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Speciation and Phylogeography of *Opsariichthys bidens* (Pisces: Cypriniformes: Cyprinidae) in China: Analysis of the Cytochrome *b* Gene of mtDNA from Diverse Populations

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Gao-Yan Li, Xu-Zhen Wang, Ya-Hui Zhao, Jie Zhang, Chun-Guang Zhang, and Shun-Ping He (2009) Speciation and phylogeography of *Opsariichthys bidens* (Pisces: Cypriniformes: Cyprinidae) in China: analysis of the cytochrome *b* gene of mtDNA from diverse populations. *Zoological Studies* **48**(4): 569-583. The cyprinid fish *Opsariichthys bidens* Günther is distributed in all major river systems of continental East Asia, and represents an attractive model for phylogeographic studies among cyprinid species or within a given species. In this study, we investigated the phylogeographic and demographic history of this species, using partial sequences of the cytochrome (cyt) *b* gene in mitochondrial (mt)DNA. Fish samples were collected from almost all major river systems where *O. bidens* is distributed in China. Sequence analysis showed remarkably high polymorphism, with 125 haplotypes in the 234 specimens examined, and with 89.8% of haplotypes occurring in only 1 specimen. A neutrality test indicated that some groups were not at mutation-drift equilibrium, suggesting a past population expansion. These results were supported by a mismatch distribution analysis. Based on our analysis, *O. bidens* consists of 4 groups belonging to 2 clades. The divergence time of the 2 clades was estimated to be 11.06-8.04 my. This value corresponds to the time of the 2nd uplift of the Qinghai-Tibet Plateau, the emergence of the East Asian monsoon, and the Epoch-6 Event. A two species scheme is proposed.
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Key words: Demographic history, Genetic divergence, Mismatch distribution analysis, Molecular clock, Neutrality test.

Cyprinid fishes represent the most extensive freshwater fish family, the Cyprinidae, which consist of more than 340 genera and more than 2000 species (Banarescu et al. 1991, Nelson 2006). They naturally occur in almost all types of aquatic habitats on all continents except for Australia and South America (Banarescu et al. 1991, Howes 1991). These fish species are a major component of the primary freshwater fish fauna of Eurasia. In China, this family is found in almost all freshwater habitats and is the largest freshwater fish family, with more than 132 genera, and 532 species and subspecies (Chen 1998). *Opsariichthys bidens* is one of the most widespread cyprinid species in East Asia. It is distributed in all major river systems in Mainland China (except for the Qinghai-Tibet Plateau and Xinjiang Uygur Autonomous Region), as well as in the Korean Peninsula and Vietnam's Red River (Chen 1998). It was once divided into *O. uncirostris amurensis* Berg,1932 and *O. uncirostris bidens* Günther, 1873 according to the numbers of lateral line scales and gill rakers (Wu et al. 1964). But a statistical analysis of lateral line scales revealed that their numbers decrease

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with the fish's distribution from north to south, which was considered to be related to temperature gradients. Hence, they were then considered to be a monotypic species (Chen 1982). This treatment has been widely accepted. But recent molecular biological approaches have revealed a more-complicated scenario. Perdices et al. (2005) showed that populations of *O. bidens* in the Yangtze, Pearl, and Hai River systems seemed to be mixtures of 5 species. Berrebi et al. (2006) suggested that there were at least 3 subspecies in *O. bidens* or even 3 species within the complex in the Yangtze and Pearl River systems. Similar situations also occur for other cyprinid species. As pointed out by Su et al. (2001), such broadly distributed species might simply not represent single taxa. Moreover, phylogeographic studies have revealed multiple mitochondrial lineages inside different cyprinid taxa from China's major drainages (Xiao et al. 2001, Perdices et al. 2004). However, there is also other molecular systematics research of the Opsariichthines (Wang et al. 2007), which tentatively concluded that there was only 1 species within the genus *Opsariichthys*, with *O. bidens* and *O. uncirostris* retained as 2 local populations of *O. uncirostris*.

Obviously, some important relations within *O. bidens* remain unclear. A further study is required for a robust phylogeography encompassing more samples distributed over the river systems of East Asia. Due to its wide distribution in East Asia, analyses of the phylogenetic and demographic history of *O. bidens* across the major river systems of China could shed light on historical connections of major river systems in Asia. Such analyses would also be helpful in understanding the evolutionary history of other cyprinid fishes. The aims of the present study were to clarify the longstanding debate on the taxonomy of *O. bidens* and to study the phylogeography and demographic history among its diverse populations. The cyt *b* gene is a useful marker for these purposes. It is probably the best-known mitochondrial gene with respect to structure and function of its protein product (Esposti et al. 1993). Some work has been done on the *O. bidens* cyt *b* gene (Perdices et al. 2005). We used the cyt *b* gene as a genetic marker for our comprehensive study.

MATERIALS AND METHODS

Sampling

Samples were collected from all major habitats of *O. bidens* in Mainland China. In total, 98 specimens were collected from 26 localities from 2000 to 2006 (Table 1, Fig 1). All specimens were first identified in the field, and then confirmed in the laboratory.

Selection of outgroups

Opsariichthys is considered to be most closely related to the genera *Zacco*, *Parazacco*, and *Candidia* in the family Cyprinidae (Chen 1982). *Opsariichthys bidens* lives in sympatry with *Z. platypus* in almost all localities, and these 2 species are very similar in gross morphology, except for the shape of the mouth (*O. bidens* has

Table 1. Summary of sample information. GenBank accession numbers of the cytochrome *b* genes are provided

Locality (no.)	Haplotype	Counts	Group (or subgroup)	Species-voucher	GenBank accession no.
Amur R. (1)	H42	1	B2	132593	FJ602011
	H46	1	B2	132602	FJ602014
	H48	1	B2	132601	FJ602013
	H50	1	B2	132600	FJ602012
Tumen R. (2)	H41	1	B2	132591	FJ601948
Liao R. (3)	H46	1	B2	132612	FJ602015
	H49	1	B2	132613	FJ602016
Hai R. (4)	H39	1	B2	BE68-1	AY646619[a]
Hai R. (5)	H46	1	B2	73121	FJ601924
	H47	1	B2	73124	FJ601925
Hai R. (6)	H40	1	B2	BE66-1	AY646618*[a]

Table 1. (Cont)

