Identifying the geographical origin of protected sea cucumbers (*Apostichopus japonicus*) in China using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)

Zhenyu Yun*, Zhao Sun, Haiyan Xu¹, Zhihong Sun¹, Yao Zhang, and Zhen Liu

Sub-Institute of Food and Agriculture Standardization, China National Institute of Standardization, Beijing 100191, China

¹College of Food Science and Engineering, Inner Mongolia Agricultural University, Hohhot, Inner Mongolia 010018, China

Received October 7, 2016
Revised December 30, 2016
Accepted January 4, 2017
Published online April 30, 2017

*Corresponding Author
Tel/Fax: +86-10-58811645
E-mail: yunzy@cniis.gov.cn

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2017

Abstract Dalian sea cucumber, Yantai sea cucumber, and Weihai sea cucumber, which belong to *Apostichopus japonicus*, are protected as geographical indications in China based on their high nutritional values and medical properties. The 26 samples, including Dalian sea cucumbers (9 samples) in Liaoning province, Yantai sea cucumbers (9 samples), and Weihai sea cucumbers (8 samples) in Shandong province, were individually collected from the designated geographical sea areas and the genetic relationships and DNA polymorphisms were evaluated by random amplified polymorphic DNA technology and gene segments sequencing. The RAPD dendrogram showed that the genetic diversity of the three types of sea cucumbers was rich. The neighbor-joining tree shows that the genetic relationship of the samples from the adjacent sea areas is closer. It demonstrates that the gene characteristics of sea cucumbers from different sea areas were obvious and the genetic diversity analysis by RAPD-PCR can be used as a rapid method for geographical discrimination.

Keywords: sea cucumber, geographical origin, random amplified polymorphic DNA, genetic diversity

Introduction

Sea cucumbers, which belong to the phylum *Echinodermata*, class *Holothuroidea*, and family *Stichopodidae*, are marine invertebrates and have long been used for food and folk medicine in some communities of Asia. With regards to the nutritional value, sea cucumbers are rich in vitamin A, vitamin B₁ (thiamine), vitamin B₂ (riboflavin), vitamin B₃ (niacin), and minerals, especially, calcium, magnesium, iron and zinc; furthermore, they are pharmacologically efficient in antiangiogenic, anticancer, anticoagulant, anti-hypertension, antiangiogenic, antimicrobial, antioxidant, antithrombotic, and antitumor activities (1). Various species of sea cucumbers have been found in Asian and other benthic areas and deep seas. *Apostichopus japonicus* (*A. japonicus*) is an important sea cucumber species found in the coastal ecosystems of China, Japan, and Korea. Dalian sea cucumber, Yantai sea cucumber, and Weihai sea cucumber, which belong to *Apostichopus japonicus*, are protected as Chinese geographical indication products. The nutritional value and medical properties of the Dalian sea cucumber are found to be highest (2). Owing to its value and reputation, the mariculture of *A. japonicus* has been developed quickly to satisfy an increasing market demand. The problem related to misleading information on the origin of sea

cucumbers has become increasingly serious and an efficient analysis method needs to be developed for identifying the true origin of *A. japonicus*. However, different ecology environments, including geographic location and climate, lead to different qualities of the product and genetic diversities; thus, the same species in different environments would possess their own special genetic characteristics. The knowledge about species diversity and phylogenetic relationships will help to understand the species evolution (3); therefore, the study on the genetic diversity of *A. japonicus* from different geographical areas is important.

Molecular techniques are used to study sea cucumber genetics, including allozymes, amplified fragment length polymorphisms (AFLP), mitochondrial DNA, single nucleotide polymorphisms (SNPs), microsatellites, and inter-simple sequence repeats (ISSRs) (4–9). These are powerful tools for distinguishing between different types of sea cucumbers, although the use of these molecular markers is time-consuming and complicated.

The RAPD method is developed to detect polymorphisms in genetic DNA and is based on polymerase chain reaction (PCR), a simple and an inexpensive technique. RAPD is characterized by low repeatability and transferability between laboratories. The RAPD-PCR method is reproducible and efficacious. Since it uses short

synthetic oligonucleotides with random sequences, the amplification protocol differs from that of the standard PCR, in that only a single random oligonucleotide primer is used and no prior knowledge of the genome subjected to the analysis is required (10,11).

