การประเมินลักษณะทางสัณฐานและความสัมพันธ์ทางพันธุกรรมของปลาเสือตอลายเล็ก (*Datnioides undecimradiatus* (Roberts & Kottelat, 1994)) กับชนิดที่อยู่ในสกุล *Datnioides* Bleeker, 1853 โดยใช้เครื่องหมายอาร์เอพีดี

Evaluation of Morphometric Characteristics and Genetic Relationship of Northeastern Siamese Tigerfish (*Datnioides undecimradiatus* (Roberts & Kottelat, 1994)) with Other Species in the Genus *Datnioides* Bleeker, 1853 Using RAPD markers

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บทคัดย่อ

ปลาเสือตอลายเล็ก (Northeastern Siamese Tigerfish; *Datnioides undecimradiatus*) จัดเป็นปลาน้ำจืดพื้นเมืองที่มีมูลค่าจากการนำมาเลี้ยงเป็นปลาสวยงาม ซึ่งถึงแม้ว่าปลาเสือตอลายเล็กจะถูกจัดจำแนกให้อยู่ใน สกุล *Datnioides* แล้วก็ตาม แต่ก็พบว่า ปลาชนิดดังกล่าวมีแนวโน้มของการเกิดความผันแปรของลักษณะภายนอกซึ่งอาจส่งผลต่อความคล้ายคลึงของลักษณะกับชนิดที่จัดอยู่ในสกุล *Datnioides* ชนิดอื่นๆ ซึ่งประกอบด้วยปลาเสือตออินโดนีเซีย (*D. microlepis*), ปลาเสือตอปาปัวนิวกินี (*D. campbelli*), ปลากะพงลาย (*D. polota*) และปลาเสือตอลายใหญ่ (*D. pulcher*) ซึ่งผลจากการศึกษาลักษณะสัดส่วนอวัยวะของลักษณะวัด จำนวน 25 ลักษณะ เพื่อตรวจสอบและจำแนกความแตกต่างระหว่างปลาในกลุ่มปลาเสือตอทั้ง 5 ชนิด พบว่า เมื่อทำการวิเคราะห์แนวโน้มความสัมพันธ์ของลักษณะสัดส่วนอวัยวะกับชนิดของปลา ด้วยวิธี PCA พบว่า สามารถแบ่งปลาในสกุล *Datnioides* ออกได้เป็น 2 กลุ่ม อย่างชัดเจน ได้แก่ กลุ่มที่ 1 ประกอบด้วย ปลาเสือตอปลาปัวนิวกินี, ปลากะพงลาย และปลาเสือตอลายใหญ่ ส่วนกลุ่มที่ 2 ประกอบด้วย ปลาเสือตออินโดนีเซียกับปลาเสือตอลายเล็ก และเมื่อทำการการวิเคราะห์การจัดกลุ่มความสัมพันธ์ของลักษณะสัดส่วนอวัยวะด้วยวิธี Cluster Analysis พบว่า ปลาเสือตอลายเล็กมีลักษณะสัดส่วนอวัยวะที่คล้ายคลึงและถูกจัดให้อยู่ในกลุ่มเดียวกันกับปลาเสือตออินโดและปลาเสือตอลายใหญ่ ส่วนผลการศึกษาความสัมพันธ์ทางพันธุกรรมระหว่างชนิดของปลาในสกุล *Datnioides* ด้วยเครื่องหมาย RAPD (randomly amplified polymorphic DNA) จำนวน 14 ไพรเมอร์ พบว่า ปลากะพงลาย เป็นชนิดที่มีค่าร้อยละของจำนวน Polymorphic band สูงที่สุด คือ 53.57% ขณะที่ชนิดที่พบ จำนวน Polymorphic band ต่ำสุด คือ ปลาเสือตออินโดนีเซีย 29.35% ซึ่งเมื่อทำการวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการ (phylogenetic tree) พบว่า ปลาเสือตอลายเล็ก เป็นชนิดที่มีความสัมพันธ์ที่ใกล้ชิดกับปลาเสือตอลายใหญ่ มากที่สุด รองลงมาเป็น ปลาเสือตออินโดนีเซีย ปลาเสือตอ

