Vol. 43 No. 2 SCIENCE IN CHINA (Series C) April 2000

Molecular phylogenetic systematics of twelve species of Acipenseriformes based on mtDNA *ND4L* —*ND4* gene sequence analysis

ZHANG Siming (张四明)[1,2*], ZHANG Yaping (张亚平)[1],
ZHENG Xiangzhong (郑向忠)[1,3], CHEN Yongjiu (陈永久)[1],
DENG Huai (邓 怀)[2], WANG Dengqiang (汪登强)[2], WEI Qiwei (危起伟)[2],
ZHANG Yunwu (张云武)[1], NIE Long (聂 龙)[1] & WU Qingjiang (吴清江)[4]

1. Key Lab of Cellular and Molecular Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China;
2. Yangtze River Institute of Fisheries, Chinese Academy of Fishery Sciences, Jingzhou 434000, China;
3. Institute of Genetics, Fudan University, Shanghai 200433, China;
4. Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

* Present address: Yangtze River Institute of Fisheries, Chinese Academy of Fishery Sciences, Jingzhou 434000, China (email: zhangsm@yfi.ac.cn).

Correspondence should be addressed to Zhang Yaping (email: zhangyp@public.km.yn.cn)

Received May 19, 1999

Abstract Acipenseriformes is an endangered primitive fish group, which occupies a special place in the history of ideas concerning fish evolution, even in vertebrate evolution. However, the classification and evolution of the fishes have been debated. The mitochondrial DNA (mtDNA) *ND4L* and partial *ND4* genes were first sequenced in twelve species of the order Acipenseriformes, including endemic Chinese species. The following points were drawn from DNA sequences analysis: (i) the two species of *Huso* can be ascribed to *Acipenser*; (ii) *A. dabryanus* is the mostly closely related to *A. sinensis*, and most likely the landlocked form of *A. sinensis*; (iii) genus *Acipenser* in trans-Pacific region might have a common origin; (iv) mtDNA *ND4L* and *ND4* genes are the ideal genetic markers for phylogenetic analysis of the order Acipenseriformes.

Keywords: Acipenseriformes, mtDNA *ND4L*, mtDNA *ND4*, molecular phylogeny, DNA sequencing.

Sturgeons and paddlefishes, which belong to the order Acipenseriformes, are the endangered primitive fishes. This group of fishes occupies a special place in the history of ideas concerning fish evolution, even in vertebrate evolution, on the other hand, the populations of sturgeons and paddlefishes worldwide have declined[1]. Though Acipenseriformes has long been regarded as an interesting group in biogeographical and phylogenetic studies since the times of Linnaeus, the classification of the fishes has been controversial. So far, the phylogenetic studies of the group remain in their infancy[2]. There are about 27 extant species worldwide. However, the exact number of species and systematic relationships among different genera and different species are still unknown. Though some data on morphology, cytogenetics and molecular biology have been accumulated, there is still a long way to go to fully understand the phylogenetic relationships.

DNA is an honest recorder of evolutionary history of organisms. Studies on DNA sequences may reveal the process of evolution and provide new clues to phylogenetic relationship. To date, information on DNA sequences of different taxa has been accumulated rapidly[3]. However, the molecular phylogeny of Acipenseriformes has lagged and is still at the level of initial surveys. Limited number of reports centered on mitochondrial cytochrome *b* and rRNA genes was available. Because Acipenseriformes is a primitive fish group and the evolution of their genes is relatively conservative, the genes mentioned could not provide enough information for phylogenetic analysis[4,5]. In recent years, the use of mtDNA *ND4L* and *ND*4 genes for phylogenetic analysis has drawn attention[6,7]. But, no reports on fishes are available. In the present investigation, the mtDNA *ND4L* and *ND*4 genes were first sequenced in 12 species of Acipenseriformes, including some endemic Chinese species and species from Europe and North America as well.

1 Materials and methods

1.1 Materials

The origins, tissues and preservation conditions of samples used in the study are presented in table 1.

Table 1 Origin, tissue and preservation condition of samples

Species	Tissue	Preservation condition	Origin or sampling sites	Date of collection
Acipenser sinensis	muscle/blood	cryopreservation	Yichang, Hubei	Nov. 1996
A. dabryanus	muscle/blood	cryopreservation	Wanxian, Chongqing	May 1997
A. schrenckii	muscle/blood	cryopreservation	Heilongjiang River	Jun. 1997
A. medirostris	muscle	95% ethanol	Russia	Mar. 1998
A. naccarii	muscle	95% ethanol	Italy	Oct. 1997
A. brevirostrum	muscle	95% ethanol	USA	Nov. 1997
A. stellatus	muscle	95% ethanol	Russia	Mar. 1998
A. gueldenstaedtii	muscle	formalin	Russia	Oct. 1997
Huso huso	muscle	95% ethanol	Russia	Mar. 1998
H.dauricus	muscle	95% ethanol	Heilongjiang River	Jun. 1998
Psephurus gladius	muscle/blood	cryopreservation	Chongqing	May 1997
Polyodon spathula	muscle/blood	cryopreservation	USA	Dec. 1996

1.2 Isolation of total DNA

For the samples preserved in ethanol, they should be washed in STE (10 mmol/L Tris-HCl, 1 mol/L EDTA, pH=8.0) three to four times for about 20 h. After that, DNA was isolated according to the routine method[8]. In the case of the samples preserved in formalin, the isolation of total DNA was carried out according to the method of Xiao et al.[9].