In this study, we sequenced 16S rDNA partial sequences and identified 26 sea cucumbers samples, examined the genetic diversity of *A. japonicus* through RAPD markers, and assessed the molecular divergence of *A. japonicus* from three areas in China: Dalian, Yantai, and Weihai; furthermore, the DNA bands of RAPD gels were sequenced and the obtained sequences were analyzed to study the genetic structure of *A. japonicus* from the three sea areas. The purpose of this study was to develop an efficient molecular-level analysis method for identifying the genotype of *A. japonicus* from different regions, hoping that it could be used as a rapid identity tool in different procedure of *A. japonicus* such as farming, processing, distribution, and marketing.

Materials and Methods

Sample collection and grouping Sea cucumbers were sampled from Dalian (DL; 9 individuals), Yantai (YT; 9 individuals), and Weihai (WH; 8 individuals) in China, desiccated, brought back to the laboratory, and stored at 4°C (Table 1). Reference sea cucumber data were obtained from GenBank (*A. japonicus*, AY852279.1 and *The lenotaananas*, FJ971387.1).

Total DNA extraction Dried sea cucumbers were soaked in normal saline for 4–5 h. DNA was purified from 30 mg of muscle tissue from the soaked sea cucumbers by following the E.Z.N.A. Mollusc DNA Kit protocol (Omega Bio-Tek) with some modifications. The tissue was placed in a mortar, poured into liquid nitrogen and rapidly ground with a pestle and the powder was placed in a clean 1.5 mL micro-centrifuge tube. ML1 buffer (350 µL) and 25 µL proteinase K being added, the mixture was not vortexed and incubated at 60°C until the entire sample was solubilized. Chloroform: isoamyl alcohol (24:1; 350 µL) was added to the lysate, which was then centrifuged at 10,000×g for 2 min at room temperature. The upper aqueous phase was transferred to a clean 1.5 mL microfuge tube. Next, 5 µL RNase A was added and incubated at room temperature for 10–30 min. The supernatant was collected and an equal volume of MBL buffer was added. Then, 0.5 equal volume of absolute ethanol was added and mixed well by vortexing. Further, 750 µL of the mixture was applied to a HiBind DNA column assembled in a 2 mL collection tube, with centrifugation at 10,000×g for 1 min. The flow-through liquid was discarded and the collection tube was re-used. The column was placed in a new 2 mL collection tube and washed by adding 500 µL HB buffer, which was centrifuged at 10,000×g for 30 s. The flow-through was discarded. The column was placed in the same 2 mL collection tube and was washed by adding 700 µL DNA wash buffer diluted with absolute ethanol and centrifuged at 10,000×g for 1 min

Table 1. Geographical origin of the sea cucumbers examined in this experiment

Samples	GenBank accession number of sequences	Regions	Location
DL1	KU183638	Dalian	Saili Island
DL3	KU183639	Dalian	Hong Island
DL4	KU183640	Dalian	Zhangzi Island
DL5	KU183641	Dalian	Dachangshan Island
DL6	KU183642	Dalian	Haiwangjiu Island
DL8	KU183643	Dalian	Hulu Island
DL9	KU183644	Dalian	Wangjia Island
DL10	KU183645	Dalian	Haiyang Island
DL12	KU183646	Dalian	Changxing Island
YT13	KU183647	Yantai	Xiaoqin Island
YT14	KU183648	Yantai	Chang Island
YT15	KU183649	Yantai	Danzi Island
YT16	KU183650	Yantai	Zhibu Island
YT17	KU183651	Yantai	Changdao Island
YT19	KU183652	Yantai	Yangma Island
YT20	KU183653	Yantai	Changdao Island
YT22	KU195415	Yantai	Bajiao Island
YT23	KU183654	Yantai	Zhibu Island
WH24	KU183655	Weihai	South sea area of Weihai
WH25	KU183656	Weihai	North sea area of Weihai
WH26	KU183657	Weihai	South sea area of Weihai
WH27	KU183658	Weihai	East sea area of Weihai
WH28	KU183659	Weihai	North sea area of Weihai
WH33	KU183660	Weihai	North sea area of Weihai
WH34	KU183661	Weihai	South sea area of Weihai
WH35	KU183662	Weihai	East sea area of Weihai

as described above. The flow-through liquid was discarded and the collection tube was re-used. The HB buffer and wash buffer steps were repeated. The liquid was discarded and the column was re-inserted into the empty collection tube and was centrifuged at 15,000×g for 2 min. Finally, to elute the DNA, 50–100 µL elution buffer preheated to 60–70°C was added directly to the HiBind matrix. The DNA samples were acquired in a clean tube and stored at –20°C until use.