ปาป้วนิวกินี และ ปลากะพงลาย ตามลำดับ สรุปได้ว่า การศึกษาลักษณะสัดส่วนอวัยวะของลักษณะวัดร่วมกับการวิเคราะห์ด้วยเครื่องหมายดีเอ็นเอ สามารถนำมาใช้เป็นวิธีการศึกษาการจัดจำแนกทางอนุกรมวิธานได้เป็นอย่างดี

คำสำคัญ: ปลาเสือตอลายเล็ก, ลักษณะทางสัณฐาน, ความสัมพันธ์ทางพันธุกรรม, เครื่องหมายอาร์เอพีดี

Abstract

Northeastern Siamese Tigerfish (*Datnioides undecimradiatus*), the native freshwater species has become the valuable ornamental fish. Although the classification of this species was ranged into genus *Datnioides*, it is likely that external characteristics may be variable due to similarity with the other species, *D. microlepis*, *D. campbelli*, *D. polota* and *D. pulcher*. The present study employed 25 morphometric measurements to investigate differentiation among 5 species. Principle component analysis clearly revealed that there were 2 groups consisted of first group (*D. campbelli*, *D. polota* and *D. pulcher*) and second group (*D. microlepis* and *D. undecimradiatus*). Meanwhile, the cluster analysis differently showed that *D. undecimradiatus* was in the same group as *D. microlepis* and *D. pulcher*. Fourteen decamer random primers were amplified to investigate the genetic relationship among *Datnioides* using randomly amplified polymorphic DNA (RAPD). It showed that the highest polymorphic content was presented in *D. polota* (53.57%), while the lowest polymorphic contents were found in *D. microlepis* (29.35%). The phylogenetic tree revealed the closest relationship between *D. undecimradiatus* and *D. pulcher* followed by *D. microlepis*, *D. campbelli* and *D. polota*. It is concluded that the morphometric measurements and analysis of DNA markers may support the taxonomic identification.

Keywords: Northeastern Siamese Tigerfish, Morphometric Characteristics, Genetic Relationship, RAPD Marker

Introduction

Northeastern Siamese Tigerfish (*Datnioides undecimradiatus*) belongs in order Perciformes and family Datnioididae. Its distribution was found in the Mekong river and its tributaries in northeastern of Thailand (Nong Khai, Nakorn Phanom, Mukdaharn and Ubon Ratchathani provinces). The common characteristics of this genus were black-lined body, long and hard spine of anal fin, spiny preoperculum bone and position of eyes at the middle of the head (Rainboth, 1996). With external characteristics, Northeastern Siamese Tigerfish was classified into Siamese Tigerfish (*D. pulcher*) which is the critically endangered (CR) species in the redlist and the valuable species

in ornamental market of the world. Currently, a large amount of Northeastern Siamese Tigerfish were captured to be the ornamental fish and ranked in the vulnerable (VU) species (Iucnredlist.org., 2014).

Genus *Datnioides* is composed of 5 main species; Northeastern Siamese tigerfish (*D. undecimradiatus* (Roberts and Kottelat, 1994)), Silver Tigerfish (*D. polota* Hamilton, 1822), Finescale Tigerfish (*D. microlepis* Bleeker, 1854), Newguinea Tigerfish (*D. campbelli* Whitley, 1939) and Siamese Tigerfish (*D. pulcher* (Kottelat 1998)). The limitation of taxonomic identification was presented in the grouping and scientific name. Siamese Tigerfish (*D. pulcher*) was grouped in the same species as Finescale Tigerfish (*D. microlepis*). Then it was separated into the new species (*D. pulcher*) in 1989 (Calacademy.org., 2014:online).

All *Datnioides* species are the popular ornamental freshwater fish, especially Siamese Tigerfish (*D. pulcher*) which are expensive and decrease in amount in the wild. It is considered similar in term of morphology to *D. microlepis,* a cheaper species. Both fish have been classified in the same species and used the same scientific name in the past. It is very confusing and difficult to distinguish between these two species. In addition, some of academic data related to the study of genus *Datnioides* were reported on the equal number of meristic counts including the number of dorsal fin rays, anal fin ray, gill racker and the lateral line scale between *D. pulcher* and *D. microlepis*. Consistently to results of the Karyotypes study, it was found that both *D. pulcher* and *D. microlepis* appearance as well as the structures' number of the Chromosome was equal (Pacharaphan, 2007) In contrast Dutrudi and Nontree (2014) reportedly revealed that *D. microlepis* had a more genetic similarity to *D. undecimradiatus*, compared to *D. pulcher.* There were also discrepancies in the data which could be used to identify these fish separately. The taxonomic information in morphometric measurements and genetic identification is essential. The objectives of this study was to determine the morphometric differentiation of genus *Datnioides* and genetic relationship within and among species of genus *Datnioides* using rapid amplified polymorphic DNA (RAPD) marker.

