J Mol Evol (2007) 65:660–667
DOI 10.1007/s00239-007-9039-3

Patterns of Concerted Evolution of the rDNA Family in a Natural Population of Zhikong Scallop, *Chlamys farreri*

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Received: 17 February 2007 / Accepted: 18 September 2007 / Published online: 1 November 2007

Abstract Using denaturing high-performance liquid chromatography (DHPLC), we screened the insertion/deletion (indel) polymorphism in the internal transcribed spacer (ITS) sequences of 40 individuals from a natural population of Zhikong scallop (*Chlamys farreri*). Surprisingly, only 7.5% of individuals were homogeneous in ITS constitution, while the others (92.5%) were heterozygous. Based on different peak types in DHPLC analysis, seven individuals were randomly chosen to investigate indel polymorphism in the ITS sequences within individuals. Furthermore, indel polymorphism in the ITS sequences of single sperms was also investigated in more individuals belonging to different peak types. Based on these results, we concluded that rapid intrachromosomal recombination drove homogenization of rDNA arrays and interchromosomal recombination might contribute to form new variants, and that it may be less rare than previously thought although it was much less frequent than intrachromosomal recombination in the homogenization process. Further, we proposed an expanded model for concerted evolution of the rDNA family in a natural population of *C. farreri*. A pathway in the new model which homogenizes a variant unit, beginning with two-peak type individuals and ends with two-peak type individuals, is a larruping pathway in the natural population of *C. farreri*. As the highest proportion in natural populations, two-peak individuals with equal peak areas can be viewed as being in a steady and balanced state which is maintained by rapid intrachromosomal recombination.

Keywords Concerted evolution · Internal transcribed spacer (ITS) · Denaturing high-performance liquid chromatography (DHPLC) · *Chlamys farreri*

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Introduction

Most eukaryotic genomes consist of a significant fraction of repetitive DNA sequences in the form of tandem repeats or interspersed repetitive elements (Elder and Turner 1995). Of functional genes, highly repetitive gene families are often organized into tandem repeats. Some examples of tandemly repeated gene families include globin genes, immunoglobulin genes, rRNA genes, tRNA genes, and histone genes. Such an arrangement is believed to be beneficial in order to provide coordinated expression.

Tandemly arranged gene families tend to exhibit concerted evolution, a term used to describe the phenomenon that multiple copies of a gene family tend to be homogeneous, leading to greater sequence similarities among the paralogues within a genome than among orthologues among species (Liao 1999). Two primary mechanisms, unequal crossover and gene conversion, were thought to

play important roles in the homogenization of multigene families (Dover 1982). In spite of the same homogenization role, these two mechanisms can act to achieve apparently opposite results with respect to molecular evolution: they can correct and eliminate new variants and they can also promote the spread of new gene variants throughout individual gene clusters, among homologous and nonhomologous chromosomes, and within an interbreeding population (Gonzalez and Sylvester 2001).

Within eukaryotic genomes, rDNA families exist in arrays [nucleolar organizer regions (NORs) on one or more chromosomes] of varying length, ranging from a single gene in the case of *Tetrahymena* to several thousand copies per genome in some cereals (Elder and Turner 1995). Different subunits in rDNA families are known to evolve at varying rates, depending upon the degree of selective constraint operating on each. Most studies on rDNA have so far focused on one type of spacer, namely the intergenic spacer (IGS). However, the internal repeat structure of the IGS complicates its homogenization behavior, and the interpretation of IGS polymorphisms may be misleading for the understanding of the homogenizing process of the whole rDNA (Dover 1989). The internal transcribed spacer (ITS) is not internally repetitive and its evolving rate is between those of genes and nontranscribed regions (Lopez-Pinon et al. 2002). Therefore, the ITS is ideal for the detailed analyses of the homogenization processes of the rDNA family (Schlotterer and Tautz 1994).

In this study, we focused on the insertion/deletion (indel) polymorphism in the ITS sequence to study the homogenization process of the rDNA family in the population of Zhikong scallop, *Chlamys farreri*. *C. farreri* is a diploid organism with 38 chromosomes (Komaru and Wada 1985). Its rDNA family is uniquely located on the telomeric region of the short arm of chromosome 5 (Wang and Guo 2004), which reduces the complexity in experimental analysis. Under optimized condition, ITS heteroduplexes caused by indels could be well identified in denaturing high-performance liquid chromatography (DHPLC) analysis. Using this technique, we have screened the indel polymorphism in the ITS sequences of 40 individuals from a natural population. Surprisingly, only 7.5% of individuals were homogeneous in ITS constitution, while the others (92.5%) were heterozygous. Based on different peak types in DHPLC analysis, several individuals were randomly chosen to investigate indel polymorphism in the ITS sequences within individuals. Further, indel polymorphism in the ITS sequences of single sperms was also investigated in additional individuals belonging to different peak types. Based on these results, we proposed an expanded model for concerted evolution of the rDNA family in a natural population of *C. farreri*.