Locality (no.)	Haplotype	Counts	Group (or subgroup)	Species-voucher	GenBank accession no.
Yalu R. (7)	H43	1	B2	60020	FJ602010
	H44	1	B2	60022	FJ601950
	H45	1	B2	60023	FJ601951
	H58	1	B2	60018	FJ601949
	H59	1	B2	60024	FJ601952
	H62	1	B2	60025	FJ601953
Biliu R. (8)	H55	1	B2	40093	FJ601919
	H56	1	B2	40095	FJ601921
	H57	1	B2	40094	FJ601920
	H60	1	B2	40096	FJ601922
	H61	1	B2	40099	FJ601923
Huai R. (9)	H51	7	B2	139547, 139548, 139549, 139550, 139553, 1395554, 139556	FJ601926, FJ601927, FJ601928, FJ601929, FJ601930, FJ601931, FJ601932
	H52	1	B2	139558	FJ601933
Yellow R. (10)	H63	4	B1	40336, 40338, 4033839, 4033840	FJ601954, FJ601955, FJ601956, FJ601957
Qing R. (11)	H116	2	A1	E12, E14	FJ601967, FJ601968
	H118	1	A1	E13	FJ601969
Han Shui (12)	H53	1	B2	X11	FJ601997
	H110	1	A1	X12	FJ601998
Li Shui (13)	H79	1	A2	LI16-3	AY646530[a]
	H103	1	A1	LI16-2	AY646529[a]
	H105	1	A1	LI16-5	AY646532[a]
	H111	1	A1	LI16-1	AY646528[a]
	H113	1	A1	LI16-4	AY646531[a]
	H114	1	A1	LI16-6	AY646533[a]
Li Shui (14)	H78	1	A2	LI20-5	AY646538[a]
	H103	6	A1	LI20-1, -3, -4, -6, -7, -8	AY646534, AY646536, Y646537, AY646539, AY646540, AY646541[a]
	H104	1	A1	LI20-2	AY646535[a]
Yuan R. (15)	H103	1	A1	YU11-5	AY646559[a]
	H113	3	A1	YU11-1, -2, -4	AY646555, AY646556, AY646558[a]
	H117	1	A1	YU11-3	AY646557[a]
Yuan R. (16)	H81	2	A1	YU14-5, -6	AY646564, AY646565[a]
	H98	1	A1	YU14-2	AY646561[a]
	H99	1	A1	YU14-7	AY646566[a]
	H100	1	A1	YU14-3	AY646562[a]
	H102	1	A1	YU14-1	AY646560[a]
	H103	1	A1	YU14-4	AY646563[a]
	H106	1	A1	YU14-9	AY646568[a]
	H113	1	A1	YU14-8	AY646567[a]
Yuan R. (17)	H54	1	B2	T23	FJ601993
	H103	1	A1	T26	FJ601996
	H113	1	A1	T25	FJ601994
	H115	1	A1	T22	FJ601995
Yuan R. (18)	H95	1	A1	YU2-4	AY646545[a]
	H103	2	A1	YU2-1, -2	AY646542, AY646543[a]
	H113	2	A1	YU2-3, -5	AY646544, AY646546[a]
	H119	1	A1	YU2-6	AY646547[a]
Yuan R. (19)	H94	1	A1	YU6-1	AY646548[a]
	H107	1	A1	YU6-4	AY646551[a]

Table 1. (Cont)

Locality (no.)	Haplotype	Counts	Group (or subgroup)	Species-voucher	GenBank accession no.
	H108	1	A1	YU6-3	AY646550[a]
	H113	3	A1	YU6-5, -6, -7	AY646552, AY646553, Y646554[a]
	H117	1	A1	YU6-2	AY646549[a]
Yongning R. (20)	H80	1	A2	YU54-1	AY646569[a]
Zi Shui (21)	H97	1	A1	ZI29-2	AY646513[a]
	H101	4	A1	ZI29-1, -4, -5, -6	AY646512, AY646515, Y646516, AY646517[a]
	H113	1	A1	ZI29-3	AY646514[a]
Zi Shui (22)	H88	1	A1	ZI32-5	AY646522[a]
	H93	1	A1	ZI32-2	AY646519[a]
	H112	4	A1	ZI32-7, -8, -9, -10	AY646524, AY646525, Y646526, AY64652-7[a]
	H113	4	A1	ZI32-1, -3, - 4, -6	AY646518, AY646520, Y646521, AY646523[a]
Xiang R. (23)	H92	1	A1	XIA37-1	AY646574[a]
Xiang R. (24)	H84	1	A1	XIA7-4	AY646573[a]
	H85	1	A1	XIA7-1	AY646570[a]
	H86	1	A1	XIA7-2	AY646571[a]
	H87	1	A1	XIA7-3	AY646572[a]
Liu R. (25)	H82	4	A1	LIU14A-1, -2, -3, -4	AY646598, AY646599, AY646600, AY646601[a]
Li R. (26)	H69	8	A2	LIJ4-2, -3, -5, -6, -9, -10, -11, -13	AY646576, AY646577, Y646579, AY646580, AY646583, Y646584, AY646585, AY646587[a]
	H70	1	A2	LIJ4-12	AY646586[a]
	H72	2	A2	LIJ4-4, -8	AY646579, AY646582[a]
	H73	1	A2	LIJ4-7	AY646581[a]
	H74	2	A2	LIJ4-1, -14	AY646575, AY646588[a]
Jiulong R. (27)	H123	1	A2	Z12	FJ602006
	H124	2	A2	Z11, Z15	FJ602005, FJ602009
	H125	2	A2	Z13, Z14	FJ602007, FJ602008
Liu R. (28)	H82	3	A1	LIU9-4-6	AY646594, AY646595, AY646596[a]
	H89	1	A1	LIU9-3	AY646593[a]
	H90	1	A1	LIU9-2	AY646592[a]
	H91	1	A1	LIU9-1	AY646591[a]
Liu R. (29)	H83	1	A1	LIU10-1	AY646597[a]
Bei R. (30)	H120	2	A2	L22, L23	FJ601982, FJ601983
	H121	1	A2	L24	FJ601984
	H122	1	A2	L21	FJ601981
Hongshui R. (31)	H96	1	A1	HO16-1	AY646602[a]
Fuchuan R. (32)	H64	3	A2	FU54-1, -3, -6	AY646611, AY646613, AY646616[a]
	H65	1	A2	FU54-5	AY646615[a]
	H66	1	A2	FU54-4	AY646614[a]
	H67	1	A2	FU54-2	AY646612[a]
	H68	1	A2	FU54-7	AY646617[a]
You R. (33)	H23	5	D	X22, X23, X24, X25, X26	FJ602003, FJ602004, FJ602002, FJ601999, FJ602000
	H25	1	D	X21	FJ602001
Liu R. (34)	H82	3	A1	40255, 40256, 40257	FJ601941, FJ601942, FJ601943
Liu R. (35)	H82	2	A1	R14, R15	FJ601986, FJ601987
	H109	1	A1	R11	FJ601985