Materials and methods

Fish samples

Forty three fish samples of 5 species in *D. undecimrediatus* were collected from the lower part of Moon River and Mekong River in Ubon Ratchathani province region. The 3 samples of *D. pulcher* and 10 samples of *D. microlepis, D. campbelli* and *D. polota* were transferred from ornamental fish shops in Bangkok, Thailand. The taxonomic identification was based on Smith (1945), Rainboth (1996) and Kottelat (2001).

Morphometric studies

The standard method of characteristic measurement was based on Hubbs and Lagler (1947). The one characteristic were compared in percentage of total lengths, nineteen characteristics were compared in percentage of standard lengths and five characteristics were compared in percentage of the head length lateral as in table 1. All of 25 morphometric measurement characters were examined using correlation matrix in principle component analysis (PCA) and cluster analysis by the R program.

Genomic DNA Extraction

The 500 µl blood samples were collected from 5 species of *Datnioides*. Genomic DNA was extracted using protein precipitation method (Endlard and Seifter, 1990). Blood samples were digested with 500 µl TNES-urea buffer and 5 µl proteinase K enzyme. Genomic DNA was washed with 200 µl protein precipitation buffer, 600 µl; isopropanol and 700 µl of 70% ethyl alcohol. DNA samples were stored in 30 µl TE buffer at -20 ^{0}C until use. The 5 µl of genomic DNA was electrophored on 1% agarose gel and checked the quantity with spectrophotometry.

RAPD-PCR Analysis

The 80 pairs of universal primers were used in RAPD-PCR technique. The PCR was carried out in a final volume 10 µl. The reaction consisted of 1.0 µl of template DNA, 1x of 10x PCR buffer, 0.6 µl of 50 mM $MgCl_2$, 0.8 µl of 0.025 mM dNTPs, 10 µM primer, 0.2 µl of 500U *Taq* polymerase and 5.6 µl sterile water. The PCR was carried out on a thermal cycler, with predenaturation at 94 ^{0}C for 3 min, followed by 40 cycles of 94 ^{0}C for 30 sec, 38 ^{0}C for 30 sec, 72 ^{0}C for 1 min, and a final extension at 72 ^{0}C for 10 min. The amplified DNA fragments were analyzed using 1% agarose gel, electrophoresis checked by UV light transilluminator and documented using the gel documentation composed of dark hood and Canon camera with UV light filter.

Statistical analysis

The morphometric data were presented as mean ± standard deviation. Differences in characteristics between the different species were analyzed by one-way ANOVA and Tukey HSD multiple comparison test (R-package). Significant values were considered at $P < 0.05$. The correlation of morphometric parameters among species were analyzed in Principle Component Analysis (PCA) and cluster analysis using R package (version 2.6.1).

The polymorphic bands of RAPD were scored as presence or absence (1 or 0, respectively). The total bands, percentage of polymorphic band, polymorphic information content (PIC), allele frequency were presented. The genetic diversity was examined following Nei and Li (1979), while the genetic distance and UPGMA dendrograms were calculated following PHYLIP Version 3.5 and Nei (1978)

Results

Morphometric measurements of *Datnioides* spp.

