ON THE TAXONOMIC STATUS OF YANGTZE STURGEON, ASIAN AND AMERICAN GREEN STURGEON BASED ON MITOCHONDRIAL CONTROL REGION SEQUENCES

ZHANG Si-Ming①② WU Qing-Jiang③ ZHANG Ya-Ping①

①Lab of Cellular and Molecular Evolution, Kunming Institute of Zoology, The Chinese Academy of Sciences, Kunming 650223, China
②Yangtze River Institute of Fisheries, Chinese Academy of Fisheries Sciences, Jingzhou 434000, Hubei, China
③Institute of Hydrobiology, The Chinese Academy of Sciences, Wuhan 430072, China

Abstract It has been controversial whether Asian Green sturgeon and American Green sturgeon (Acipenser medirostris) are valid species, and the relationship among Yangtze sturgeon (A. dabryanus) and other species, especially Chinese sturgeon (A. sinensis), has also been confusing. The control region (D-loop) of mitochondrial DNA (mtDNA) was amplified and sequenced to address these problems. Our sequence data support the following hypotheses: (a) Yangtze sturgeon may be the most closely related to the Chinese sturgeon and is likely to be a landlocked form of Chinese sturgeon; and (b) green sturgeon inhabiting North America and Far East Asia may belong to the same species, A. medirostris. However, more data are needed to test these ideas.

Key words Green sturgeon, Yangtze sturgeon (Acipenser dabryanus), Chinese sturgeon (Acipenser sinensis), Systematics, Mitochondrial control region

The order Acipenseriformes comprises an ancient order of fishes consisting of about 25 extant species of sturgeons and two species of paddlefishes. However, no agreement on the exact number of species has been reached. Relationships among species in this order, especially within the genus Acipenser Linnaeus, 1758, are still open to question. The nomenclature and species status of the green sturgeon (Acipenser medirostris Ayres, 1854) inhabiting northeast Pacific in Asia (hereinafter referred to as Asian Green sturgeon) and the Pacific Northwest of America (hereinafter referred to as American Green sturgeon) have been particularly confusing. As early as 1854, the American Green sturgeon was described as A. medirostris by Ayres (1854). Later, an Asiatic form of the species was named A. mikadoi by Hilgendorf (1892) and Schmidt (1904). However, Berg (1911, 1948) suggested that the Asian Green sturgeon should be considered to be conspecific with the American Green sturgeon, A. medirostris. In addition, Lindberg et al. (1965) regarded the Asian Green sturgeon as a subspecies, A. medirostris mikadoi based on morphological data, however, Artyukhin et al. (1990) disagreed on the subspecies status of the Asiatic form. Recently, Birstein et al. (1993) found that the DNA contents of Asian Green sturgeon were 14.2 pg whereas Blacklidge et al. (1993) noted that the DNA contents of American Green sturgeon were 8.82 pg. And so, Birstein et al. (1993) suggested that the Asiatic and American forms of the species should be described into two subspecies, A. m. mikadoi and A. m. medirostris, respectively, instead of two species, A. mikadoi and A. medirostris (Birstein et al., 1997; Birstein et al., 1998). The taxonomic status of the two forms of sturgeon needs further investigations.

The relationship between the non-anadromous Yangtze sturgeon (A. dabryanus Dumeril, 1864) in-
habiting the upper Yangtze river and an anadromous species, the Chinese sturgeon (A. sinensis Gray, 1834) mainly inhabiting the Yangtze River has also been confusing. According to Bemis et al. (1997) and Birstein et al. (1998) the Yangtze sturgeon is not only very distantly related to the Chinese sturgeon, but it is also distantly related to other sturgeons in the Trans-Pacific region. Contrary to said opinion, biogeographic analysis suggests that the Yangtze sturgeon is closely related to species of the Trans-Pacific region, and most closely related to the Chinese sturgeon (Artuykhin, 1995). Our recent studies using random amplified polymorphic DNA (RAPD) (Zhang et al., 1999a), mtDNA ND4L and ND4 gene sequence analysis (Zhang et al., 2000a) and repeat sequences of mtDNA control region (D-loop) (Zhang et al., 2000b) were consistent with the viewpoint of Artuykhin (1995).

Mitochondrial DNA (mtDNA) is a useful marker in studies of phylogenetics and population genetics because of its rapid evolutionary rate and almost complete maternal inheritance. The application of nucleotide sequencing in phylogenetic reconstruction is becoming popular due to its high resolving power (Avise et al., 1987; Zhang, 1996). Comparative studies on D-loop of mtDNA in fishes revealed that it is sufficiently variable to examine relationships among closely related populations (Bernatchez et al., 1992), subspecies (Ong et al., 1996), species (Meyer et al., 1990; Turner, 1997), and genera (Sturmbauer et al., 1992; Turner, 1997).