Materials and Methods

Scallop Materials, DNA Extraction, and Sperm Isolation

Forty adult individuals of *C. farreri* were sampled from a natural population around Changdao Island, Shandong Province, China. Genomic DNA of these individuals was extracted from muscle tissues with the phenol/chloroform extraction method (Sambrook et al. 1989).

The male scallops were induced to produce sperms using the thermal stimulation method. When a little high-concentration sperm solution was freshly obtained, it was washed two to three times with phosphate buffer solution (PBS, 145 mM NaCl, 4 mM NaH_2PO_4, and 6 mM Na_2HPO_4, pH 7.2). Several drops of a 1% eosin solution were then added to stain the sperm for 15 minutes. Subsequently, the sperm solution was diluted serially by PBS, until only one sperm/μL was achieved in the diluted solution. The diluted sperm solution (1 μL) was checked using a microscope under 100 × magnification to assure inclusion of only one sperm. If it contained only one sperm (i.e., only one red dot was found in all eye shots), the 1 μL diluted sperm solution was placed in a 0.2 μL tube for polymerase chain reaction (PCR) amplification. Sperm DNA can be released at the initial denaturation step.

PCR amplification, Cloning, Sequencing and Sequence Alignment

PCR amplifications were set up in a volume of 20 μL composed of 100 ng adult genomic DNA or 1 μL sperm solution, 0.2 μM each of the forward and reverse primers, 2 mM $MgCl_2$, 0.2 mM each dNTP, 1×PCR reaction buffer, and 1 U *Taq* polymerase (Promega, Shanghai, China). A pair of primers (forward: 5′ GTTTCTGTAGGTGAA CCTG 3′; reverse: 5′ CTCGTCTGATCTGAGGTCGGA 3′) was used to amplify ITS1, the 5.8S gene, and ITS2, which has also been used in our previous study (Wang et al. 2006). A PTC-100 thermocycler (MJ, USA) was used for PCR amplification. The expected fragment length was about 740 bp. All PCR amplifications began with an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

PCR products of selected individuals were ligated into pMD18-T (Takara, Dalian, China) and subsequently transformed into *Escherichia coli* DH5α cells. Positive colonies were sequenced using a 3730 automatic sequencer (ABI, USA). Sequence alignments were performed using the program ClustalX 1.83 (Thompson et al. 1997). Sites

shown to be PCR-error prone were omitted from subsequent analysis.

DHPLC analysis

DHPLC was performed on a 3500HT WAVE^{TM} DNA fragment analysis system (Transgenomic, USA). The PCR products were loaded on a C_{18} reverse-phase column based on alkylated polystyrene-divenylbenzene particles (DnaSep^{TM} column). The temperature of the separation column was fixed at 50°C. DHPLC analysis was carried out with an acetonitrile gradient formed by mixing buffers A (0.1 M triethylammonium acetate, 0.025% acetonitrile) and B (0.1 M triethylammonium acetate, 25% acetonitrile). The optimized gradient was 0.0 min in 71% buffer A/29% buffer B, 0.5 min in 66% buffer A/34% buffer B, 5.1 min in 43% buffer A/57% buffer B, 9.7 min in 37% buffer A/63% buffer B, 14.2 min in 35% buffer A/65% buffer B, and 18.8 min in 34% buffer A/66% buffer B. Flow rate was 0.9 mL/min and DNA was detected with a UV detector at 260 nm. Analysis per sample took 21.4 min, including column regeneration and equilibration.

Results

Indel Polymorphism in the ITS Sequences of 40 Individuals from a Natural Population

In previous experiments, conditions of DHPLC analysis were established for detecting indel polymorphisms in the ITS sequences. Under the optimized conditions, ITS heteroduplexes caused by even a 1 bp indel could be well identified in DHPLC analysis. Using this technique, we screened indel polymorphism in the ITS sequences of 40 individuals from a natural population of *C. farreri*. Surprisingly, only three (7.5%) individuals were homogeneous in ITS constitution, while the others (92.5%) were heterozygous (Fig. 1). Among the heterozygous individuals, 25 (62.5%) had two peaks, 11 (27.5%) had three peaks, and one (2.5%) had four peaks in DHPLC analysis. For one-peak individuals, even with the increase of temperature, they still showed a single peak in DHPLC analysis, suggesting no polymorphism in the ITS sequences of one-peak individuals. For two-peak individuals, the peak area of heteroduplexes was almost equal to that of homoduplexes in 23 (57.5%) individuals (e.g., Fig. 1, b and c), with the exception of two (5.0%) individuals in which the peak area of heteroduplexes was significantly lower than that of homoduplexes (e.g., Fig. 1d).