Twenty five morphometric characters of genus *Datnioides* were examined. The highest values were LCP, BWD, BWA, PecFL, HWN, HWO and IOS in *D. polota;* HLD and ED in *D. microlepis;* HLL, PDL, PPelL, PAnalL, PAnusL, PelFBL, CFL and SNL in *D. campbelli;* CPD, DFH, PelFH and AFH in *D. undecimradiatus* and SL, BD, DFBL and AFBL in *D. pulcher*. While the lowest values were HLD, BD, CPD, DFH, AFH and SNL in *D. polota*; PAnusL, BWD, PelFBL and HWO in *D. microlepis*; SL, LCP, DFBL and AFBL in *D. campbelli*; HWN and IOS in *D. undecimradiatus* and HLL, PDL, PPelL, PAnalL, BWA, PecFL, PelFH, CFL and ED in *D. pulcher*. There were the greatest amounts of BWD, BWA, HWN, HWO and IOS in *D. polota*; PPelL, PAnalL, CFL and SNL in *D. campbelli* and DFBL in *D. pulcher*. While there were the lowest number of parameters were HLD, BD, DFH and AFH in *D. polota*; PAnusL and PelFBL in *D. microlepis* and HLL, PPelL and BWA in *D. pulcher* ($P<0.05$) (Table1). Among the 25 characters, PCA revealed the significant differentiation of genus *Datnioides*. It was placed into 2 separate groups; 3 species of *D. pulcher*, *D. campbelli*, and *D. polota* and 2 closest relationship species of *D. microlepis* and *D. undecimradiatus* have similar characters compared to the other species (Figure 1A).

Between all of *Datnioides* species, there were the 4 highly significant morphometric characters included IOS, BWA, PPelL and DFBL (Table 1 and Figure 1B).

For cluster analysis of 25 characters, grouping of genus *Datnioides* differently showed 2 separate groups; the first group: *D. campbelli* and *D. polota* and the second group: *D. pulcher*, *D. microlepis* and *D. undecimradiatus* (Figure 2).

Table1 The proportional values of morphometric measurements of 5 *Datnioides* species

Morphometric	Dmi	Dun	Dca	Dpo	Dpu
	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD
(In % of TL)					
SL	84.341±0.942^{a}	83.462±0.590^{ab}	82.786± 0.383^{b}	83.624±0.807^{ab}	84.930±0.872^{a}
(In % of SL)					
HLD	33.941±0.720^{a}	31.548±0.906^{b}	31.693±0.970^{b}	22.427±0.556^{c}	33.473±0.391^{a}
HLL	41.203±0.882^{a}	40.054±0.513^{ab}	41.512±0.893^{a}	39.084±0.953^{b}	35.588±0.824^{c}
PDL	47.976±0.840^{a}	45.643±0.794^{b}	48.976±0.610^{a}	44.080±0.861^{c}	42.793±0.953^{c}
PPelL	45.037±0.694^{b}	43.445±0.734^{c}	49.337±0.535^{a}	45.131±0.845^{b}	39.339±0.408^{d}
PAnalL	72.467±0.921^{c}	74.342±0.842^{b}	76.891±0.816^{a}	74.599±0.899^{b}	72.032±0.843^{c}
PAnusL	63.962±0.936^{c}	66.439±0.397^{b}	69.702±0.874^{a}	69.204±0.904^{a}	65.864±0.362^{b}
BD	45.511±0.715^{a}	44.297±0.526^{b}	43.962±0.731^{b}	39.073±0.962^{c}	46.714±0.818^{a}
CPD	12.214±0.451^{a}	12.278±0.493^{a}	11.939±0.610^{ac}	11.538±0.256^{bc}	11.602±0.061^{ab}
LCP	13.649±0.422^{ab}	13.377±0.986^{b}	12.149±0.436^{c}	14.536±0.507^{a}	13.655±0.822^{ab}
BWD	16.562±0.467^{c}	18.506±0.298^{b}	18.764±0.926^{b}	22.031±0.573^{a}	17.200±0.262^{c}
BWA	13.374±0.468^{c}	16.131±0.684^{b}	12.712±0.484^{c}	18.546±0.411^{a}	10.921±0.890^{d}
DFH	23.138±0.753^{bc}	24.664±0.555^{a}	24.286±0.809^{ab}	16.341±0.785^{d}	21.737±0.745^{c}
DFBL	56.740±0.884^{c}	59.953±0.665^{b}	53.089±0.644^{d}	54.145±0.942^{d}	61.786±0.953^{a}
PecFL	20.760±0.776^{bc}	21.669±0.878^{ac}	21.88±0.632^{ab}	22.196±0.751^{a}	19.274±0.528^{d}
PelFH	24.907±0.676^{b}	26.612±0.954^{a}	26.432±0.846^{a}	23.472±0.925^{c}	21.832±0.642^{c}
PelFBL	6.1360±0.284^{d}	6.577±0.151^{c}	7.251±0.305^{a}	6.807±0.207^{bc}	7.053±0.086^{ab}
AFH	30.191±0.544^{ab}	30.831±0.924^{a}	28.766±0.895^{c}	26.516±0.479^{d}	28.817±0.913^{bc}
AFBL	21.491±0.728^{a}	21.289±0.737^{ab}	19.371±0.890^{c}	20.117±0.692^{bc}	22.272±0.923^{a}
CFL	19.571±0.791^{b}	19.398±0.879^{b}	23.366±0.867^{a}	19.942±0.670^{b}	18.742±0.679^{b}
(In % of HLL)					
HWN	19.108±0.460^{b}	18.499±0.371^{b}	19.401±0.452^{b}	21.412±0.812^{a}	19.277±0.250^{b}
HWO	43.893±0.716^{d}	44.806±0.539^{cd}	53.466±0.756^{b}	56.631±0.912^{a}	45.864±0.120^{c}
IOS	36.340±0.828^{c}	33.000±0.493^{d}	37.722±0.547^{b}	42.296±0.989^{a}	33.148±0.813^{d}
ED	25.432±0.530^{a}	21.836±0.437^{c}	24.452±0.807^{ab}	23.324±0.894^{b}	17.350±0.943^{d}
SNL	29.805±0.622^{b}	30.413±0.863^{b}	32.222±0.330^{a}	27.436±0.595^{c}	31.937±0.959^{a}