1. 3 Primer sequences and PCR amplification

Regions amplified covered the mtDNA *ND4L* gene and part of *ND*4 gene. Two pairs of primers were used and their sequences are as follows: Larg (L): 5'-AAGACCTCTGATTTCGGCTC-3'; ND4F2 (L): 5'-CTCCAAATCTAAACCTCCTAC-3'; ND4R2 (H); 5'-AACAGCATTAGTGTCGGGAT-3'; ND4H (H): 5'-CCTCATCGTGTGATAATAAT-3'.

The polymerase chain reaction (PCR) was performed in total reaction volume of 50 μL. PCR amplification was carried out using the following cycling parameters: one cycle of pre-denaturation at 94℃ for 3 min, 40 cycles of denaturation at 94℃ for 1 min, primer annealing at 52℃ for 45 s and extension at 72℃ for 1 min with the final extension at 72℃ for 8 min after 40 cycles.

DNA sequencing was performed on ABI 377 automated sequencer after purification of PCR products.

1.4 Data analysis

DNA sequence alignment was carried out using the DNA star software package. UPGMA and NJ trees were constructed by using the MEGA software package based on the Kimuar-2-Parameter genetic distances. Construction of MP tree using heuristic search method was made using software PAUP 3.1.1.

2 Results

2.1 Sequence variations of mtDNA *ND4L* and *ND4* genes

A total of 703 bp-length *ND4L* and *ND4* genes were sequenced. The contents of A, T, G, C are 26.9%, 24.5%, 34.9% and 13.7% respectively. The content of A + T is almost the same as that of G + C. However, the content of G is higher than that of C. The ratio of transitions to transversions is 4.4 ∶ 1. A total of 166 variation sites were detected in the 703 bp-length fragment, accounting for about 23.6% of the total DNA sequenced. Fig. 1 is the aligned sequences of *ND4L* and *ND4* genes of 12 species of Acipenseriformes.

```
                                                                                          90
01   TGTGGCTCAAATCCACAGAGACTTAATGACCCATATCATATTCACCTTCTCAACCGCCTTTATACTAGGATTATCAGGCCTAACTTTCAA
02   TATGGTGCAAGTCCATAGTCGTCTTATGACCCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGATTAACCTTCCA
03   TATGGTGCAAGTCCATAGTCGTCTTATGACCCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGATTAACCTTCCA
04   TATGGTGCAAGTCCATAGTCGTCTTATGACCCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGATTAACCTTCCA
05   TATGGTGCAAGTCCATAGTCGTCTTATGAACCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGACTAACCTTCCA
06   TATGGTGCAAGTCCATAGTCGTCTTATGAACCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGACTAACCTTCCA
07   TATGGTGCAAGTCCATCGTCGTCTTATGACCCCAGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATGGGATTAACCTTCCA
08   TATGGTGCAAGTCCATAGTCGTCTTATGACCCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGATTGACCTTCCA
09   TATGGTGCAAGTCCATAGTCGTCTTATGACCCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGATTAACCTTCCA
10   TATGGTGCAAGTCCATAGTCGTCTTATGACCCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGATTAACCTTCCA
11   TATGGTGCAAGTCCATAGTCGTCTTATGACCCCTGTACACTTCAGCTTCAGCTCCGCCTTCATGTTAGGACTAATAGGATTAACCTTCCA
12   TATGGTGCAAGTCCATCGTCGTCTTATGACCCCAGTACACTTCAGCTTCAGCTCTGCCTTCATACTAGGACTTATAGGATTGACCTTCCA
13   TATGGTGCAAATCCATAGTCGTCTTATGACCCCAGTACACTTCAGCTTCAGCTCCGCCTTCATACTAGGACTTATGGGACTAACCTTTCA
                                                                                         180
01   TCGCACTCACCTACTCTCTGCCCTGCTATGTTTAGAGGGTATAATATTATCCTTATTTATTGCCCTAGCAATATGATGCACCCAAAATGA
02   CCGAACCCACCTCCTCTCTGCCCTTCTCTGCCTAGAAGGAATGATACTATCTTTGTTTATTGCCCTCTCCCTCTGATCCCTTCAATTAGA
03   CCGAACCCACCTCCTCTCTGCCCTTCTCTGCCTAGAAGGAATAATACTATCTTTATTTATTGCCCTCTCCCTCTGATCCCTTCAACTAGA
04   CCGAACCCACCTCCTCTCCGCCCTTCTCTGCCTAGAAGGAATGATACTGTCTTTATTTATCGCCCTCTCCCTCTGATCACTTCAACTAGA
05   CCGAACCCACCTCCTCTCTGCCCTTCTCTGCCTAGAAGGAATAATACTATCTTTATTTATTGCCCTCTCCCTCTGATCCCTTCAACTAGA
06   CCGAACCCACCTCCTCTCTGCCCTTCTCTGCCTAGAAGGAATAATACTATCTTTATTTATTGCCCTCTCCCTCTGATCCCTTCAACTAGA
07   CCGAACCCACCTCCTCTCTGCCCTTCTCTGCCTAGAAGGAATAATACTATCTCTATTTATCGCCCTCTCCCTCTGATCCCTTCAACTAGA
08   CCGAACCCACCTCCTCTCCGCCCTTCTCTGCCTAGAAGGAATGATACTATCTCTATTTATCGCCCTCTCCCTCTGATCCCTTCAACTAGA
09   CCGAACCCACCTCCTCTCCGCCCTTCTCTGCCTAGAAGGAATGATACTATCTCTATTTATCGCCCTCTCCCTCTGATCCCTTCAACTAGA
10   CCGAACCCACCTCCTCTCTGCCCTTCTCTGCCTAGAAGGAATGATACTATCTTTGTTTATTGCCCTCTCCCTCTGATCTCTTCAACTAGA
11   CCGAACCCACCTCCTCTCCGCCCTTCTCTGCCTAGAAGGAATAATACTATCTTTATTTATCGCCCTCTCCCTCTGATCCCTTCAACTAGA
12   TCGAACCCACCTTCTCTCTGCCCTCCTCTGCCTAGAAGGAATAATATTATCCCTATTTATTGCCCTCTCCCTCTGGTCCCTCCAATTAGA
13   CCGAACCCACCTTCTCTCTGCCCTTCTCTGCCTAGAAGGAATAATACTATCTCTATTTATTGCTCTCTCCCTCTGGTCCCTTCAATTAGA
```