Table 1. (Cont)

Locality (no.)	Haplotype	Counts	Group (or subgroup)	Species-voucher	GenBank accession no.
Nanpan R. (36)	H17	4	C	40389, 40390, 40391, 40392	FJ601944, FJ601945, FJ601946, FJ601947
Gui R. (37)	H71	1	A2	GUI52-1	AY646610[a]
Liu R. (38)	H75	1	A2	J22	FJ601972
	H76	3	A2	J23-5	FJ601973, FJ601974, FJ601975
	H77	1	A2	J21	FJ601971
Hongshui R. (39)	H24	2	D	HO18-1, -7	AY646603, AY646609[a]
	H28	2	D	HO18-2, -4	AY646604, AY646606[a]
	H34	2	D	HO18-3, -5	AY646605, AY646607[a]
	H35	1	D	HO18-6	AY646608[a]
Hongshui R. (40)	H34	1	D	D15	FJ601966
	H36	1	D	D11	FJ601964
	H37	1	D	D14	FJ601965
You R. (41)	H26	1	D	YO22-2	AY646621[a]
	H27	1	D	YO22-6	AY646625[a]
	H28	1	D	YO22-1	AY646620[a]
	H31	1	D	YO22-4	AY646623[a]
	H32	1	D	YO22-3	AY646622[a]
	H33	1	D	YO22-5	AY646624[a]
Beiliu R. (42)	H1	1	C	BE50-1	AY646649[a]
You R. (43)	H22	3	D	YO24-1, -2, -4	AY646626, AY646627, AY646629[a]
	H23	1	D	YO24-3	AY646628[a]
Beiliu R. (44)	H1	1	C	BE46-5	AY646646[a]
	H2	2	C	BE46-1, -2	AY646642, AY646643[a]
	H7	1	C	BE46-3	AY646644[a]
	H8	1	C	BE46-4	AY646645[a]
	H9	1	C	BE46-7	AY646648[a]
	H10	1	C	BE46-6	AY646647[a]
Nanliu R. (45)	H7	1	C	B12	FJ601959
	H9	1	C	B13	FJ601960
	H11	1	C	B11	FJ601958
Ming R. (46)	H21	2	D	MI31A-1, -2	AY646630, AY646631[a]
Ming R. (47)	H21	3	D	MI33-2, -3, -5	AY646633, AY646634, AY646636[a]
	H29	2	D	MI33-1, -4	AY646632,5[a]
Ming R. (48)	H21	3	D	MI34-1, - 3, -4	AY646637, AY646639, AY646640[a]
	H30	1	D	MI34-2	AY646638[a]
	H38	1	D	MI34-5	AY646641[a]
Lancang R. (49)	H17	3	C	141174, 141176, 141177	FJ601938, FJ601939, FJ601940
	H20	2	C	141166, 141170	FJ601936, FJ601937
Lancang R. (50)	H19	1	C	J11	FJ601970
Lancang R. (51)	H17	1	C	141153	FJ601934
	H18	1	C	141154	FJ601935
Hainan (52)	H3	1	C	S16	FJ601988
	H4	1	C	S14	FJ601991
	H5	3	C	S12, S13, S17	FJ601989, FJ601990, FJ601992
Hainan (53)	H6	1	C	L15	FJ601980
	H15	3	C	L12, L13, L14	FJ601977, FJ601978, FJ601979
	H16	1	C	L11	FJ601976
Hainan (54)	H12	1	C	B24	FJ601962
	H13	1	C	B22	FJ601961
	H14	1	C	B26	FJ601963

[a]Indicates sequences from Perdices et al. (2005).

a clear zigzag form of the upper and lower jaws, which does not exist in *Z. platypus*). The cyt *b* gene sequences of these 2 species also share a very high similarity (Perdices et al. 2006). So we chose *Parazacco* and *Candidia*, but not *Zacco*, as outgroups. We also included *Leuciscus* as a more-distant cyprinid outgroup.

DNA extraction and sequencing of cyt *b* genes

DNA was extracted from fin tissue using a standard phenol: chloroform method (Sambrook et al. 2004). The partial cyt *b* gene was amplified by a polymerase chain reaction (PCR) using primers L14724 (5'-GACTTGAAAAACCACCGTTG-3') and H15915 (5'-CTCCGATCTCCGGATTACAAGAC-3') (Schmidt et al. 1993, Brito et al. 1997). For some cases we also used primers LCB1 (5'-AATGACT TGAAGAACCACCGT-3') and HA (5'-CAACGAT CTCCGGTTTACAAGAC-3') (Wang 2005b). The PCR was run in 60 μl reactions, each containing 10× buffer, 2.5 mM of the dNTP mixture, 20 μM of each primer, 1 μl of template DNA, and 1 unit Taq polymerase (TaKaRa Bio INC, Japan). The conditions were as follows: initial preheating at 92°C for 5 min, 92°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 70 s, and a final extension at 72°C for 10 min. In total, 30-35 amplification cycles were carried out on an Applied Biosystem GeneAmp® 9700 thermocycler (USA).

