Study on phylogenetic relationship of freshwater planarians (Turbellaria: Tricladida: Paludicola) in nine Chinese localities using RAPD method☆

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Abstract

Objective. China is rich in freshwater planarian resource. In order to investigate phylogenetic relationships, Random Amplified Polymorphic DNA (RAPD) technique was applied to analyze RAPD pattern variations in planarians collected from nine Chinese localities, which belong to *Dugesia japonica* and *Seidlia sinensis*, respectively. *Methods.* Among 60 arbitrary primers (10 bp) under predetermined optimum reaction conditions, 27 primers were informative and yielded a total number of 314 clear and reproducible bands. The patterns show polymorphic variations among and within species. RAPD data were used to calculate Nei and Li similarity coefficient and genetic distance. Molecular systematic evolution tree was reconstructed by Nearest-Neighbor method via program SPSS 10.0. *Results.* The results showed that samples of *D. japonica* collected from eight localities clustered first, and then clustered with *S. sinensis*. In the clade of *D. japonica*, three branches are further divided. *Conclusion.* This clustering pattern coincides with the results drawn from the cytogenetical study, and also relates to their geographic distribution. [Life Science Journal. 2009; 6(2): 84 – 89] (ISSN: 1097 – 8135).

Keywords: freshwater planarian; RAPD; phylogenetic relationship; China

1 Introduction

Planarians are the earliest free-living platyhelminthes with triploblast and bilateral-symmetry, and also the important animal group which transits from water-inhabitation to land. Therefore, they play an important role in studying the systematic evolution of animals. Moreover, because of their powerful regenerative abilities, they are the ideal materials for studying the molecular mechanism of cell differentiation and redifferentiation. Researches on planarian have already been carried out to molecular level in Europe and the United States recently. For example, the molecular phylogenetics on the higher level were studied[1,2]. And the origin and evolution of planarian brain have been studied[3] and some genes about regeneration, such as Hox gene, Pax-6 gene have been cloned and expressed[4,5]. Though planarians distribute widely in China and the resources are rich, it is a pity that the study is weak in our country and few scientists are working in this field. Till now, we have just made some progress in Paludicola (Tricladida) and reported 14 species belonging to five genera, three families[6].

As a method for DNA fingerprinting based on the Polymerase Chain Reaction (PCR), the Random Amplified Polymorphic DNA (RAPD), which developed by Williams *et al*[7], Welsh & McClelland[8], and Welsh *et al*[9], is currently receiving particular attention. Because of its simplicity, it has been extensively used as a powerful tool for gene-mapping, population and pedigree analysis, phylogenetic study and bacterial strain identification[10–12]. The basic strategy involves the PCR amplification of random fragments of genomic DNA with single

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or multiple primers of arbitrary sequence. Polymorphism between individuals (or strains) is detected as differences between the patterns of DNA fragments amplified from the different DNAs using a given primer.

The taxonomic standard of planarian is mainly based on the morphology of copulatory organs[13]. This study used RAPD technique with enough random primers and the evidence of genome level for the phylogenetic and taxonomic research of freshwater planarians in nine Chinese localities was obtained.

2 Materials and Methods

2.1 Experimenal animals

The experimental planarians are collected from nine localities in China, which belong to *Dugesia japonica* (*D. joponica*) and *Seidlia sinensis* (*S. sinensis*), respectively (Figure 1 and Table 1).

Figure 1. The geographic distribution of samples.

2.2 Reagents

The reagents include Genomic DNA Minipreps kit, 10 bp random primers, TaqDNA polymerase, 10 × PCR buffer, 25 mmol/L $MgCl_2$, dNTP Mix, RNase A and Proteinase K. RNase A was from Beijing DingGuo Biotech. Co. Ltd. (Beijing, China). Proteinase K was from Qiagen. Other reagents were all from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

2.3 Preparation of DNA template

Genomic DNA was extracted with the genomic DNA Minipreps kit (Shanghai, China). Then DNA was resuspended in TE solution after being dried in air, keeping storage in 4 ºC for use.