Fig. 1

(*To be continued on the next page*)

(Continued)

```
                                                                                              270
01   GACTATAATATTCTCTTCCGCCCCCCTACTATTACTAGCCCTCTCGGCGTGCGAAGCAGGCCTCGGCCTAAGCCTACTTGTCGCCACTGC
02   ATCTACCACCTACGCCACCGCCCCAATACTGCTACTAGCATTCTCAGCATGCGAGGCCGGAGCGGGCTTGGCCCTCCTTGTAGCTACTAC
03   ATCTACTACCTACGCCACCGCCCCAATACTGCTACTAGCATTCTCAGCATGTGAAGCCGGGGCAGGCTTGGCCCTCCTTGTAGCTACTAC
04   ATCCACCGCCTACGCCACCGCCCCAATACTGCTACTAGCATTCTCGGCATGTGAAGCCGGAGCAGGCTTGGCCCTCCTTGTAGCTGCCAC
05   ATCTACTACTTACGCCACCGCCCCGATACTGCTACTAGCATTCTCAGCATGTGAAGCCGGAGCGGGCTTGGCCCTCCTTGTAGCTACTAC
06   ATCTACTACCTACGCCACCGCCCCGATACTGCTACTAGCATTCTCAGCATGTGAAGCCGGAGCGGGCTTGGCCCTCCTTGTAGCTACTAC
07   ATCTACTACCTACGCCACCGCCCCAATACTGCTACTAGCATTCTCAGCATGTGAAGCTGGGGCAGGCTTGGCCCTCCTTGTCGCTACTAC
08   ATCCACCACCTACGCCACCGCCCCAATACTGCTACTAGCATTCTCGGCATGTGAAGCCGGAGCAGGCTTGGCCCTCCTTGTAGCTACCAC
09   ATCCACCACCTACGCCACCGCCCCAATACTGCTACTAGCATTCTCGGCATGTGAAGCCGGAGCAGGCTTGGCCCTCCTTGTAGCTACCAC
10   ATCTACCACCTACGCCACCGCCCCAATACTGCTACTAGCATTCTCAGCATGTGAGGCCGGAGCAGGCTTGGCCCTCCTTGTAGCTACTAC
11   ATCCACCACCTACGCCACCGCCCCAATACTGCTACTAGCATTCTCGGCATGTGAAGCTGGGGCAGGCTTGGCCCTCCTTGTAGCTACCAC
12   ATCAACCACCCATGCCACCGCCCCCATACTCCTACTTGCATTTTCGGCATGCGAAGCCGGAGCAGGCCTGGCCCTCCTCGTTGCCGCCAC
13   ATCAACTACCTATGCCACCGCCCCCATACTCCTACTAGCATTTTCAGCATGCGAAGCTGGAGCAGGCCTGGCCCTCCTTGTCGCCACCAC
                                                                                              360
01   CCGCGCCCACGGGTCTGATCACCTTCAAAACCTTAACCTCCTACAATGTTAAAACTACTAATTCCAACCATTATACTATTCCCAATAATT
02   ACGTACACACGGCACAGACCACCTCCAAAATCTAAACCTCCTACAATGCTAAAAATTATAATCCCGACACTAATGCTGTTCCCAACGACT
03   ACGCACACACGGCACAGACCACCTCCAAAATCTAAACCTCCTACAATGCTAAAAATTATAATCCCAACACTAATGCTATTCCCAACGACT
04   GCGTACACACGGCACAGACCACCTCCAAAATCTAAACCTCCTACAATGCTAAAAATTTTAATCCCAACACTGATGCTATTCCCAACGACC
05   GCGTACGCACGGCACAGACCACCTCCAAAATCTAAACCTCCTACAATGCTAAAAATTATAATTCCAACACTAATGCTATTCCCAACGACC
06   GCGTACGCACGGGACAGACCACCTCCAAAATCTAAACCTCCTACAATGCTAAAAATTATAATTCCAACACTAATGCTATTCCCAACGACC
07   ACGCACACACGGCACAGATCACCTCCAAAATCTAAACCTCCTACAATGCTAAAAATTATAATCCCAACACTAATGCTATTCCCAACGACT
08   ACGTACACATGGCACAGACCACCTCCAAAATCTAAATCTCCTACAATGCTAAAAATTATAATCCCGACACTAATGCTATTTCCAACGACC
09   GCGTACACATGGCACAGACCACCTCCAAAATCTAAATCTCCTACAATGCTAAAAATTATAATCCCAACACTAATGCTATTTCCAACGACC
10   ACGTACACACGGCACAGACCACCTCCAAAATCTAAACCTCCTACAATGCTAAAAATTATAATCCCAACACTAATGCTATTCCCAACGACT
11   ACGTACACACGGGACAGACCACCTCCAAAATCTAAATCTCCTACAATGCTAAAAATTTTAATCCCAACGCTAATGCTATTCCCAACGACC
12   GCGAACGCACGGCACAGATCACCTCCAAAACCTAAACCTACTACAATGCTAAAAATCTTAATCCCGACATTAATGCTATTCCCCACAACA
13   ACGCACACACGGCACAGATCACCTCCAAAACCTAAACCTGCTACAATGCTAAAAATTTTAATCCCGACACTAATGCTGTTTCCCACAACA