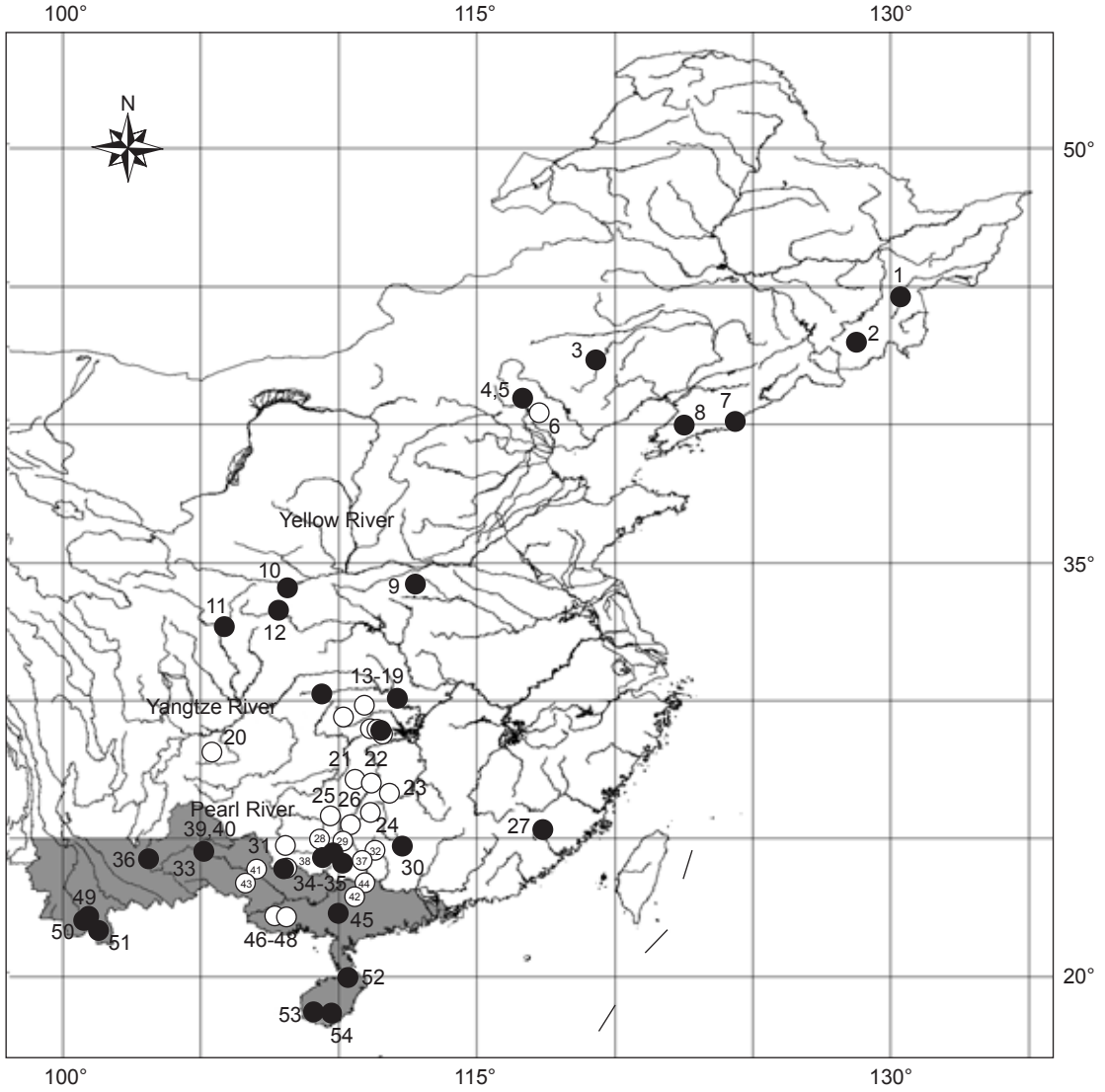

Fig. 1. Map of China showing the sampling sites. Black dots denote sites from where the authors collected fish samples, and circles denote Perdices et al.'s (2005) sampling sites. The shadowed area indicates clade 2 and other area includes clade 1.

The PCR products were precipitated in ethanol at -20°C for 2 h and then collected by centrifugation at 12,000 rpm for 10 min. The pellets were washed twice with 70% ethanol. Purified PCR products were sequenced at Invitrogen Corp. (USA). Vector NTI 10.0.1 software (Lu et al. 2004) was used to manually splice and edit the sequencing result. As there were some ambiguous nucleotides at the end of the sequences, we only used a region of 1071 bp that did not contain ambiguous nucleotides for further analysis. Sequences were aligned using Clustal X 1.83 (Thompson et al. 1997). Sites that showed nucleotide substitutions at the population level were re-examined by visual inspection of each individual's raw chromatographic data.

Phylogenetic analyses

Phylogenetic relationships of cyt *b* gene sequences for the 98 in-group specimens obtained were analyzed. From GenBank, 136 in-group sequences and 3 outgroup sequences (*Candidia barbatus*, AY958200, *Parazacco spilurus fasciatus*, AY958195, Wang et al. 2007; Wang H.Y., unpubl.; *Leuciscus leuciscus*, AY509823, Ketmaier et al. 2004) (Table 1; Perdices et al. 2005) were also included in the analysis. The total number of sequences was 237.

Hierarchical series of likelihood ratio tests were performed using Modeltest 3.7 (Posada et al. 1998) to identify the appropriate nucleotide substitution models. Phylogenetic trees for the complete dataset were analyzed by Neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods in PAUP* 4.0b10 (Swofford 1998). For the NJ analysis, the Tamura-Nei + G (0.2123) substitution model was used to calculate divergence distances. For the MP analysis, heuristic searches with TBR branch swapping were carried out, and 10 replicates of random addition of taxa were used; only the most-parsimonious trees were retained, and 0-length branches were collapsed. The weighting scheme of *ti/tv* = 10 was employed to adjust for transitional saturation. A bootstrap analysis was performed to assess the relative robustness of the NJ branches (with 1000 replicates) and MP trees (with 100 replicates).

The Tamura-Nei model with Gamma correction was used for the ML searches in PAUP* (Swofford 1998). PhyML (Guindon et al. 2003 2005) was employed to assess the relative robustness of the branch support in the ML tree with 1000 replicates of the bootstrap analysis. Bayesian inference (BI) was implemented with MrBayes 3.1.2 (Huelsenbeck et al. 2001, Ronquist et al. 2003). One million generations were run under the TrN + G model of evolution (print tree frequency every 100 generations, sample frequency every 100 generations, and starting tree burn-in of 1000 generations). Posterior probability values were used as support for the Bayesian topology.

Molecular diversity indices, including the numbers of haplotypes, polymorphic sites, transitions, transversions, and indels, were obtained using Arlequin 3.1 (Schneider et al. 2000). Haplotype diversity, nucleotide diversity, the mean number of pairwise differences, and the corresponding variances of all values were calculated following a gamma correction as implemented in Arlequin. Genetic distances were generated for the phylogenetic reconstruction with MEGA 3.1 (Kumar et al. 2004) using the model of Tamura et al. (1993). The among-site rate heterogeneity was corrected with the shape parameter of a gamma distribution.