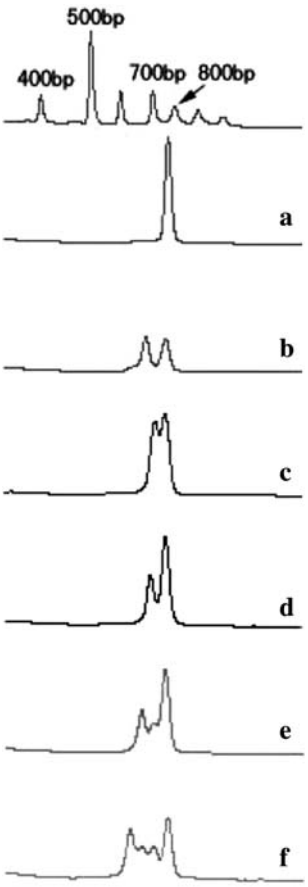

Fig. 1 Indel polymorphism in the ITS sequences of representative individuals from a natural population. (a) One-peak individual. (b)–(d) Two-peak individuals. (e) Three-peak individual. (f) Four-peak individuals

Indel Polymorphism in the ITS Sequences Within Individuals Belonging to Different Peak Types

In order to investigate the indel polymorphism in the ITS sequences within individuals belonging to different peak types, seven individuals (two of the one-peak type, two of the two-peak type, two of the three-peak type, and one of the four-peak type) were randomly chosen and their PCR products cloned. For individuals of the multipeak type, PCR products of two clones randomly chosen were mixed, denatured, and renatured. If the peak of heteroduplexes appeared in DHPLC analysis, the two clones were then selected for sequencing. A total of eight clones from each individual were selected for sequencing. As shown in Fig. 2, the polymorphism in the ITS sequences was caused by indels and nucleotide substitutions. All polymorphic sites were located in the central regions of ITS1 and ITS2. A total of 10 types of ITS sequences were found in 56 clones. ITS sequences were highly divergent among these types even though they were evaluated within peak types (two-peak, three-peak, etc.). In our previous study, high

divergence in ITS1 sequences mainly caused by indels was common among species in Pectinidae (Wang et al. 2007). Among these types, one type (C.F1-1) was the most common and it had been found in all seven individuals. The ITS sequences of different clones were consistent in one-peak individuals but inconsistent in individuals belonging to multipeak types. There were two types of ITS sequences in two-peak individuals, three types in three-peak individuals, and four types in the four-peak individual. Recombinant clones were surprisingly found in a three-peak individual (e.g., C.F5-5 may be a recombinant of C.F5-6 and C.F5-7). Further, potential relevant bases around the polymorphic sites were identified in the ITS1 and ITS2 sequences (see Table 1). All polymorphic sites and their potential relevant bases coexisted in a form of tandem $(SA_n)_n$, where S represents C and G bases.

Indel Polymorphism in Single Sperms of Individuals Belonging to Different Peak Types

Based on the results that there were only two types of ITS sequences in a two-peak individual and the peak area of heteroduplexes was almost equal to that of homoduplexes in most of the two-peak individuals, we speculated that one haploid genome of a two-peak individual with an equal peak area might contain only one type of ITS sequences. In order to validate this speculation, indel polymorphisms in the ITS sequences were examined in single sperms of individuals belonging to different peak types (see Fig. 3). In total, two one-peak type (including C.F1), five two-peak type (including C.F3), and five three-peak type individuals (including C.F5) were chosen and 20 single sperms of each individual were used to examine indel polymorphism in the ITS sequences. For one-peak individuals, no indel polymorphism in the ITS sequences was detected in all sperms from an individual (e.g., Fig. 3e). For two-peak individuals with equal peak areas, some sperms contained one type of ITS sequence (e.g., Fig. 3, g and h), while the others contained the other type (e.g., Fig. 3, i). For three-peak individuals, some sperms contained two types of ITS sequences (e.g., Fig. 3m), while the others contained only one type of ITS sequence (e.g. Fig. 3, n and o). Occasionally, when PCR products of two sperms, each of which contained one type of ITS sequences, were mixed, denatured, and renatured, a peak of heteroduplexes appeared (e.g., Fig. 3p). It seemed that the proportion of two types of ITS sequences was not invariable but dynamic in a sperm. One type of ITS sequence could be completely converted to the other type in the process of homogenization.