                                                                                              450
01   TGGACACTAAATCCTAAATGATTGTGATCAGCTACCACAACACATAGCTTAATTATTGCTTCCTTATCTCTCACACTATTTAAATGCTAC
02   TGACTTGTAACCCCAAAATGACTTTGAACCACAACAACAGCCCAGGCCCTAGTTATTGCCACCGCAAGCCTGACTTTACTTAACTGAAAT
03   TGACTTGTAACCCCAAAATGACTTTGAACCACAACAACAGCCCAAGCCCTAGTCATTGCCACCGCAAGCCTGACCTTACTTAACTGAAAT
04   TGACTTGCAACCCCAAAATGACTTTGAACCACAACAACAGCCCAAGCCTTGACCATTGCCACCGCAAGCCTGACCCTACTTAACTGAAAC
05   TGACTTGTGACCCCAAAGTGACTTTGAACCACAACAACAGCCCAAGCCCTAGTCATTGCCACCGCAAGCCTAACCCTACTTAACTGAGAC
06   TGACTTGTGACCCCAAAGTGACTTTGAACCACAACAACAGCCCAAGCCCTAGTCATTGCCACCGCAAGCCTAACCCTACTTAACTGAGAC
07   TGACTCGTAACCCCAAAATGACTTTGAACCACAACAACAGCCCAAGCCCTAGTCATTGCCACCGCAAGCCTGACCTTACTTAACTGAAAT
08   TGACTTGCAACCCCAAAATGACTTTGAACCACAACAACAGCCCAAGCCCTAGTCATTGCCACCGCAAGCCTGACTCTACTTAACTGAAAC
09   TGACTTGCAACCCCAAAATGACTTTGAACCACAACAACAGCCCAAGCCCTAGTCATTGCCACCGCAAGCCTGACTCTACTTAACTGAAAC
10   TGACTTGTAACCCCAAAATGACTTTGAACCACAACAACAGCCCAGGCCCTAGTCATTGCCACCGCAAGCCTGACTTTACTTAACTGAAAT
11   TGACTTGTGGCCCCAAAATGACTTTGAACTACAACAACAGCCCAAGCCCTAGTCATTGCCACCGCAAGCCTGACTCTACTTAACTGAAAC
12   TGACTTGCAACACCAAAATGACTATGAACCACAACAACAGCCCAAGCCTTAATCATTGCCACCGCAAGCCTAACCCTACTCAACTGGAGC
13   TGAATCACAACCCCAAAATGACTGTGAACCACAATAACAGCCCAAGCCTTAGCCATTGCTACCGCAAGCCTAACCCTGCTTAACTGAGAC
                                                                                              540
01   TCCACAACCCAATGATCAAATCTTAATTATATACTAGCCACCGACATAATCTCCACCCCTTTAATTATCCTAACCTGCTGACTACTTCCA
02   TCAGAAACCGGTTGAACCTCCTCAAACCCCTACCTAGGCACAGACCCCTTATCCACTCCCCTACTCGTCTTGACATGCTGACTTCTCCCC
03   TCAGAAACCGGTTGAACCTCCTCAAACCCCTACCTAGGCACGGACCCCCTCTCCACCCCCCTGCTCGTCTTGACATGCTGACTTCTCCCC
04   TCAGAAACCGGCTGAACCTCCTCAAATCCCTACCTGGGCGCAGACCCACTCTCCACACCCCTGCTCGTCTTAACATGCTGACTTCTCCCC
05   TCAGAAACCGGTTGAGCCTCATCAAACCCCTACCTAGGCACGGACCCCCTCTCCACCCCCCTGCTCGTCTTAACATGCTGACTTCTCCCC
06   TCAGAAACCGGTTGAGCCTCATCAAACCCCTACCTAGGCACGGACCCCCTCTCCACCCCCCTGCTCGTCTTAACATGCTGACTTCTCCCC
07   TCAGAAACCGGTTGAACCTCCTCAAACCCCTACCTAGGCACGGACCCCCTCTCCACCCCCCTGCTCGTCTTGACATGCTGACTACTCCCC
08   TCAGAAACCGGTTGAACCTCCTCAAACCCCTACCTGGGCACAGACCCGCTCTCCACACCCCTACTCGTCTTGACATGCTGACTTCTCCCC
09   TCAGAAACCGGTTGAACCTCCTCAAACCCCTACCTGGGCACAGACCCGCTCTCCACACCCCTACTCGTCTTGACATGCTGACTTCTCCCC
10   TCAGAAACCGGTTGAACCTCCTCAAACCCCTACCTAGGCACAGACCCCCTATCCACCCCCCTACTCGTCTTGACATGCTGACTTCTCCCC
11   TCAGAAACCGGTTGAACCTCCTCAAACCCCTACCTGGGCACAGACCCGCTCTCCGCACCCCTACTCGTCTTAACGTGCTGACTTCTCCCC
12   TCAGAAACTGGCTGAACTTCCACTAACCCCTACCTAGGCACGGATCCACTCTCCACCCCCCTACTAGTCCTAACATGCTGACTACTACCC
13   TCGGAAACTGGTTGAACTTCCACCAACCCCTACCTGGGCACAGATCCGCTCTCCACCCCTCTGCTCGTCCTAACATGCTGACTACTACCC
```