Demographic history analysis

The population genetic structure was evaluated with F_{ST} statistics (Wright 1931). The TrN + G model selected by ModelTest was employed with a gamma correction of nucleotide substitutions for heterogeneity of mutation rates. The significance of the F_{ST} was tested by 10,000 permutations in Arlequin. The population structure and geographical pattern of population subdivision of *O. bidens* were further investigated using the analysis of molecular variance (AMOVA) software package in Arlequin (Schneider et al. 2000). Genetic differentiation between populations and taxa was estimated by calculating pairwise distances, which included information on haplotype frequency and molecular distance (TrN + G distance). For the hierarchical analysis, populations were grouped according to either the mtDNA lineages (n = 4) recovered in the phylogenetic analysis or by geography as populations (n = 54), tributaries (n = 30), and drainages (n = 15). The significance of the covariance components associated with the different possible levels of genetic structure was tested using 1000 permutations by Arlequin.

The demographic history of the populations was evaluated by a mismatch distribution analysis. Mismatch distribution is the distribution of the

number of mutation differences between pairs of sequences (Rogers et al. 1992). For populations that have recently undergone a demographic expansion, a unimodal and approximately Poisson-distributed mismatch distribution is expected, and the time when the population expansion began can be estimated. The expansion parameter (τ) and the mutation parameter before expansion were calculated with Arlequin 3.1 (Schneider et al. 2000). Harpending's raggedness index (Harpending 1994) and the sum of the squared deviation (SSD) between the observed and expected mismatches were used to test for the validity of the estimated expansion model. Two neutrality tests (Tajima's selective neutrality test and Fu's neutrality test) were used to check for deviation from neutrality. The Tajima test (Tajima 1989) calculates the value of D, a specifically developed statistic, and assesses its departure from 0. Zero is the value expected in the case of mutation-drift equilibrium. Events of population growth produce significant negative D-values (Aris-Brosou et al. 1996).

RESULTS

Molecular characterization of the cyt *b* gene

We analyzed 1071 bp of the cytochrome *b* gene for 98 *O. bidens* specimens. For the purpose of comparison with published data, 136 specimens from GenBank were also analyzed. It was found that the majority of variable (298) and parsimoniously informative sites (258) were 3rd position substitutions (80.87% and 87.21%, respectively). Similar nucleotide compositions were found across individuals with an anti-G bias (16.5%), which is typical for the mitochondrial genome (Cantatore et al. 1994). Plots of transitions and transversions against uncorrected pairwise distances indicated an absence of nucleotide saturation (Fig. 2). The TrN + G model was chosen for the NJ and ML analyses according to the ModelTest results: a transition/transversion ratio (*ti/tv*) of 10; base frequencies of A = 0.2708, C = 0.2950, G = 0.1417, and T = 0.2926; the number of estimated parameters of 6; substitution rates of A-C = 1.00, A-G = 22.83, A-T = 1.00, C-G = 1.00, C-T = 8.02, and G-T = 1.00; and a gamma distribution shape parameter of 0.2123.

Sequence divergence and partitioning

A 2 clade scheme was established in this study, which was strongly supported by bootstrapping (100%) and posterior probability values (80%) (Fig. 3), regardless of the phylogenetic reconstruction method used. Both clades showed high support (bootstrap > 97% and posterior probability > 78%) (Fig 3). Clade 1 consisted of 2 groups (A and B), and clade 2 also contained 2 groups (C and D). Within clade 1, group A was divided into 2 subgroups (A1 and A2) grouped in a monophyletic cluster with high bootstrap values (99, 100, and 85 for the respective NJ, MP and ML methods, and

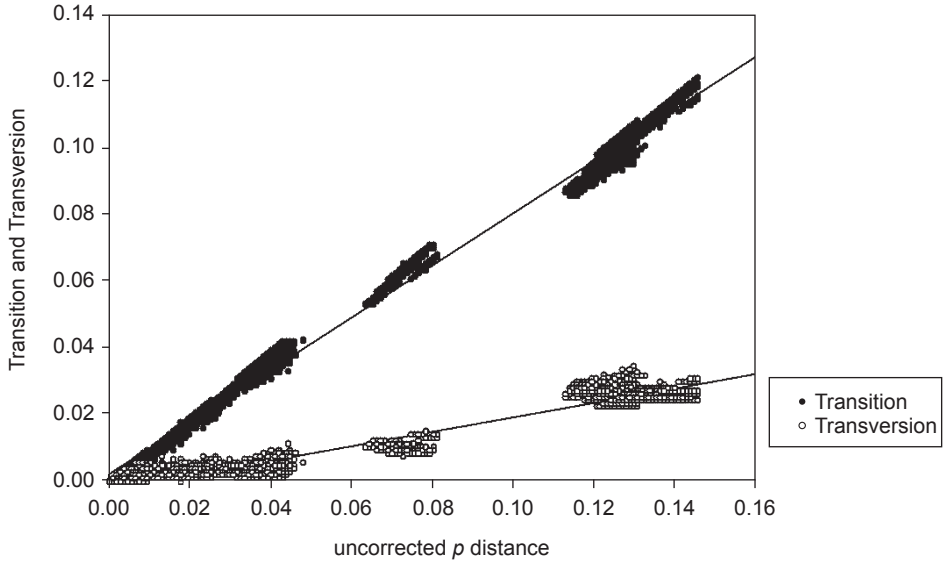

Fig. 2. Nucleotide substitution saturation analysis.

79 for posterior probabilities of the BI). Group B was divided into 2 subgroups (B1 and B2) grouped in a monophyletic cluster with high to moderate bootstrap values (76, 100, and 73 for the respective NJ, MP and ML methods, and 75 for posterior probabilities of the BI). Within clade 2, the 2 groups (C and D) were highly supported by all analyses (NJ, MP, ML of 100, and posterior probability of the BI of 78).