Table 1. List of localities of samples

Locality	Code	Species
Yingtaogou, Beijing City	D-1	*Dugesia japonica*
Luanchuan County, Henan	D-2	*Dugesia japonica*
Hangzhou City, Zhejiang	D-3	*Dugesia japonica*
Stone-man Mountain, Henan	S-4	*Seidlia sinensis*
Lushan County, Henan	D-5	*Dugesia japonica*
Xiuwu County, Henan	D-6	*Dugesia japonica*
Yangcheng County, Shanxi	D-7	*Dugesia japonica*
Jiyuan City, Henan	D-8	*Dugesia japonica*
Xin County, Henan	D-9	*Dugesia japonica*

2.4 Amplification conditions

According to the pre-experiments, the optimum conditions are as follows: amplification reactions were performed in a total volumes of 25 μl, containing 1 × PCR buffer, 2.0 mmol/L $MgCl_2$, 0.2 μmol/L primer, 0.2 mmol/L dNTPs, 20 ng of DNA template and 1.0 U of Taq DNA polymerase. Samples were subjected to PCR in a Little Genious Thermal Cycler (Guangzhou Fangtong Biotech. Co. Ltd. Guangzhou) using 40 cycles of 1 min at 94 ºC, 1 min at 37 ºC, 2 min at 72 ºC with pre-denaturation at 94 ºC for 5 min before the first cycle and final extention at 72 ºC for 5 min after the last cycle[14].

2.5 Agarose gel electrophoresis

Amplification products were analyzed on a 1.5% agarose gel containing EB (ethidium bromide) with 1 × TAE buffer for 2.5 h under the condition of 5 v/cm and room temperature. Then the electropherograms photographed under GIS-1000 Gel Auto-phtotograph System.

2.6 Statistical analysis

Presence and absence of a given fragment that amplified from genomic DNA were represented by "1" and "0" characters, respectively. Clear or weak and reproducible bands were recorded as "1". No or weak and non-reproducible bands were recorded as "0". Thus a matrix of characters was obtained for phylogenetic analysis as initial data. According to the formula $S = 2Nxy/(Nx+Ny)$[15], the similarity coefficients were calculated, where Nxy is the number of similar bands between two samples. The coefficient allows a value of the similarity between any two samples. This analysis provided data about the genomic distances between different samples. The coefficient was used because it is recommended for routine computation of genetic similarities using RAPD data[16]. The coefficients gave similarity values in the range 0 to 1 and were converted to genetic distance (D) as $D = 1 - S$.

Cluster analysis was made for these nine samples and the molecular systematic tree was reconstructed by Nearest Neighbor method via SPSS 10.0.

3 Results

3.1 RAPD results

Nine samples were screened using 60 10-mer primers, 27 of them generated clear and reproducible fingerprintings interest. See Table 2 for sequences of these 27 primers and numbers of RAPD bands.

The total number of bands amplified with 27 primers is 314, and the average number of each primer is 11.6. Primers S10, S90 amplified the most bands, both are 15. Primers S13, S66 and S77 all amplified the fewest bands with the number of eight. Therefore, there is notable difference in amplification products among different primers. Figure 2 shows profiles obtained with primers S10 and S82, respectively.

3.2 Genetic distance and cluster analysis

To show genetic affinity, the genetic distances among these nine samples were calculated via the formula D = 1 – S, and the distance matrix was shown in Table 3. Figure 3 shows the dendrogram reconstructed through Nearest Neighbor method via SPSS 10.0.

4 Discussion

Researches on the molecular systematics of planarians

Table 2. List of 27 primer sequences and numbers of RAPD bands amplified with each primer for nine samples