total length (TL), standard length (SL), head length dorsal (HLD), head length lateral (HLL), pre-dorsal length (PDL), pre-pelvic length (PPelL), pre-anal length (PAnalL), pre-anus length (PAnusL), body depth (BD), caudal peduncular depth(CPD), length of caudal peduncle (LCP), body width dorsal (BWD), body width anal (BWA), dorsal fin height (DFH), dorsal fin base length (DFBL), pectoral fin length (PecFL), pelvic fin height (PelFH), pelvic fin base length (PelFBL), anal fin height (AFH), anal fin base length (AFBL), caudal fin length (CFL), head width nare (HWN), head width opercle (HWO), interorbital space (IOS), eye diameter (ED) and snout length (SNL)

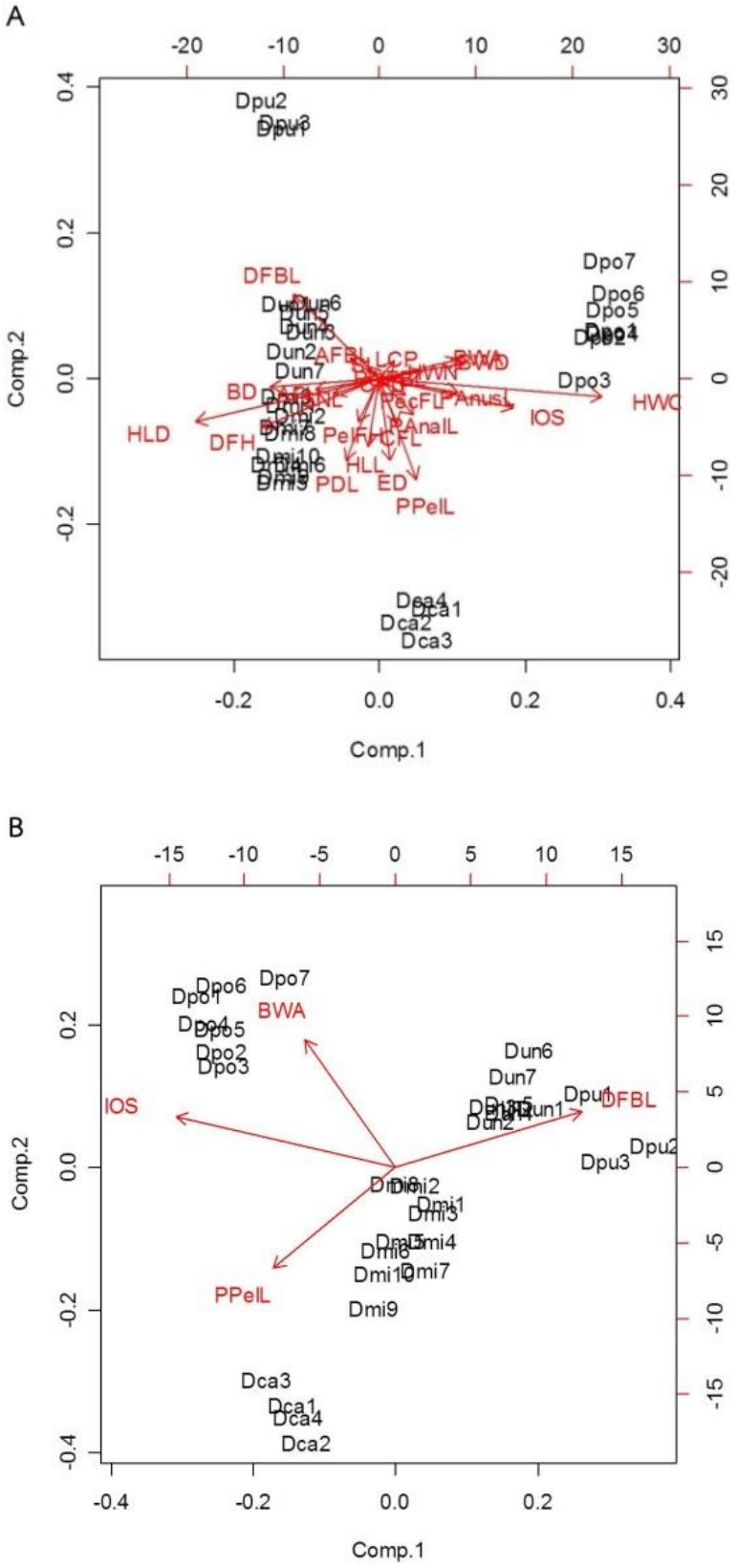

Figure1 PCA plot of morphometric parameters (A) 25 morphometric characters and (B) the 4 significant morphometric characters between all of *Datnioides* species

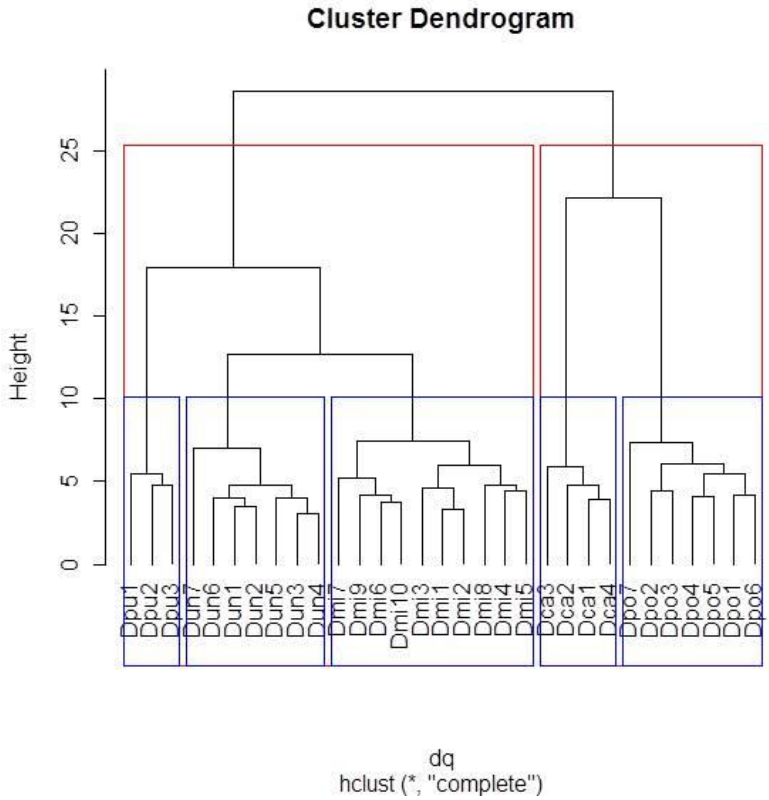

Figure 2 Cluster analysis dendrogram based 25 morphometric characters of 5 Datnioides's species: Dpu=*D. pulcher*, Dca=*D. campbelli*, Dun=*D. undecimradiatus*, Dmi=*D. microlepis* and Dpo=*D. polota*