A closer examination of these groups revealed interesting geographical information. Subgroup A1 includes almost all individuals collected from the Yangtze River basin (collection localities 11-24) (Fig. 1), and some individuals from the Liu River (collection localities 25, 28, 29, 34, and 35), a tributary of the Pearl River system. Subgroup A2 consists of individuals from the Liu River (collection locality 38), Gui River (collection locality 37), He River (collection locality 32), and Bei River (collection locality 30) (all northern

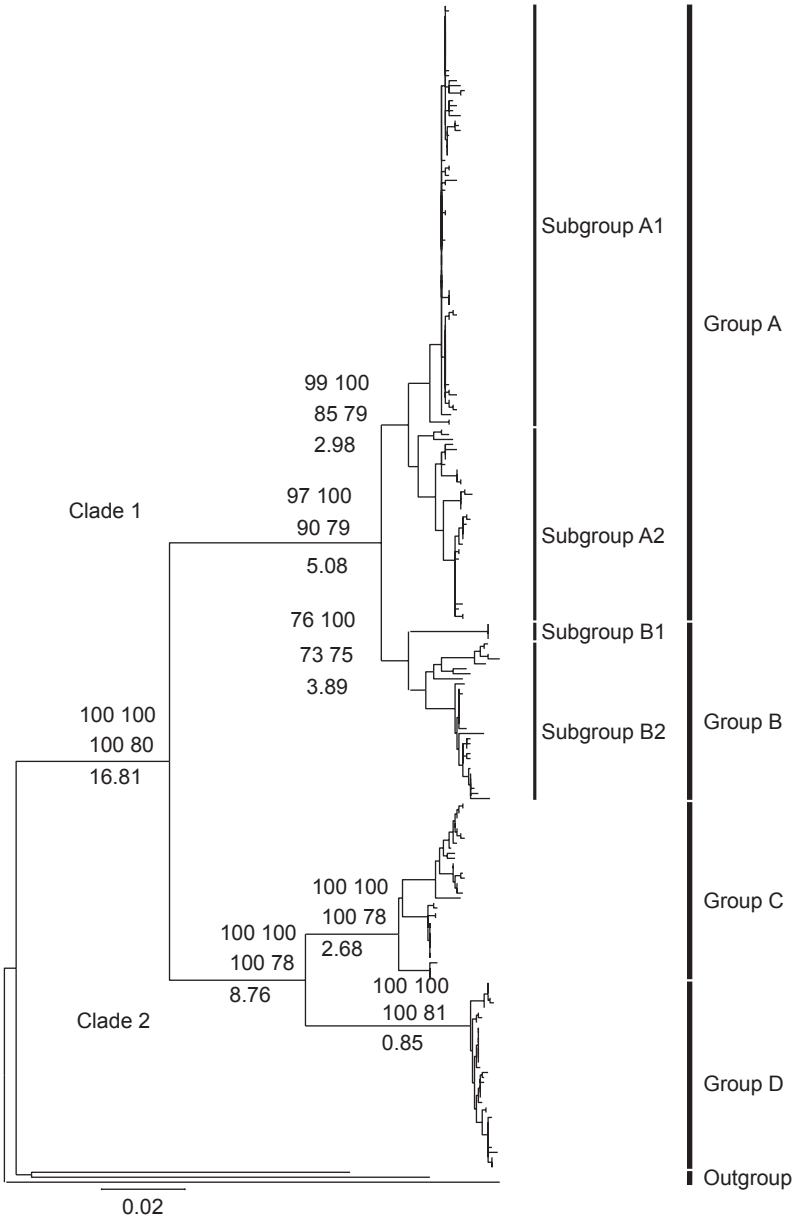

Fig. 3. Phylogenetic relationships of *Opsariichthys bidens* based on the cytochrome *b* gene sequences using Neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). Values below the branches are TrN + G distances. Numbers above the branches are (from top to bottom, from left to right) bootstrap values of 1000 replicates for NJ, 100 replicates for MP, 1000 replicates for ML, and posterior probability values for BI.

tributaries of the Pearl River system), Jiulong River (collection locality 27) (which directly drains into the Taiwan Strait), and 3 other specimens from the Yangtze River (collection localities 13, 14, and 20), each forming a haplotype. Subgroup B1 consisting of all individuals from the Yellow River (collection locality 10) formed only 1 haplotype. Subgroup B2 included all individuals from the Huai River (collection locality 9), Hai River (collection localities 4-6), Liao River (collection locality 3), Biliu River (collection locality 8), Yalu River (collection locality 7), Tumen River (collection locality 2), and Amur River (collection locality 1) (all to the north of the Yangtze River system). Group C included individuals from the Lancang River basin (collection localities 49-51), Nanpan River (collection locality 17), and Beiliu River (collection localities 42 and 44) (upper tributaries of the Xi River of the Pearl River system), Nanliu River (collection locality 45) (which directly drains into Beibu Bay of the South China Sea), and all individuals from Hainan I. (collection localities 52-54). Group D consisted of individuals collected from the Hongshui River (collection localities 31, 39, and 40), Zuo River (collection localities 46-48), and You River (collection localities 33, 41, and 43) (middle-lower reaches of the Xi River).

The genetic divergence between the 2 clades was 16.81%. Within clade 1, the between-group (A and B) genetic divergence was 5.08%, and within-group genetic divergences were 2.98% (group A) and 3.89% (group B). For the 2 groups (C and D) within clade 2, the between-group genetic divergence was 8.76%, and within-group genetic divergences were 2.68% (group C) and 0.85% (group D).

Phylogenetic implications

We obtained a total of 125 haplotypes among the 234 individuals of *O. bidens*, 98 from our collection and 136 from published research (GenBank). All haplotypes were drainage specific, except for H17 and H46 (Table 1). H17 was found in both the Nanpan and Lancang Rivers, and H46 was shared by the Amur, Liao, and Hai Rivers. Furthermore, all haplotypes found in a given locality were included in the same monophyletic lineage, except for those in localities 12, 14, 15, 18, and 27 (Table. 1). Although a large number of haplotypes are present in *O. bidens*, 89.76% of them occurred only once. The intra-population haplotype diversity ranged from 1.00 ± 0.50 to 0.00 ± 0.00. Nucleotide diversity ranged from 0.03 ± 0.02 (for the population of locality 12 of the Han-Shui River, a tributary of the Yangtze River) to 0.00 ± 0.00 (4 other populations, at localities 10, 26, 35, and 37). Analysis of the haplotype frequencies indicated that 54 different populations displayed high levels of genetic differentiation. A comparison of groups indicated that diversity was the lowest in group D (haplotype diversity of 0.93 ± 0.03 and nucleotide diversity of 0.00 ± 0.00), while the highest haplotype diversity was observed in group A (0.96 ± 0.01) and the highest nucleotide diversity was observed in group B (0.02 ± 0.00). If all samples were considered as a single population, the haplotype diversity and nucleotide diversity were 0.98 ± 0.00 and 0.07 ± 0.00, respectively.