Fig. 1

(To be continued on the next page)

(*Continued*)

```
                                                                                           630
01  CTAATAATTATTGCTAGCCAAAACCATATATCCACGGAACCCATCAACCGACAACGAAGTTACATTACACTACTAGTATCCCTGCAAGCC
02  TTAATAATTCTAGCAAGCCAAAACCACATCTCCCCAGAACCAATCAGCCGCCAACGAACCTACATTACCCTACTAGTGTCCCTCCAACTA
03  TTAATGATCCTAGCAAGCCAAAACCACATCTCCCCGGAACCAATCAGCCGCCAACGAACCTACATCACCCTACTAGTGTCCCTCCAGCTA
04  TTAATAATTCTAGCAAGCCAAAACCACATCTCCCCAGAGCCTATTAGCCGCCAACGAACCTACATCACCCTACTAGTATCCCTCCAACTA
05  CTAATGATCCTAGCAAGCCAAAACCACATCTCCCCAGAACCAATCGGCCGCCAACGAACCTACATCACCCTACTAGTGTCCCTCCAACTA
06  CTAATGATCCTAGCAAGCCAAAACCACATCTCCCCAGAACCAATCGGCCGCCAACGAACCTACATCACCCTACTAGTGTCCCTCCAACTA
07  TTAATGATCCTAGCAAGCCAAAACCACATCTCCCCGGAACCAATCAGCCCCCAACGAACCTACATCACCCTACTAGTGTCCCTCCAGCTA
08  TTAATAATTCTAGCAAGCCAAAACCACATCTCCCCAGAACCAATCAGCCGCCAACGAACCTACATCACCCTACTAGTGTCCCTCCAACTA
09  TTAATAATTCTAGCAAGCCAAAACCACATCTCCCCAGAACCAATCAGCCGCCAACGAACCTACATCACCCTACTAGTGTCCCTCCAACTA
10  TTAATAATCCTAGCAAGCCAAAACCACATCTCCCCAGAACCCATCAACCGCCAACGAACCTACATCACCCTACTAGTGTCCCTCCAACTA
11  TTAATAATTCTAGCAAGCCAAAACCACATCTCCCCAGAACCAATCAACCGCCAACGAACCTACATTACCCTACTAGTGTCCCTCCAACTA
12  CTAATGATTCTAGCAAGCCAAAACCACATTTCTCCAGAGCCCGTTAGTCGTCAACGAACCTACATCACCCTTCTAGTGTCTCTCCAACTA
13  CTAATAATTCTAGCAAGCCAAAACCATATCTCCCCAGAACCCATTAATCGCCAACGAACCTACATTACCCTTCTAGTGTCCCTCCAGCTG
                                                                        703
01  CTACTTATTATAGCCTTCAGCGCCACAGAAATTATTTTATTTTATATTATATTTGAATCCACCTTAATCCCTA
02  TTTTTAATTATAGCCTTCGGAGCCACCGAAATTATCCTGTTCTACATTATGTTCGAAGCCACACTAATCCCAA
03  TTTTTAATTATAGCCTTCGGGGCCACCGAAATTATCCTGTTCTATATCATGTTTGAGGCCACACTAATCCCAA
04  TTCCTAATTATAGCCTTTGGGGCCACCGAAATCATCCTGTTCTATATCATGTTTGAAGCCACACTAATCCCGA
05  TTTTTAATTATAGCCTTCGGAGCCACCGAAATTATCCTATTCTACATCATGTTTGAAGCCACACTAATCCCAA
06  TTTTTAATTATAGCCTTCGGAGCCACCGAAATTATCCTATTCTACATCATGTTTGAAGCCACACTAATCCCAA
07  TTCTTAATTATAGCCTTCGGGGCCACCGAAATTATCCTGTTCTATATCATGTTTGAGGCCACACTAATCCCAA
08  TTCCTAATTATAGCCTTTGGGGCCACCGAAATCATCCTATTTTATATCATATTTGAAGGCACACTAATCCCAA
09  TTCCTAATTATAGCCTTTGGGGCCACCGAAATCATCCTATTTTATATCATATTTGAAGCCACACTAATCCCAA
10  TTTTTAATTATAGCCTTCGGAGCCACCGAAATTATCCTATTCTACATTATGTTCGAAGCCACACTAATCCCAA
11  TTCCTAATTATAGCCTTCGGGGCCACCGAAATTATCCTGTTCTACATCATGTTTGAGGCCACACTAATCCCAA
12  TTCTTAATTATAGCCTTCGGGGCCACCGAAATCATCTTATTCTACATCATATTTGGAACCACACTAATCCCCA
13  TTCTTAATCATAGCCTTCGGGGCCACCGAAATTATTTTATTTTATATCATATTTGAAGCCACACTGATCCCCA
```

Fig. 1. Sequence alignment of 703 bp segment from *ND4L-ND4* genes of 12 species of Acipenseriformes. The DNA sequences from line 1 to line 13 present *P. ornatipinnis*, *A. medirostris*, *A. schrenckii*, *A. stellatus*, *A. sinensis*, *A. dabryanus*, *A. gueldenstaedtii*, *A. brevirostrum*, *A. naccarii*, *H. dauricus*, *H. huso*, *P. spathula* and *P. gladius*, respectively.