Primer sequence	D-1	D-2	D-3	S-4	D-5	D-6	D-7	D-8	D-9	Total
S5 (5’-TGCGCCCTTC-3’)	0	3	4	2	3	2	1	2	6	10
S8 (5’-GTCCACACGG-3’)	2	3	5	4	6	2	1	3	5	12
S10 (5’-CTGCTGGGAC-3’)	2	5	7	5	6	8	5	4	5	15
S13 (5’-TTCCCCCGCT-3’)	0	3	3	2	5	1	2	1	2	8
S15 (5’-GGAGGGTGTT-3’)	3	1	3	3	3	4	4	3	4	12
S16 (5’-TTTGCCCGGA-3’)	1	2	2	3	3	1	2	2	5	10
S17(5’- GGGAACGAG-3’)	4	2	4	1	4	5	4	4	6	10
S18 (5’-CCACAGCAGT-3’)	4	8	6	5	3	7	6	5	3	12
S20 (5’-GGACCCTTAC-3’)	1	3	7	5	3	8	5	6	2	11
S62 (5’-GTGAGGCGTC-3’)	3	4	3	2	3	5	2	5	2	11
S64 (5’-CCGCATCTAC-3’)	3	4	4	2	4	6	4	5	3	11
S65 (5’-GATGACCGCC-3’)	4	3	5	5	3	8	10	8	2	14
S66 (5’-GAACGGACTC-3’)	2	2	2	3	3	4	4	2	3	8
S70 (5’-TGTCTGGGTG-3’)	4	4	6	4	4	5	3	4	5	11
S71(5’-AAAGCTGCGG-3’)	2	1	3	2	2	3	3	3	1	8
S75 (5’-GACGGATCAG-3’)	5	3	4	8	5	5	2	4	2	13
S78 (5’-TGAGTGGGTG-3’)	3	2	6	5	3	3	4	4	3	12
S79 (5’-GTTGCCAGCC-3’)	3	1	3	5	4	3	3	3	5	11
S80 (5’-ACTTCGCCAC -3’)	4	4	3	3	3	4	5	2	5	12
S82(5’-GGCACTGAGG-3’)	2	5	5	6	7	3	5	6	3	13
S83 (5’-GAGCCCTCCA-3’)	3	1	7	5	5	5	4	5	2	12
S84 (5’-AGCGTGTCTG-3’)	4	6	6	5	6	7	5	5	3	12
S85(5’-CTGAGACGGA-3’)	3	0	4	5	5	6	5	6	5	13
S88 (5’-TCACGTCCAC-3’)	4	5	3	4	6	4	5	4	3	12
S89 (5’-TGCCCAGCCT-3’)	4	5	8	5	4	4	7	8	5	14
S90 (5’-AGGGCCGTCT-3’)	3	4	3	5	3	7	8	6	3	15
S93 (5’-CTCTCCGCCA-3’)	7	7	7	3	7	6	6	6	8	12
Total	80	91	123	105	115	126	115	116	101	314

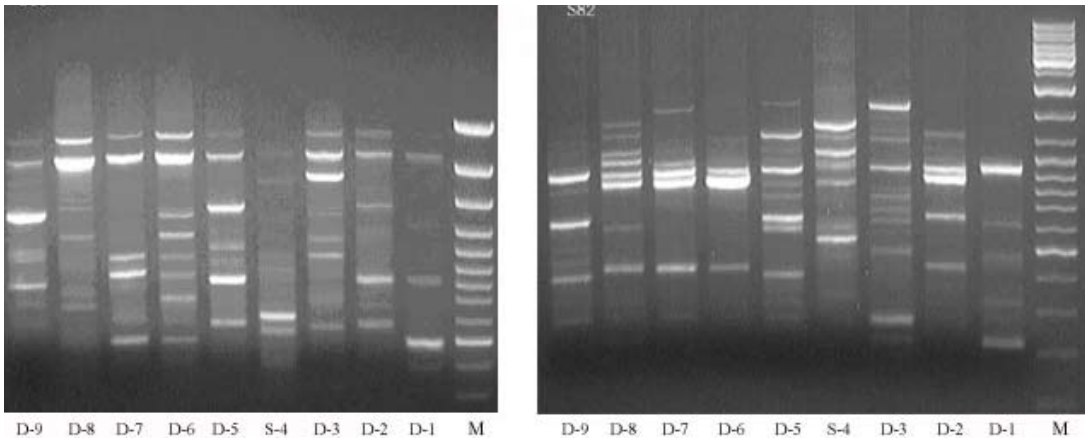

Figure 2. Electrophoresis patterns of RAPD products of freshwater planarian from nine localities in China (primer S10, S82, respectively). M: Molecular size marker, Gene RulerTM 100 bp DNA Ladder Plus.

Table 3. Genetic distance between freshwater planarians from nine localities in China

	D-1	D-2	D-3	S-4	D-5	D-6	D-7	D-8	D-9
D-1	0								
D-2	0.4791	0							
D-3	0.6355	0.5607	0						
S-4	0.6865	0.6429	0.6228	0					
D-5	0.6615	0.4369	0.6303	0.7545	0				
D-6	0.6602	0.5853	0.5422	0.5844	0.6515	0			
D-7	0.6821	0.5340	0.5798	0.6182	0.6087	0.2863	0		
D-8	0.5816	0.5362	0.5732	0.6199	0.6450	0.3471	0.3247	0	
D-9	0.6133	0.5000	0.6429	0.7184	0.4074	0.6476	0.6296	0.6959	0