The main objective of this study was to (1) address the taxonomic status of A. medirostris inhabiting Northern America (American Green sturgeon) and Far East Asian (Asian Green sturgeon), and (2) test the systematic relationships among Yangtze sturgeon and the other species of Acipenser, particularly Chinese sturgeon using mtDNA control region sequence analysis.

1 Materials and Methods

1.1 Sample collection and DNA extraction

All tissue samples were obtained from fishes caught by fishermen accidentally. Three samples of Chinese sturgeon and one sample of Yangtze sturgeon were caught from the Yangtze river, China in 1997. One sample of Amur sturgeon (A. schrenckii Brandt, 1869) was obtained from the Amur River in 1997. One sample of Asian Green sturgeon was collected from the Tumnin river in Russia in 1996. The counterpart DNA sequences of American Green sturgeon and White sturgeon (A. transmontanus Richardson, 1836) cited from Brown et al. (1993) was used for comparative study.

Total genomic DNA was isolated from frozen or 95% ethanol preserved muscle or fin tissue. About 0.5 g tissue was homogenized after adding 1 ml STE (10 mmol/L Tris·HCl, pH = 8.0; 0.1 mol EDTA, pH = 8.0). After homogenization, 30 μl Proteinase K at concentration 10 mg/ml and 150 μl SDS at concentration 10% (v/v) were added and the tissue was incubation at 50 °C for more than 5 h. Phenol extraction was performed. 95% ethanol was used to precipitate DNA. Double distilled water was added to dissolve the precipitated DNA.

1.2 PCR amplification and sequencing of mtDNA

Since we found that different numbers of tandem repeats exist in D-loop of individual Chinese sturgeon (Zhang et al., 1999b), it is difficult to compare the repeat regions. We designed primers to amplify the fragment, which did not include the repeat region of D-loop in this study. The primer sequences follow: DL (L): 5′-CAA GAAC ACAA GTAA TGAG ′3′, H740 (H): 5′-GAT CAA GTGT A TGC AT GACA-3′; H740 was from Brown et al. (1993).

PCR was performed in a total reaction volume of 50 μl containing 5 μl of 10 × buffer, 4 μl of each dNTP (4 mmol/4dNTPs), 1 μl of each primer (10 pmol), 0.3 μl of Tag DNA polymerase (5 U/μl), proper template DNA and ddH2O to 50 μl volume. Each PCR cycle consisted of 45 sec denaturation at 94 °C 1 min annealing at 50 °C and for 1 min extension at 72 °C, repeated 40 times. Pre-denaturation at 94 °C for 3 min before the normal cycles and a final extension at 72 °C for 8 min after normal cycles were included.

DNA sequencing was performed with an ABI 377 automated DNA sequencer.
1.3 Data analysis

Genetic differences (percent divergences) were estimated using the "Molecular Evolutionary Genetic Analysis" (MEGA, 1.02) software package (Kumar et al., 1993) based on Kimura 2 Parameter genetic distance. Also, using same genetic distance an unweighted pair group method with arithmetic means (UPGMA) and neighbor-joining (NJ) method in MEGA were used to construct phylogenetic trees. Maximum parsimony (MP) tree analysis was performed using the "Phylogenetic Analysis Using Parsimony" (PAUP, 3.1) software package (Swofford, 1993) based on heuristic search. The bootstrap values (percentage of 1000 replicates) were given for each node.

Fig. 1 Nucleotide sequences of mtDNA D-loop from several species of genus Acipenser

1. A. dabryanus (GenBank accession No. AF362129)
2. A. medirostris (North America, NA; GenBank accession No. L1509)
3. A. medirostris (Asian Far East, AFE; GenBank accession No. AF362130)
4. A. schrenckii (GenBank accession No. AF362131)
5. A. sinensis (GenBank accession No. AF362132)
6. A. sinensis (GenBank accession No. AF362133)
7. A. transmontanus (GenBank accession No. AF362134)
8. A. transmontanus (GenBank accession No. L01517)
9. A. transmontanus (GenBank accession No. L01512)

[Dots (.) denote identity with No. 1 sequence, and dashes (-) represent gaps inserted for maximum alignment]
Table 1: Interspecific and intraspecific genetic divergence and genetic similarity