Genetic differentiation of genus *Datnioides*

From 80 pairs of primer in RAPD technique, only 14 pairs of primer showed the 398 polymorphic bands in genus *Datnioides* (Figure 3). The greatest polymorphic bands were found in *D. polota* (45 bands; 53.57 %) followed by *D. undecimradiatus* (41 bands; 46.07 %), *D. pulcher* (23 bands; 38.98 %), *D. campbelli* (28 bands; 37.84 %) and *D. microlepis* (27 bands, 29.35 %) (Table 3). The average polymorphism information content of genus *Datnioides* reached 0.74, while the average allele frequencies ranged between 0 to 0.38.

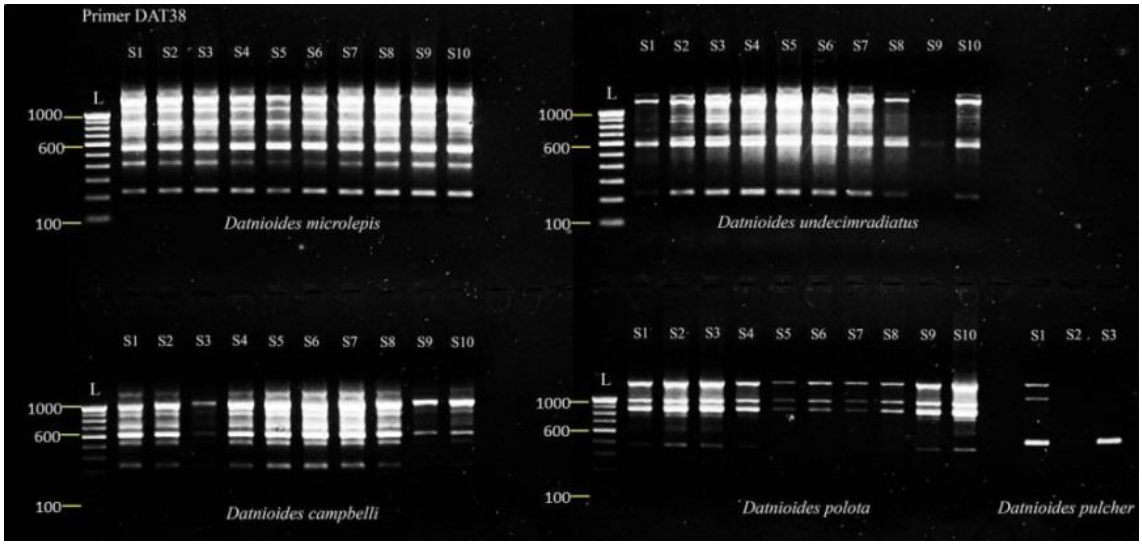

Figure 3 RAPD pattern with Primer DAT38 of 5 *Datnioides* species on the 1% agarose gel electrophoresis (100V-90min.). L=100 bp ladder, S=Numbers of sample

The genetic relationship of genus *Datnioides* was allocated into different clusters using UPGMA dendrogram. The cluster showed the close relationship of *D. undecimradiatus* and *D. pulcher* followed by *D. microlepis*, *D. campbell* and *D. polota* (Figure 4).