16S rDNA sequences To identify the sea cucumber species, in this study, 16S rDNA partial sequences were amplified using echinoderm-specific primers [16Sar, 5'-CGCC-TGTTTATCAAAAACAT-3'; 16Sbr, 5'-CTCCGGT-TTGAAGTCAGATCA-3'] (12). The cycling conditions were as follows: initial denaturation for 30 s at 95°C; followed by 40 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min and then a final extension at 72°C for 4 min.

RAPD-PCR amplification and sequencing of RAPD bands Ten primers (Table 2) were designed by Inner Mongolia Agriculture University, China. Genomic DNA was used as a template for PCR amplification of RAPD using an automatic thermal cycler (PTC-200; MJ Research, Waltham, MA, USA). The thermal cycling conditions were as follows: 94°C for 2 min; 42 cycles at 94°C for 30 s, 37°C for

Table 2. Primers used for RAPD-PCR of sea cucumbers

Primer	Sequence (5'→3')	Temperature
C11	5'-AAAGTCGCGG-3'	35°C
C12	5'-TGTCATCCCC-3'	37°C
C15	5'-GACGGATCAG-3'	33°C
C16	5'-CACACTCCAG-3'	37°C
C20	5'-ACTTCGCCAC-3'	35°C
D12	5'-CACCGTATCC-3'	33°C
D13	5'-GGGGTGACGA-3'	35°C
D15	5'-CATCCGTGCT-3'	35°C
D16	5'-AGGGCGTAAG-3'	35°C
D20	5'-ACCCGGTCAC-3'	35°C

30 s, and 72°C for 80 s; and a final elongation step at 72°C for 10 min. Each PCR system was performed for a final volume of 50 µL containing 150 ng genomic DNA, 8 mM of each dNTP, 10 pmol of each primer, and 2.5 U Taq polymerase in 1×PCR buffer (with Mg²⁺). PCR products were electrophoresed in 1.5% agarose gels. The 16S rDNA PCR products were sequenced at Shanghai Majorbio Bio-Pharm Technology. The same primers for 16S rDNA were used for PCR and DNA sequencing.

Blocks of agarose gels containing selected RAPD bands were excised and dissolved according to the method reported by Lacumin *et al.* (13). Dissolved products were clone sequenced by Shanghai Majorbio Bio-pharm Technology Corporation (Shanghai, China).

Data analysis A consensus 16S-like DNA sequence of sea cucumbers was obtained using DNASTAR software. The 16S-like DNA sequences were analyzed by the BLAST algorithm (14) (<http://www.ncbi.nlm.nih.gov/BLAST/>) and were submitted to NCBI (<http://www.blast.ncbi.nlm.nih.gov>). Phylogenetic analysis of the 16S rRNA gene sequences was conducted using the neighbor-joining method with MEGA 5.0 software (15).

The electrophoretic profiles of RAPD products were analyzed using the NTsys2.10t software to examine the genetic relationships among the individuals from the three regions. Genetic similarity coefficients were calculated and a dendrogram was constructed using the unweighted pair group method by means of arithmetic (UPGMA).

The sequences of agarose gels bands were trimmed and aligned using MEGA 5.0 software package (version 5.0, www.megasoftware.net). The neighbor-joining tree was formed using the MEGA 5.0 software on the basis of the sequences of agarose gel bands from RAPD.

Nucleotide sequence accession numbers The sequences from 16S-like DNA sequences were deposited in the GenBank database under accession numbers from KU183638 to KU183662 and KU195415, and the sequences accession numbers of five common genes were from KX697731 to KX697860.

Fig. 1. Phylogenetic relationship tree based on 16S rDNA gene sequence analyses of sea cucumbers from three regions.