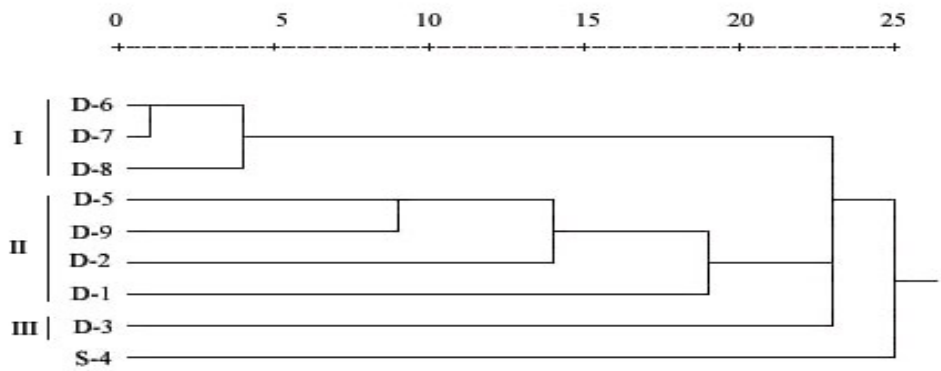

Figure 3. Dendrogram reconstructed for freshwater planarians from nine localities in China.

using RAPD method have not been reported in China yet. In this paper, we studied the phylogenetic relationship among nine samples belonging to *D. japonica* and *S. sinensis*, respectively, using RAPD technique.

There are two clades in the dendrogram. The first clade can be divided into three branches. In branch I, D-6 (from Xiuwu County, Henan Province) and D-7 (from Yangcheng County, Shanxi Province) clustered first, and then clustered with D-8 (from Jiyuan City, Henan Province). In branch II, D-2 (from Luanchuan County, Henan Province) clustered with D-5 (from Lushan County, Henan Province) and D-9 (from Xin County,

Henan Province) after the latter two clustering together, then they three clustered with D-1(from Beijing City). At last, branch I and II clustered with branch III (D-3 from Hangzhou City, Zhejiang Province), forming the first clade of *D. japonica*. Then the first clade clustered with the second clade of S. sinensis (from the Stone-man Mountain, Henan Province). Populations of the same species are closer in relationship than those belonging to different genera, which indicate indirectly that the taxonomic positions of these samples are correct at least at genus level.

The clustering pattern within *D. japonica* also coincides with their geographic distribution. Xiuwu County, Yangcheng County and Jiyuan City are close in latitude, belonging to Taihang Mountain Area. As all of them belong to Palearctic realm in fauna, they are similar to a great extent and planarians from these three localities are close in relationship. Lushan County, Luanchuan County and Xin County belong to Funiu Mountain Area and Dabie Mountain Area, respectively. They are close in latitude, and lower than the former three. They belong to the transitional area of Palearctic region to Oriental region, so the planarians in these three areas are close. Beijing City belongs to Palearctic region, higher in latitude. Hangzhou City belongs to subtropical zone, Oriental region in the fauna, lower in latitude. So the populations from these two cities are remotest in relationship than to the other six populations.

Furthermore, the results from cytogenetics also support this RAPD analysis. In branch II, *D. japonica* from Lushan County, Xin County and Luanchuan County are all triploid, having 24 chromosomes. And *D. japonica* with the karyotype formula of 2n=2x=16=16m forming branch III. In branch I, the cluster results also coincide with cytogenetical results, but there is a little disagreement. The karyotype formulae of planarians from Xiuwu County are 2n=2x=16=16m and 2n=3x=24=24m, while that of planarians from Yangcheng County is 2n=3x=24=3m+15sm+3st+3T. Those from Jiyuan City are 2n=3x=24=3m+18sm+3st (more), and 2n=2x=16 (fewer)[17]. While S. sinensis possesses 42 chromosomes which differentiate greatly from those of *D. japonica* in quantity and shape, constructing the second clade of the dendrogram.

From above, it is confirmed that the relationships of different populations of *D. japonica* are closer than those between Dugesia and Seidlia. Our research, at genome level, supports the results drawn from morphology and cytogenetics. Meanwhile, it also indicates that RAPD technique holds considerable promise for the population genetic, systematic evolution and classification of freshwater planarians.

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