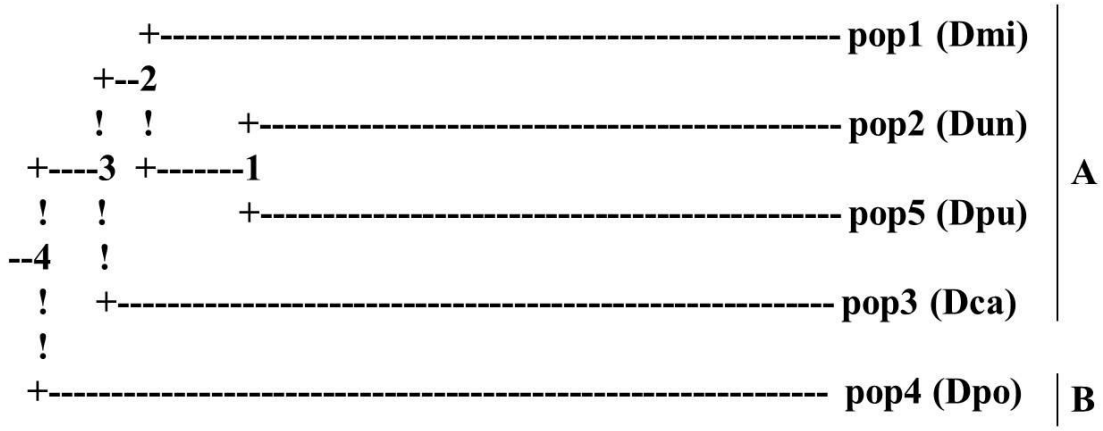

Figure 4 UPGMA dendrogram based on Nei's Genetic distance

Conclusions and Discussion

The results of morphological characteristics measured in this study which were educational methods and were suitable for practical applications, especially with alive fish revealed the measured proportions of the morphometric characteristics. These were analyzed for their relationships with fish species by both PCA method and Cluster analysis. The results revealed that the *D. microlepis* had the same proportions of morphometric and related to *D. undecimradiatus* more

than *D. pulcher*. The Eco-morphometric data can be used to distinguish between *D. pulcher* and *D. microlepis*.

RAPD technique has been shown the significant difference in allelic frequency and polymorphic content among genus *Datnioides*. The greatest allelic frequency overall loci was found in *D. microlepis*. The allelic frequency of *D. microlepis* (0.239), *D. undecimradiatus* (0.221) and *D. polota* (0.214) showed higher values than *D. Campbell* (0.185) and *D. pulcher* (0.142). RAPD marker revealed to detect the genetic diversity of genus *Datnioides*. Several studies reported to monitor the genetic diversity and distance using RAPD technique e.g. eight cyprinid fish species included *Barbus xanthopterus*, *B. kersin*, *B. barbulus*, *B. grypus*, *B. sharpeyi*, *B. luteus*, *Aspius vorax* and *Cyprinus carpio* (Mustafa *et al*., 2012); *Rasbora sumatrana*, *R. paucicerforata*, *R. enthovaneii* and *R. cephalotaenia* (Zohrah, 2002); *Clinostomum complanatum* and *Neutraclinostomum intermedialis* (Grobler *et al.,* 1999); Indian Coldwater Fishes (Sivaraman *et al*., 2010); six Labeo species (Paramananda *et al*., 2005); five Indian sciaenids (Wazir *et al*., 2007); and four fish species (*Pimelodus maculatus, Prochilodus lineatus, Salminus brasiliensis* and *Steindachneridion scripta*) (Micheline *et al.,* 2006)

The genetic distance and phylogenetic analysis of RAPD marker demonstrated significant difference among *Datnioides*. The lowest genetic distance was found between *D. pulcher* and *D.undecimradiatus*. Both of them was close to *D. microlepis*. These results were presented slightly differences from clustering analysis using morphometric measurements which showed close relationship of *D. undecimradiatus* and *D. microlepis* and next to *D. pulcher.*

This phenomenon may be attributed to representatives of morphometric parameters and number of fish samples in RAPD analysis. Arulraj *et al.* (2011) reported the morphometric measurements and genetic diversity of genus *Garra* using RAPD marker and found that both methods can detect the genetic distance and higher relationship of *G. mullya* and *G. kalakadensis* than *G. gotyla stenorhynchus*. In addition, there was reported to Combining Morphology and Genetics in Resolving Taxonomy- A Systematic Revision of Spined Loaches (Ivana *et al*., 2014); Morphometric and Genetic Analyzes of Indian Mackerel (*Rastrelliger kanagurta*) (Jayasankar *et al*., 2004) and Morphometric and Genetic Variations of *Etroplus suratensis* (Bloch) (Dhanya *et al.,* 2013). All of this study founded that the morphometric measurements methods and RAPD technique can detect the genetic distance and relationship of that fishes.

The morphometric measurements are essential to identify initially the genus *Datnioides* through principle component and clustering analysis. In case of genus *Datnioides*, *D. pulcher* has been grouped to be the rare (critically endangered) species, while *D. undecimradiatus* has been

reported to be valuable species. It is difficult to sacrifice or investigate in the internal fragment of head and body. There for molecular technique with DNA marker can be useful that clarify and present genetic variability.

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