Results and Discussion

16S rDNA partial sequence identification To identify sea cucumber species, a 16S rDNA partial sequence was amplified and analyzed. A 540 bp fragment was amplified using the primers 16Sar and 16Sbr in each sea cucumber. Consensus sequences were obtained using DNASTAR. The sequences were then compared with those of the related species in the GenBank and the sequence similarities were checked using BLAST. The similarity of the 16S rDNA sequences of the 26 sea cucumbers and *A. japonicus* was 99%. A phylogenetic analysis was performed to investigate the relations between the 16S rDNA partial sequences among the 26 sea cucumbers, *A. japonicus*, and *T. ananas* (Fig. 1). The samples and related reference sea cucumbers were divided into two large clusters (Fig. 1). In the neighbor-joining tree, group 1 comprised 26 samples and *A. japonicus*, whereas group 2 included only *T. ananas*. Therefore, all samples were identified as *A. japonicus*.

A. japonicus is a type of valuable seafood in Asia, particularly in China. Moreover, because of the differences in the gene, the quality and nutritive value of *A. japonicus* from different sea areas differ. *A. japonicus* from Dalian in China is better than that from other regions. As early as 2007, Yao *et al.* (8) had researched the genetic variation and relations of *A. japonicus* between specimens from Qingdao and Penglai in China using an ISSR method. Liao *et al.* (16) developed 20 microsatellite DNA marker sands and used them to investigate polymorphisms of 60 *A. japonicus* individuals collected from China. This revealed that the markers would be useful for future breeding and assessing genetic variation within sea cucumbers. In present

Fig. 2. Dendrogram based on the UPGMA cluster analysis of 26 sea cucumbers RAPD fingerprint patterns (Marker is 2,000 bp).

study, the genetic differences of samples from Dalian, Yantai, and Weihai were investigated based on RAPD-PCR.

In this study, we used the 16S rDNA sequence to identify 26 sea cucumbers as *A. japonicus*. Kerry et al. (12) demonstrated the reliability of this method for sea cucumber identification. Obviously, the 26 samples were classified into two groups in the resulting phylogenetic tree (Fig. 1), which indicated more than 99% similarity in 16S rDNA sequence when compared with the reference *A. japonicus*.

RAPD-PCR analysis The RAPD-PCR technique is based on the amplification of a random DNA segment using a single primer with an arbitrary nucleotide sequence (11,17). RAPD polymorphisms result from the DNA sequence variation at primer binding sites and from the DNA length differences between the primer binding sites (18). RAPD has been utilized recently for genetic similarity estimation. Similar to other molecular techniques, RAPD has been successful at identifying markers that distinguish many species, such as tilapia (19) and *Wuchereriabancrofti* (20), although the inter-population variation detected can differ significantly. The method of RAPD has been successfully applied to the differentiation of *Lactococcus lactis* subsp. *lactis* and *cremoris* (21). Weihai is located in the Yellow Sea, Yantai is located at the boundary of the Bohai Sea and the Yellow Sea of China, and the growth conditions of Weihai sea cucumber and Yantai sea cucumber are considered to be similar. So, the two types of the sea cucumbers may have similar genetic characteristics. The high selectivity of RAPD-PCR enables the technique to discriminate between species. In this study, ten RAPD primers were screened. Since amplification with primer D20 gave clear and reproducible bands, it was chosen for RAPD assays. RAPD-PCR of 26 *A. japonicus* with primer D20 gave products ranging in size from 200 to 2,000 bp. The results were shown in a dendrogram generated with NTSys2.10t (Fig. 2). The primer generated 4–12 products per sample, which

revealed good diversity among the 26 samples.