<table>
<thead>
<tr>
<th>Species</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. dabr anus (A)</td>
<td>60.9</td>
<td>61.2</td>
<td>70.8</td>
<td>91.1</td>
<td>91.6</td>
<td>90.5</td>
<td>70.5</td>
<td>69.8</td>
<td></td>
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<tr>
<td>A. medirostris (NA) (B)</td>
<td>24.3</td>
<td>94.1</td>
<td>70.0</td>
<td>63.5</td>
<td>64.7</td>
<td>65.0</td>
<td>80.8</td>
<td>81.2</td>
<td></td>
</tr>
<tr>
<td>A. medirostris (AFE) (C)</td>
<td>25.4</td>
<td>5.6</td>
<td>70.3</td>
<td>64.7</td>
<td>63.8</td>
<td>63.2</td>
<td>80.2</td>
<td>80.5</td>
<td></td>
</tr>
<tr>
<td>A. schrenkii (D)</td>
<td>19.6</td>
<td>21.7</td>
<td>23.7</td>
<td>71.6</td>
<td>71.6</td>
<td>72.2</td>
<td>85.1</td>
<td>83.8</td>
<td></td>
</tr>
<tr>
<td>A. sinensis 1 (E)</td>
<td>5.5</td>
<td>24.8</td>
<td>24.8</td>
<td>20.7</td>
<td>98.9</td>
<td>97.7</td>
<td>70.8</td>
<td>69.2</td>
<td></td>
</tr>
<tr>
<td>A. sinensis 2 (F)</td>
<td>5.2</td>
<td>24.2</td>
<td>25.4</td>
<td>20.7</td>
<td>0.9</td>
<td>98.0</td>
<td>71.1</td>
<td>68.8</td>
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<tr>
<td>A. sinensis 3 (G)</td>
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<td>23.6</td>
<td>26.0</td>
<td>20.1</td>
<td>2.0</td>
<td>1.7</td>
<td>70.1</td>
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</tr>
<tr>
<td>A. transmontanus 1 (H)</td>
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<td>15.8</td>
<td>17.8</td>
<td>10.1</td>
<td>18.8</td>
<td>18.8</td>
<td>18.2</td>
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<tr>
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<td>15.1</td>
<td>18.4</td>
<td>10.7</td>
<td>20.1</td>
<td>19.5</td>
<td>18.2</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

The numbers above diagonal are percent similarity, and the numbers below diagonal are percent divergence.

NA: Northern America (NA); AFE: Asian Far East (AFE)

2 Results

2.1 Nucleotide sequence variations and genetic divergences

The mtDNA D-loop sequences spanning 352 bases were aligned for nine individuals from six species (Fig. 1). Among them, sequences from six specimens are given the GenBank accession numbers, which are presented in Fig. 1. Extensive deletion is found among the nine sequences, especially in interspecific comparison.

The genetic similarity and divergence are presented in Table 1. The highest divergence of 24.8% to 26.0% is observed between Asian Green sturgeon and Chinese sturgeon. The intraspecific divergences within Chinese sturgeon varies from 0.9% to 2.0%. Chinese sturgeon and Yangtze sturgeon differ by 5.2% to 6.1%, averaging 5.5%. The level of divergence between Asian Green sturgeon and American Green sturgeon, 5.6%, falls within range of divergence found between Chinese sturgeon and Yangtze sturgeon (5.2% to 6.1%).

2.2 Phylogenetic trees

The topologies of UPGMA and NJ trees (Fig. 2) are similar to each other. Differences were observed among the three individuals of Chinese sturgeon. Yangtze sturgeon and Chinese sturgeon are more closely related to each other than either is to the other species. Asian Green sturgeon and American Green sturgeon cluster together regardless of whether the Yangtze sturgeon or the Amur sturgeon was used as outgroup in the MP phylogenetic trees (Fig. 3). The bootstrap value after 1000 replicates was 100 at the node containing the Asian Green sturgeon and the Northern American Green sturgeon.
Discussion

3.1 Relationship among the Yangtze river sturgeon and other species of Acipenser

Regarding the relationship among the Yangtze sturgeon and other species of Acipenser, Bemis et al. (1997) suggested that the Yangtze sturgeon was distantly related to the Chinese sturgeon although no convincing reasons were provided as to why. Recently, a similar conclusion was made based on cytochrome b (Cyt b) gene sequences and the combined "weighted" data for the Cyt b gene, 12S and 16S mtRNA genes although only the Cyt b sequence was obtained from the Yangtze sturgeon (Birstein et al., 1998). Data from morphology, ecology and biogeography support a close relationship between the Yangtze sturgeon and the Chinese sturgeon (Artyukhin, 1995), which is congruent with the present result. In fact, our studies by RAPD (Zhang et al., 1999a), sequences of ND4L-ND4 genes (Zhang et al., 2000a) and mitochondrial D-loop repeat sequences (Zhang et al., 2000b) also support that the two kinds of sturgeons (Yangtze and Chinese sturgeon) within the genus Acipenser from the Yangtze river are each other's closest relatives.