No fossil record for *Opsariichthys* has yet been discovered to date, but there is a molecular clock rate of European cyprinids for cyt *b* (0.76% of divergence per pairwise comparison per million years (my)) (Zardoya et al. 1999). On the basis of geological events, a molecular clock of 1.3% was estimated for the freshwater fish *Luciobarbus* (Machordom et al. 2001). These molecular clocks are congruent with the estimate of 1.05% divergence/my calculated using fossil data by Dowling et al. (2002). This molecular clock (1.05%) was used to estimate the divergence dates for *Chondrostoma* (Durand et al. 2003, Doadrio et al. 2004) and *Squalius aradensis* (Mesquita et al. 2005). Using these studies as a reference and molecular clock calibration rates of 0.76%-1.05%/bp/my for *Opsariichthys*, we were able to estimate the divergence time of the 2 clades to be about 11.06-8.04 my ago (Ma).

Population structural characteristics

The results of the AMOVA showed significant genetic structure at all hierarchical levels examined (Table 2). Most of the mtDNA molecular variance could be attributed to differences among lineages (F_{CT} = 0.884), and it was assumed to be the most probable geographical subdivision. The observed molecular variation among *Opsariichthys* mtDNA lineages was 88.41% with an overall F_{ST} = 0.972, which suggests that the populations are highly structured within lineages, whereas 8.78% of differences were attributable to the variability among populations within groups, and only 2.81% reflected variability within populations.

The overall mismatch distribution was clearly multimodal, which indicates that, overall, populations of *O. bidens* are stable. The mismatch

distributions, however, significantly varied among lineages. The mismatch distributions within subgroup A1 and group D (Fig. 4) well fit the predicted distributions under a model of sudden expansion, but this did not apply to other groups. For subgroups A1 and group D, small values for Harpending's raggedness index (0.0190 and 0.0197, respectively) along with a lack of significance (0.994 and 0.704) further support a unimodal interpretation of the mismatch distributions in these groups.

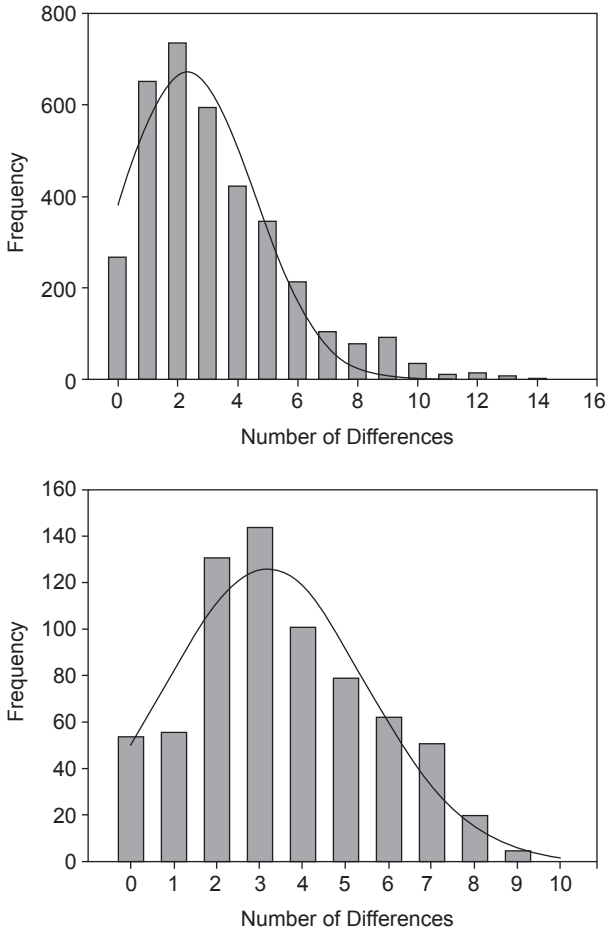

Fig. 4. Mismatch distribution of *Opsariichthys bidens* mitochondrial (mt)DNA lineages with unimodal distributions (upper for subgroup A1, lower for group D).

Both Tajima's D neutrality test (subgroup A1: D = -2.332, p = 0.000; group D: D = -0.984, p = 0.157) and Fu's neutrality test (subgroup A1: F_s = -26.320, p = 0.000, and group D: F_s = -7.698 with p = 0.003) gave negative values. This suggests that these populations have experienced demographic expansion.

The peak of the mismatch distribution provides an estimate of τ, the starting time of the expansion in units of $1/(2\ \mu)$ generations (where μ is the mutation rate per locus per generation). Its relationship with the absolute time in years (t_e) is $t_e = \tau/(2\ \mu_y)$ (Zane et al. 2006), where μ_y is the mutation rate per locus per year. The observed values of the age expansion parameter (τ) were 2.3343 for subgroup A1 and 3.1649 for group D. According to the above equation and the molecular clock of 0.76%-1.05%/bp/my, we calculated the starting time of the expansion to be at 1.5357-1.1116 Ma for subgroup A1 and 2.0822-1.5071 Ma for group D.

DISCUSSION

Speciation of *O. bidens*

There is a long-lasting debate on the speciation of *O. bidens*. According to the numbers of lateral line scales and gill rakers, Wu et al. (1964) proposed a 2 subspecies treatment: *O. uncirostris amurensis* Berg and *O. uncirostris bidens* Günther. But a statistical analysis of lateral line scales revealed that their numbers decrease with the fish's distribution from north to south, which was

Table 2. Analysis of molecular variance of populations of *Opsariichthys bidens*. Groups are rearrangements tested in the hierarchical analysis with significant values (p). Fixation indices include the total variance (F_{ST}), among-group variation (F_{CT}), and within-group variation among populations (F_{SC})