There were two clusters in the RAPD-PCR dendrogram: cluster 1 contained 11 samples and cluster 2 contained 15. Cluster 1 consisted of one subcluster (formed from DL1, DL5, DL12, DL9, DL4, DL6, and DL10) and four single branches (YT15, DL3, YT13, and DL8). All samples from Dalian were in cluster 1. The subcluster comprised seven samples from Dalian, which indicated the ability of RAPD-PCR to differentiate between regions; each branch comprised one sample; among the four branches, two samples were collected from Yantai and the other two from Dalian. Cluster 2 consisted of three subclusters and one branch. Subcluster 1 consisted of two groups; one consisted of three Yantai samples (YT16, YT17, and YT23) and the others were obtained from Yantai and Weihai. In the latter group, YT14, YT19, and WH25 were closely related to WH28, whereas YT22 and WH26 were closer to WH33. Subcluster 2 consisted of two samples from Weihai (WH24 and WH35). WH24 was on a branch by itself that was close to subclusters 1 and 2. Subcluster 3 comprised YT20 and WH27. Our results showed that RAPD-PCR was a powerful and useful technique for origin discrimination of *A. japonicus*. Cluster 1 comprised samples obtained from Dalian and two samples from Yantai, although one subcluster consisted solely of samples from Dalian; probably because the merchant who sold the samples made a mistake in identifying their origin. The remaining *A. japonicus* samples obtained from Yantai and Weihai were classified into the other cluster; it might be caused by the fact that Yantai and Weihai are relatively much closer in terms of their geographical location. This cluster could be subdivided into three much smaller clusters, which indicated that it was a complicated genetic structure. Specifically, subcluster 1 consisted of three samples (YT16, YT17, and YT23) from Yantai, and subcluster 2 comprised two samples from Weihai. These results indicated that the genotypes of the tested samples are related to their original regions. Until now, RAPD-PCR method was not adopted as an extensive approach for sea

Fig. 3. Neighbor-joining tree of 26 samples based on five bands' sequences.

cucumber classification. In this study, an analytical method was developed on the basis of RAPD-PCR, and all the data indicated that it was a rapid and reliable method for region characterization of *A. japonicus*. In addition, the RAPD dendrogram showed that the genetic diversity of *A. japonicus* from the three areas was rich.

Furthermore, the neighbor-joining tree was constructed by sequences of 5 RAPD bands from all samples. 132 bands were excised from the RAPD agarose gel. We selected five bands' sequences as five common genes, which were included in all samples. The length of the sequences was 198–364 bp. The neighbor-joining tree based on the 5 genes sequences demonstrated the genetic relationship among the *A. japonicus* from different sea areas in the study in Fig. 3. In this tree, the 26 samples were separated into 2 clusters and were designated A and B. Most samples of cluster A belonged to Yantai and Weihai, except DL4 and DL6; whereas, the samples of cluster B were merely sampled from Dalian, with the exception of YT17 and WH28. Cluster A was further divided into 2 subclusters. Subcluster 1 consisted of 6 samples from Weihai, and 1 sample from Yantai and Dalian, respectively. Cluster B2 predominantly comprised 6 samples from Yantai as well as 1 sample from Weihai and 1 sample from Dalian. Specifically, YT19 belongs to a single

sample, which is far away from the other samples in Cluster A in terms of distance, whereas YT17 is closer to the samples from Dalian than to those from Yantai in Cluster B. According to the neighbor-joining tree, we found the genetic relationship among the samples from the three sea areas. Except individual samples, most samples from the identical areas were clustered in the same branch. Therefore, we found that the genetic relationship of *A. japonicus* from the adjacent area is closer; meanwhile, the samples from Yantai and Weihai are closer than those from Dalian, which is in line with the results of RAPD. However, few samples were clustered in accordance with the geographical location as it was likely that the samples were bought from different shops and were mixed. The evolution of the gene content and its chromosomal organization is stimulated by the selective conditions in different environments. The plasticity in the genetic repertoire is essential for adaptation to specific environmental habitat; therefore, the evolution reflects niche-specific adaptation (22) so that the genes of *A. japonicus* from different sea areas have been included into their identification marks.

16S rDNA sequence analysis is proved as an efficient and accurate method for identifying different species of sea cucumbers. Using RAPD-PCR, we find that the samples from Weihai and Yantai are more closely related than those from Dalian, probably because of the geographic proximity of Weihai and Yantai. Here, RAPD-PCR approach is a promising tool for analyzing genetic characterization and also possesses a desired capacity for identifying the geographical origin of *A. japonicus*. All the experimental results suggest that such genetic diversity analysis using RAPD-PCR will be a useful strategy in identifying the true geographical origin of *A. japonicus* in China.

Acknowledgments This study was funded by the Scientific and Technological Program of AQSIQ (2015QK233), the Fundamental Research Funds of CNIS (562014Y-3352), the National Science and Technology Support Program (2015BAD17B04) and Special Fund for Quality Scientific Research (201210010 and 2012104019).

Disclosure The authors declare no conflict of interest.