2.2 Genetic divergences among 12 species of Acipenseriformes

Among 12 species of Acipenseriformes the greatest genetic divergence has taken place between either species of Polyodontidae and any of ten species of Acipenseridae in the range of 11.2%—14.2%. Among the 10 species of Acipenseridae the genetic divergences range from 0.3% to 7.7%. The smallest divergence occurs between *A. sinensis* and *A. dabryanus* with a value of 0.3%. The greatest genetic divergence, 7.7% was found in the following species pairs: *A. stellatus-A. sinensis*, *A. stellatus-A. dabryanus* and *A. stellatus-A. gueldenstaedtii* with 7.7% percent divergence. The genetic divergence between two species in genus *Huso* is within the range from 0.3% to 7.7%. No difference was detected between genus *Huso* and *Acipenser* according to the genetic divergences. The greatest divergence occurs between *P. spathula* and *P. gladius* at the level of 10.2%, which belong to different genera (table 2).

2. 3 Construction of molecular phylogenetic trees of 12 species of Acipenseriformes

The counterpart sequence was selected from Bichir (*Polypterus ornatipinnis*) as an outgroup for molecular phylogenetic analysis. The NJ and UPGMA molecular phylogenetic trees were constructed, as shown in figs. 2 and 3 by using the MEGA software package. MP tree was set up when transversions were weighted five times transitions using PAUP software (fig. 3). Bootstrap values after 1 000 replicates for MP and UPGMA trees were provided.

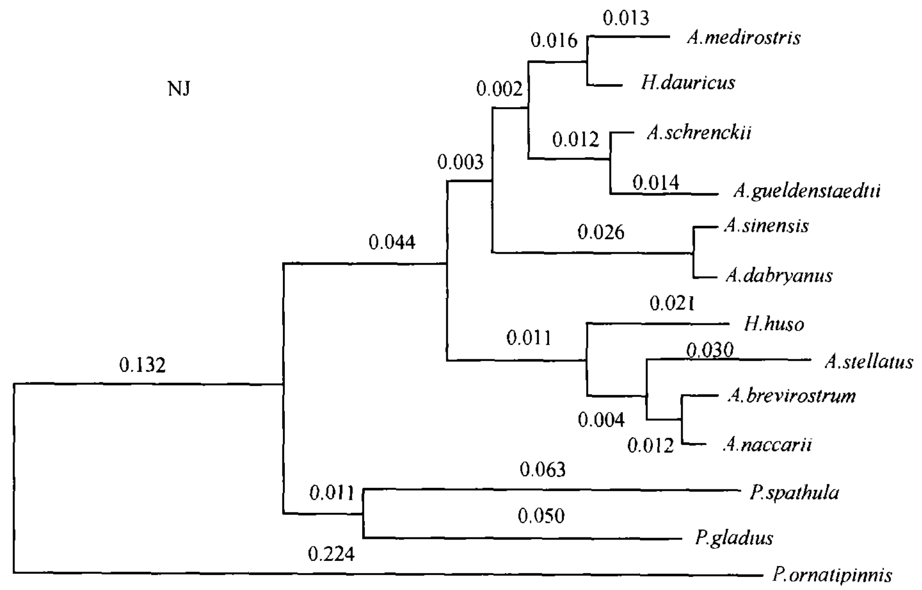

Fig. 2. NJ molecular phylogenetic tree of 12 species of Acipenseriformes based on mtDNA *ND4L-ND4* gene sequence. Branch lengths are indicated at each branch.

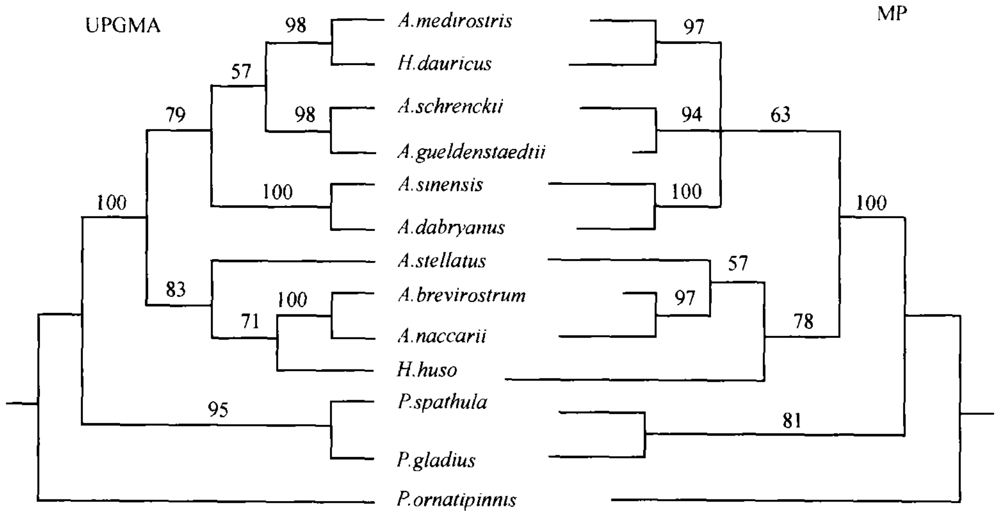

Fig. 3. UPGMA and MP molecular phylogenetic trees of 12 species of Acipenseriformes based on mtDNA *ND4L-ND4* gene sequences. The number at each node is Bootstrap values after 1 000 replicates.