Group	F_{ST}	F_{CT}	F_{SC}	Among groups (%)	Among populations within a group (%)	Within a population (%)	p
mtDNA lineages	0.972	0.884	0.758	88.41	8.78	2.81	0 ± 0
Drainages	0.938	0.416	0.893	41.62	52.15	6.23	0 ± 0
Tributaries	0.932	0.871	0.475	87.08	6.13	6.79	0 ± 0
Populations	0.930			93		7	0 ± 0

considered to be related to temperature gradients. Hence, the 2 subspecies were considered to be only a monotypic species (Chen 1982). Recent analyses of molecular data led to multi-species schemes for populations in the Yangtze, Pearl, and Hai River basins (Perdices et al. 2005, Berrebi et al. 2006). In the present study, sampling was expanded to cover all major river systems in East China. According to the data for cyt *b* gene sequences, 2 major clades were identified, with a genetic distance of 16.81%, which is much higher than published values for many other fish species. For example, the value for intraspecific nucleotide sequence divergence is 4.5% for *Leuciscus peloponnensis* (from the Thiamis (England) and Alphios (Greece) Rivers; Zardoya et al. 1999), 4.6% for *Telestes pleurobipunctatus* (from the Arachthos River, Greece) and *T. p. alfiensis* (from the Alphios River) (Zardoya et al. 1999), and 6.3% for *L. leuciscus* (Costedoat et al. 2006). It is worth noting that our value is even higher than a few interspecific divergences. For example, values between *Barbus callensis* and other *Barbus* spp. are 8.3% with *B. bocagei*, 8.3% with *B. graellsii* and *B. guiraonis*, 7.5% with *B. steindachnerii*, 7.6% with *B. comizo*, 8.2% with *B. microcephalus*, and 8.5% with *B. sclateri* (Zardoya et al. 1999).

It seems that the current findings are not in agreement with a morphological study of *O. bidens* done in this laboratory (Li 2007). A meristic analysis of 28 characters did not show significant differences between the 2 clades proposed by the present work, although we did note some differences in the numbers of lateral line scales (40-48 vs. 36-43) and predorsal scales (16-22 vs. 13-17) between clades 1 and 2, which is consistent with Chen's (1982) results. However, it is quite common that the morphologies of closely related species do not necessarily differ. Morphological similarities among recently diverged sibling species can be explained by a lack of either proper selection on morphological characters or sufficient time for morphological divergence. But why are the 2 clades of *O. bidens* so similar morphologically although they diverged a long time ago (11.20-8.04 Ma, as estimated above)? A clue to this question can be found in similarities of both their diet and habitat selection. All *O. bidens* populations inhabit the upper reaches of river branches in mountains, and all feed mainly on fish, shrimp, and aquatic insects (Su et al. 1993). Among the groups within either clade, there are no clear morphological differences and little genetic differentiation. Judging from morphological characters and nucleotide cyt *b* data, we propose a 2 species scheme for the *O. bidens* complex in China, with the Nanling Mountains as a natural barrier between the 2 species. Perdices et al.'s (2005) mtDNA lineage study and Johansson's (2006) geometric morphometrical study are both helpful contributions to the study of *O. bidens*, but their conclusions were based on samples from limited distribution areas and population numbers.

A consideration of the demography history

Perdices et al.'s (2005) study, using a nested clade analysis and mismatch distribution method, suggested that there may be historical processes in the observed genetic differentiation, such as contiguous range expansion and long-distance colonization. But geological considerations were lacking, which otherwise can be very helpful in elucidating the differentiation of *O. bidens*. In our scheme, the 2 clades are divided by the Nanling Mountains, which are located in the region between southern Hunan-Jiangxi Provinces and northern Guangxi-Guangdong Provinces (111°-117°E and 23°20'-26°40'N, Fig. 1). The elevation of the mountains is about 1000 m on average, and the Nanling Mountains are the divide between the watersheds of the Yangtze and Pearl River systems (Shu et al. 2006).

According to records of early tertiary fossil fishes from the Sanshui Basin, Guangdong Province, China, 6 species of the Danioninae (Cyprinidae) were found, one of which is *Zacco honggangensis* (Wang et al. 1981). *Zacco* is a sister group of *Opsariichthys*. Fossils of *Zacco* were found in Japan from the Miocene (Wang 2005b). We speculated that *O. bidens* should have been distributed all over East China, at least before the Miocene (23 Ma), and clades 1 and 2 were not yet separated from each other at that time. Although the Nanling Mountains were formed far before 11.06-8.04 Ma (Ministry of Geology and Mineral Resources "Nanling Project" Special Group 1988), its elevation at that time might not have been as high as it is today for forming a barrier for *O. bidens*. Populations of *O. bidens* usually live in mountainous tributaries, where river capture might have occurred. The latter may potentially be a key geomorphological driver for range expansion and cladogenesis in freshwater taxa (Burridge et al. 2006), and the expansion and dispersal of *O. bidens* may have been promoted by river capture events. The rise of the Nanling Mountains was accelerated along

with the uplift of the Qinghai-Tibet Plateau (Ministry of Geology and Mineral Resources "Nanling Project" Special Group 1988). The Asian Modern Monsoon appeared 10-8 Ma (An et al. 2001) as a result of uplift of the Qinghai-Tibet Plateau, and the climate turned cool and dry. About 8-7 Ma, the temperature of the world rapidly decreased, in a period called the "Epoch-6 Event" (Sun et al. 1998, Shi et al. 1999). Rivers shrank as rainfall decreased, which would have greatly reduced the effects of river capture on the expansion and dispersal of *O. bidens*. As a result, populations of *O. bidens* may have been divided into 2 clades due to shrinking rivers and uplift of the Nanling Mountains.

The *O. bidens* populations distributed in northern tributaries (Liu, Gui, and Bei Rivers) of the Pearl River system are grouped into clade 1, and they are close to the populations distributed in southern tributaries of the Yangtze River, which is a very common pattern observed in many other cyprinids distributed in neighboring tributaries of these 2 river systems (Wang 2005a). River capture in these regions may have changed the water systems of the upper reaches of these rivers. Although there is no direct geological evidence, we can speculate that the upper reaches originally among southern tributaries of the Yangtze River were captured and became upper reaches of northern tributaries belonging to the Pearl River system. In this process, *O. bidens* entered the tributaries of the Pearl River system from the Yangtze River system.

Hydraulic engineering may be another factor affecting the distribution of *O. bidens*. The Ling Canal located in the Nanling Mountains of northern Guangxi Province was built more than 2,200 yr ago (Zhong 1990), and connects the Xiang River (a southern tributary of the Yangtze River) and the Li River (a northern tributary of the Pearl River). This connection may have played a major role in the expansion and dispersal of *O. bidens* between the Yangtze and Pearl River systems.

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