According to our present data we hypothesize that the Yangtze sturgeon is a landlocked form of the Chinese sturgeon. In fact, single species possessing both anadromous and non-anadromous forms (landlocked form) have been commonly found in fishes. Genetic divergence within the Chinese sturgeon ranged from 0.2% to 4.8% based on 418 bp of D-loop sequences from 44 individuals (Zhang, 1998). A similar result was also found in White sturgeon which showed intraspecific divergences between 0.01% and 4.29% across 466 bp of D-loop sequence from 27 individuals of White sturgeon (Brown et al., 1993). The present study shows that the Chinese sturgeon diverge from 5.2% to 6.1%, which is very close to the ranges of intraspecific variation found within either the Chinese sturgeon (Zhang, 1998) or the White sturgeon (Brown et al., 1993). The level of divergences is much lower than that measured between the closest species pair, Green sturgeon and White sturgeon. In other words, the divergence between the Chinese sturgeon and the Yangtze sturgeon is within ranges between Yangtze river population of Chinese sturgeon and species pair of White sturgeon and Green sturgeon. The present finding regarding the close relationship between the Chinese sturgeon and the Yangtze sturgeon is also supported by our previous studies using techniques such as RAPD (Zhang et al., 1999a), mtDNA ND4L and ND4 genes sequences (Zhang et al., 2000a) and D-loop tandem repeat se-

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sequences (Zhang et al., 2000b). The Yangtze sturgeon is a non-anadromous fish inhabiting the upper Yangtze river only. The Chinese sturgeon is an anadromous fish historically distributed in many rivers, however, mainly in the Yangtze river at present. The body size of Yangtze sturgeon is much smaller than that of Chinese sturgeon at matured age. Despite this difference the two kinds of sturgeon have very similar morphological characters and it is hard to distinguish the two forms from a morphological point of view (The Changjiang aquatic resources survey group, 1988). Our recent studies (Zhang, 1998; Zhang et al., 1999a; Zhang et al., 2000a; Zhang et al., 2000b) along with the present data indicate that genetic similarity between the two forms is very high. Cytological investigation suggested that the two forms possessed a similar chromosome number and belonged to same ploidy level (octoploid) (Zhang et al., 1999c). This also differs from Birstein et al. (1998), who implied that Yangtze sturgeon might be tetraploid. In addition, the hybrids between Chinese sturgeon and Yangtze sturgeon are not only viable, but also fertile (Zhuang et al., 1997). Accordingly, we hypothesize that the Yangtze sturgeon is a landlocked form of the Chinese sturgeon. More data is now needed to test this hypothesis.

3.2 Taxonomic status of Asian Green sturgeon and American Green sturgeon

The taxonomic status of the Asian Green sturgeon and the American Green sturgeon has long been debated. Different researchers have made different conclusions based on analysis of morphological characters and merit elements. For example, based on morphological studies Asiatic and American members of the species were made conspecific (Artyukhin et al., 1990). This differs from the result made by Lindberg et al. (1965), which suggested that the Asiatic form was a subspecies, A. m. mikadoi. It is difficult to solve the problem using morphological data only. Recently, results obtained from the DNA contents and sequences of three mitochondrial regions, the Cyt b gene, and fragments of 12S and 16S rRNA genes indicated that the most closely related species to American Green sturgeon was White sturgeon, not Asian Green sturgeon (Britain, et al., 1997; Birstein et al., 1998). A profound genetic difference was noted between Asian Green sturgeon and American Green sturgeon (Birstein et al., 1998). In contrast, the present study reveals that the two kinds of Green sturgeon (Asian and American Green sturgeon) are more closely related to each other than either is to the White sturgeon, with bootstrap support of 100%. Furthermore, the genetic difference (5.6%) between the two kinds of Green sturgeons is much lower than that measured between other species, with the exception of that between Chinese sturgeon and Yangtze sturgeon. As discussed earlier, the Yangtze sturgeon may be a landlocked form of the Chinese sturgeon. In other words, Yangtze sturgeon may be renamed as A. sinensis. The divergence values between the two kinds of Green sturgeon and between the two kinds of sturgeon inhabiting the Yangtze river are similar. To date only DNA contents have been compared for the two forms of Green sturgeon, and no direct chromosome count has been made. Therefore, it is premature to conclude how the two forms of Green sturgeon should be described. According to our analysis we agree with Berg (1911, 1948), regarded the two forms of Green sturgeon as a single species, A. medirostris. However, more work such as a cytogenetic study and more DNA sequences are needed to address the relationship of Asiatic and American forms of Green sturgeon.

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