Table 2 Genetic divergence and similarity between different species

Species	A	B	C	D	E	F	G	H	I	J	K	L
A. medirostris (A)		95.7	91.7	93.9	93.9	93.9	94.0	94.0	98.0	93.7	83.9	85.5
A. schrenckii (B)	4.1		92.9	95.9	95.9	98.3	94.5	94.9	96.7	94.7	85.5	86.8
A. stellatus (C)	7.7	6.5		91.6	91.7	91.6	95.0	95.6	92.5	94.0	85.9	86.3
A. sinensis (D)	5.8	4.0	7.7		99.7	94.2	93.0	93.7	94.9	93.3	85.5	86.5
A. dabryanus (E)	5.8	1.7	7.7	0.3		94.2	93.0	93.7	94.9	93.6	85.5	86.8
A. gueldenstaedtii (F)	5.8	1.7	7.7	5.7	5.7		93.5	93.9	94.9	93.9	85.3	87.6
A. brevirostrum (G)	5.7	5.4	4.7	6.8	6.8	6.3		99.4	94.6	95.9	86.2	87.3
A. naccarii (H)	5.7	5.1	4.1	6.3	6.3	6.0	0.6		94.9	96.3	86.2	87.5
H. dauricus (I)	2.0	3.3	7.0	5.0	5.0	5.0	5.3	5.0		94.3	84.5	85.9
H. huso (J)	6.0	5.1	5.5	6.5	6.3	6.0	4.0	3.7	5.5		84.5	86.6
P. spathula (K)	14.2	13.4	12.7	13.2	13.2	13.2	12.8	12.8	13.9	14.2		89.3
P. gladius (L)	13.1	12.1	13.1	12.7	12.7	11.2	11.8	11.8	13.1	12.7	10.2	

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

3 Discussion

3. 1 Regarding whether *Huso* can exist as an independent genus

That *H. huso* and *H. dauricus* belong to genus *Huso* is a classical conception that appears in all journals and books. But, whether *Huso* could exist as a genus separated from *Acipenser* was unclear to many ichthyologists in the 19th century, who considered it as a subgenus of *Acipenser*. Recently, Findeis provided additional characteristics separating *Huso* from all other extant Acipenseridae[10]. More recently, cytogenetic and molecular genetic studies indicated that the two species of *Huso* should be ascribed to *Acipenser*[4,5], which provoked the debate.

In the present study UPGMA, NJ and MP trees based on mtDNA *ND4L* and *ND4* gene sequences show that the ten species of Acipenseridae are divided into two clades. *H. huso* and *H. dauricus* do not cluster together, but nest within each branch. The genetic divergence between two species of *Huso* is much bigger than that between *Huso* and *Acipenser*. Judging by genetic difference the divergence of 10.2% is found between two genera (*Polyodon* and *Psephurus*) of Polyodontidae. No species pair in Acipenseridae reaches such value. It suggests that divergences among the ten species of Acipenseridae do reach the divergence at level of genus differentiation in the same evolutionary course. In other words, the ten specie of Acipenseridae should belong to the same genus. The highest divergence of 7.7% in the ten species of Acipenseridae is found in *A. stellatus——A. sinensis, A. stellatus——A. dabryanus* and *A. stellatus-schrenckii*, species pair from all *Acipenser*. But, such data are not found between *Huso* and *Acipenser*. From this point of view, since *A. sinensis, A. stellatus, A. dabryanus* and *A. schrenckii* were accepted to belong to *Acipenser* (with no doubt), we must accept that the two species of *Huso* should belong to *Acipenser*. Therefore, the genus *Huso* should be abolished and ascribed to genus *Acipenser*. We can provide more evidence concerning the close relationship between the *huso* and *Acipenser*. The following hybrids between the species from *Huso* and the species from *Acipenser* are not only viable, but also fertile: *H. huso*×*A. ruthenus*, *H. Huso*×*A. stellatus*, *H. huso*×*A. nudiventris*, *H. dauricus*×*A. schrenckii* (data provided by Dr. Artyukhin).

3.2 *A. daybryanus* is most likely the landlocked form of *A. sinensis*

In this work 12 species are involved, including all the six species inhabiting in East China waters. They are *H. dauricus*, *A. schrenckii*, *A. medirostris* and the three species endemic to Yangtze River. The latter three species are *A. sinensis*, *A. dabryanus* and *P. gladius*, which are under first rank protection in China. Regarding the relationship of *A. dabryanus* to other species of *Acipenser*, there exist different viewpoints. Artyukhin (1995) pointed out that *A. dabryanus* and *A. sinensis* were the most closely related to another from biogeographic point of view[11]. However, the systematic relationship proposed by other workers was that *A. sinensis* and *A. dabryanus* are not the closely related species[5,12]. Our present data derived from random amplified polymorphic DNA (RAPD)[7] and mtDNA D-loop sequence analysis (unpublished data) strongly support that the *A. sinensis* and *A. dabryanus* are the most closely related to another and that divergence time was not early. We agree to Artyuhkin's viewpoint. It is most likely that the *A. dabryanus* is the landlocked form of *A. sinensis*. The formation might take place at early time when *A. sinensis* adapted to the ecological condition which had changed in different parts of the Yangtze River or some of its population was blocked in some branches of Yangtze River or its neighboring water bodies. We deduced that *A. sinensis* had a body size similar to or smaller than that of *A. dabryanus* nowadays before divergence had taken place. In fishes, especially in anadromous fishes such as salmons and trouts, it is very common that one species possessed two forms, the landlocked and the anadromous. Usually, the body size of landlocked form is smaller than that of anadromous form. As a matter of fact, the morphological characteristics of *A. dabryanus* are very similar to those of *A. sinensis* and the body size of former is much smaller than that of the latter when both reach maturity. In addition, DNA content analysis suggested that both were at same ploidy level (8n)[13]. Hybrid between two species produced progeny not only viable, but also fertile[14].