References

- Bordbar S, Anwar F, Saari N. High-value components and bioactives from sea cucumbers for functional foods-A review. *Mar. Drugs* 9: 1761–1805 (2011)
- Liao CY. Metamorphosis and living habit of the larvae of the sea cucumber *Stichopus japonicus* selenka. *J. Ocean Univ. Qingdao* 17: 85–94 (in Chinese with English abstract) (1987)
- Diancourt L, Passet V, Chervaux C, Garault P, Smokvina T. Multilocus sequence typing of *Lactobacillus casei* reveals a clonal population structure with low levels of homologous recombination. *Appl. Environ. Microb.* 73: 6601–6611 (2007)
- Kanno M, Kijima A. High genetic variability of isozymes in Japanese sea cucumber *Stichopus japonicus*. *Fish Gen. Breed. Sci.* 31: 7–12 (in Japanese with English abstract) (2002)
- Li Q, Chen L, Kong L. A genetic linkage map of the sea cucumber, *Apostichopus japonicus* (Selenka), based on AFLP and microsatellite markers. *Anim. Gen.* 40: 678–685 (2009)

6. Sun XJ, Li Q, Kong LF. Comparative mitochondrial genomics within sea cucumber (*Apostichopus japonicus*): Provide new insights into relationships among color variants. *Aquaculture* 309: 280–285 (2010)
7. Sun WJ, Li Q, Kong LF. Characterization of thirteen single nucleotide polymorphism markers in the sea cucumber (*Apostichopus japonicus*). *Conserv. Genet. Resour.* 2: 141–144 (2010)
8. Yao B, Hu XL, Bao ZM, Lu W, Hu JJ. Genetic variation in two sea cucumber (*Apostichopus japonicus*) stocks revealed by ISSR markers. *Chinese J. Ocean. Limn.* 25: 91–96 (2007)
9. Kanno M, Suyama Y, Li Q. Microsatellite analysis of Japanese sea cucumber, *Stichopus (Apostichopus) japonicus*, supports reproductive isolation in color variants. *Mar. Biotechnol.* 8: 672–685 (2006)
10. Erlich HA. PCR technology. *Princ Applic DNA Amplification*. New York, NY, USA. p. 246 (1989)
11. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.* 18: 6531–6535 (1990)
12. Kerry AM, Janies DA, Clouse RM, Samyn Y, Kuszak J, Kim J. Molecular phylogeny of coral-reef sea cucumbers (Holothuriidae: Aspidochirotida) based on 16S mitochondrial ribosomal DNA sequence. *Mar. Biotechnol.* 7: 53–60 (2004)
13. Lacumin L, Cecchini F, Manzano M, Osualdini M, Boscolo D, Orlic S, Comi G. Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. *Food Microbiol.* 26: 128–135 (2009)
14. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucl. Acids Res.* 25: 3389–3402 (1997)
15. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software ver. 4.0. *Mol. Biol. Evol.* 24: 1596–1599 (2007)
16. Liao M, Wang Y, Rong X, Zhang Z, Li B, Wang L, Chen G. Development of new microsatellite DNA markers from *Apostichopus japonicus* and their cross-species application in *Parastichopus parvimensis* and *Pathallus mollis*. *Int. J. Mol. Sci.* 12: 5862–5870 (2011)
17. Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.* 18: 7213–7218 (1990)
18. Zhang JC, Wang LF, Xu HY, Yu J, Liu WJ, Bao QH, Sun ZH, Zhang HP. Comparison of six molecular methods for differentiation of *Lactococcus lactis* subsp. *lactis* and *cremoris*. *Acta Microbiol. Sin.* 50: 1670–1676 (2010)
19. Bardacki F, Skibinski DOF. Application of the RAPD technique in tilapia fish: Species and subspecies identification. *Heredity* 73: 117–123 (1994)
20. Nuchprayoon S, Junpee A, Poovorawan Y. Random amplified polymorphic DNA (RAPD) for differentiation between Thai and Myanmar strains of *Wuchereria bancrofti*. *Filaria J.* 6: 1–8 (2007)
21. Chatti K, Salhi HA, Mars M, Marrakchi M, Trifi M. Genetic diversity and phylogenetic relationships in Tunisian fig (*Ficus carica* L.) cultivars mediated by RAPD. *Hereditas* 144: 149–157 (2007)
22. Barraclough T, Balbi K, Ellis R. Evolving concepts of bacterial species. *Evol. Biol.* 39: 148–157 (2012)