biology, Agriculture and Healthcare 2: 10.
3. Bonda-Reantaso MG, Subasinghe RP, Josupeit H, Cai J, Zhou X (2012) The role of crustacean fisheries and aquaculture in global food security: Past, present and future. J Invertebr Pathol 110(2): 158-165.
4. Mashaii N, Rajabipour F, Shakouri A (2011) Feeding Habits of the Scalloped Spiny Lobstrer, *Panulirus homarus* (Linnaeus, 1758) (Decapoda: Palinuridae) from the South East Coast of Iran. Turkish Journal of Fisheries and aquatic Sciences 11(1): 45-54.
5. Ptacek MB, Sarver SK, Childress MJ, Herrnkind WF (2001) Molecular phylogeny of the spiny lobster genus *Panulirus* (Decapoda: Palinuridae). Mar Freshw Res 52(8): 1037-1047.
6. Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identification through DNA barcodes. Proc Biol Sci 270(1512): 313-321.

7. Inova N, Grainger C CCDB protocols.

8. Hall TA (1999) BioEdit: a user friendly biological sequence alignment editior and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

9. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30(12): 2725-2729.

10. Tourinho JL, Sole-Cava AM, Lazoski C (2012) Cryptic species within the commercially most important lobster in the tropical Atlantic, the spiny lobster *Panulirusargus*. Mar Biol 159(9): 1897-1906.

Citation: Al-Marzouqi A, Al-Amry w, Al-Hadabi A, Al-Senaidi R (2015) DNA Barcoding of Panulirus homarus from Oman and Yemen. J Aquac Mar Biol 2(5): 00043. DOI: 10.15406/jamb.2015.02.00043