3.3 Systematic relationship analysis of the order of Acipenseriforme

The molecular phylogenetic tree of the 12 species was divided into two branches, one including two species of family Polyodontidae, and the other containing 10 species of family Acipenseridae. This agrees with the classical classification. The genetic differences between *P. spathula* and *P. gladius* belonging to different genera are much larger than those between any two species pairs within Acipenseridae. The ten species of Acipenseridae are further divided into two groups, one comprising *H. huso*, *A. stellatus*, *A. brevirostrum* and *A. naccarii*, and the other possessing *A. sinensis*, *A. dabryanus*, *A. schrenckii*, *A. gueldenstaedtii*, *A. medirostris* and *H. dauricus*. All the species of Acipenseridae distributed in trans-Pacific region clustered together, with the exception of *A. gueldenstaedtii*, which is not from trans-Pacific region. It suggests that the species of Acipenseridae in trans-Pacific region might have common origin.

3.4 MtDNA *ND*4 and *ND*4*L* genes are ideal genetic markers for phylogenetic study of

Acipenseriformes

Some studies revealed that genes such as mtDNA cytochrome *b* gene and rRNA gene were not ideal genes for phylogenetic study in some taxa, especially in sturgeons and paddlefishes. The genes possess a limited number of informative sites and are not enough to determine the systematic relationships. Therefore, it is important to seek for new gene(s) for phylogenetic study in the order of Acipenseriformes. More recent studies in other taxa showed that the evolution rate of mtDNA *ND4L* and *ND*4 genes is at moderate level, and might be ideal genetic markers for evolutionary study[6,7]. However, no reports on fish were available. In this report we have sequenced 703 bp-length fragment that covers *ND4L* gene and part of *ND*4 gene. The three molecular phylogenetic trees, namely UPGMA, NJ and MP are consistent with each other, and high bootstrap values after 1000 replicates were noted. Therefore, the mtDNA *ND4L* and *ND*4 genes are ideal genes for molecular phylogenetic study in the order of Acipenseriformes.

Acknowledgements The authors are extremely grateful to all colleagues who provided them with the samples: Dr. E. N. Artyukhin, Central Lab for Fish Reproduction (Russia), Dr. B. Kynard, Conte Anadromous Fish Research Center (USA) and Dr. F. Fontana, University of Ferrara (Italy). They also thank Prof. Chao Wenxuan for his valuable suggestions. This work was supported by the National Natural Science Foundation of China (Grant No. 39600112) and Cellular and Molecular Lab of Kunming Institute of Zoology, the Chinese Academy of Sciences.

References

1. Bemis, W. E., Findeis, E. K., The sturgeon's plight, Nature, 1994, 370: 602.
2. Bemis, W. E., Findeis, E. K., Grande, L., An overview of Acipenseiformes, Envl. Fish Biol., 1997, 48: 25.
3. Zhang, Y. P., From DNA sequences to organism tree, Zoological Res. (in Chinese), 1996, 17: 247.
4. Birstein, V. J., Hanner, R., Desall, R., Phylogeny of the Acipenseriformes: cytogenetic and molecular approaches, Envl. Fish Biol., 1997, 48: 127.
5. Birstein, V. J., Desalle, R., Molecular phylogeny of Acipenserinae, Mol. Phylogent Evol., 1998, 9(1): 141.
6. Arevalo, E., Davis, S. K., Site, J. W., Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosomes races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico, Syst. Biol., 1994, 43: 387.
7. Forstner, M. R. J., Davise, S. K., Arevalo, E., Support for the hypothesis of Anguimorph ancestry for the suborder serpents from phylogenetic analysis of mitochondrial sequences, Mol. Phylogenet. Evol., 1995, 4: 93.
8. Zhang, S. M., Deng, H., Wang, D. Q. et al., Systematic relationships among seven species of Acipenseriformes inferred from random amplified polymorphic DNA, Progress in Natural Sciences (in Chinese), 1999, 9 (9): 818.
9. Xiao, W. H., Wu, Q. H., Su, B. et al., DNA extracted from formalin-fixed Xenocypris yunnanensis and sequence analysis of its cytochrome b gene, Zoological Res. (in Chinese), 1997, 18(3): 242.
10. Findeis, E. K., Osteology and phylogenetic interrelationships of sturgeons (Acipenseridae), Envl. Fish Biol., 1997, 48: 73.
11. Artyukhin, E. N., On biologeography and relationships within the genus Acipenser, The Sturgeon Quarterly, 1995, 3(2): 6.
12. Bemis, W. E., Kynard, B., Sturgeon river: an introduction to acipenseriformes biogeography and life history, Envl. Fish Biol., 1997, 48: 167.
13. Zhang, S. M., Yan, Y., Deng, H. et al., Genome size and ploidy characteristics of several species of sturgeons and paddlefishes with comments on cellular evolution of Acipenseriformes, Acta Zoologica Sinica (in Chinese), 1999, 45(2): 200.
14. Zhuang, P., Ke, F., Wei, Q. et al., Biology and life history of Dabry's sturgeon, *Acipenser dabryanus*, in the Yangtze River, Envl. Fish Biol., 1997, 48: 257.