Discussion

Application of DHPLC in Detecting Indel Polymorphisms in the ITS Sequences

Because of high sensitivity and specificity, DHPLC, a recently developed technique, has been widely applied to fast-screen known and unknown heterozygous gene

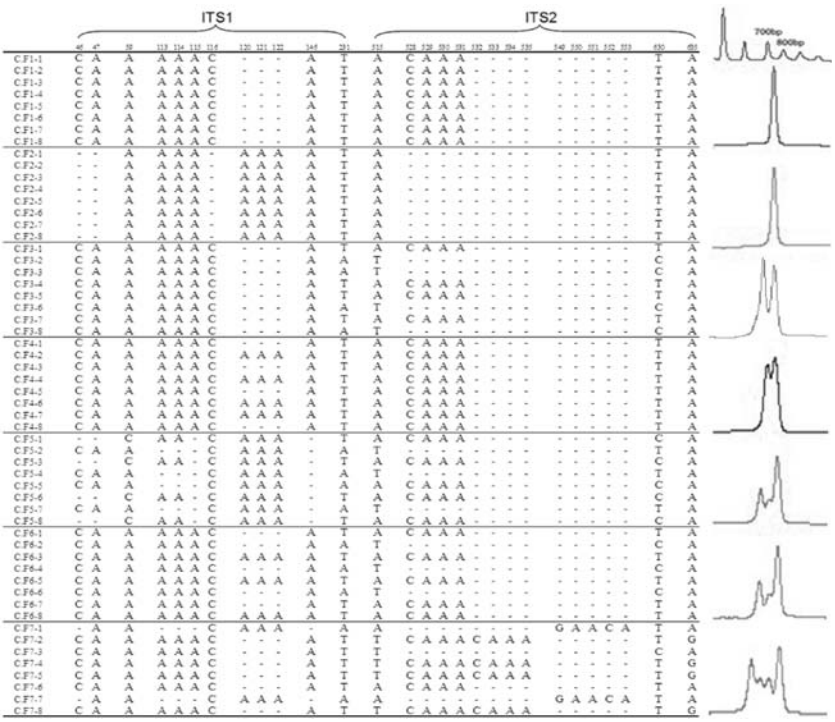

Fig. 2 The polymorphic sites in the ITS sequences of 56 clones from seven individuals belonging to different peak types. ITS1: 1–260 bp; 5.8S: 261–417 bp; ITS2: 418–699 bp

Table 1 The polymorphic sites and potential relevant bases in the ITS1 and ITS2 sequences

Region	Sequence*
ITS1	CCGAACTCCTAAAAAACACCGGGTTACGCCCGCGTGTGAA AAACACAAATCGGACAAACAAAAACAGCCGAAGGAGAT CAGGGCCAGACTCTGCCTCCTAGCTCGGCAAAAAAACAAA AAACTGTGACAACGCAAAACTTGCAAACCTTTTCGGGGTA CCTGGCATCGGTTTAATGAGCAACGGCCGCCCCGACATTA TATTTTCACGTAACAATTTGTAACCAAAAAAATTGTTGTCT CTAACGAGATGATCTCATTA
ITS2	AAAACATCTATCGCAACCTGCATGGCACAGCAGCATTGCG CCTTGGACCGTCTCGTCTCCCCGGCGAGCGGTCTTAAATGA GGAATCAGAGTCTCCAATCGAACCAAAAACAAACAAACC AGTACGGAACAGAACAAATTACAAGAGACGCAGTTCGATT AAAACACAAAATGCGCTCTCGGTTCCGTGAAACTGTAGGT TCGCCTTTTACACGTGAGAAAAAAGGTATGATGGTGTAAA TTTCTCTGGTCGACACAACACTTTGTGTTTGTCTTTTCACAC

* Polymorphic sites are underlined and potential relevant bases are indicated with a black background

mutations (Xiao and Oefner 2001; Frueh and Noyer-Weidner 2003). Cremonesi et al. (2003) demonstrated that heteroduplexes caused by indels reduced the molecular stability more than that caused by nucleotide substitutions, and thus could be more easily identified by DHPLC. In their experiment, 12 DNA samples from four different genes containing one to 29 indels could be well identified by DHPLC at 50°C. In this study, optimized condition in DHPLC analysis had been established in a previous experiment for detecting indel polymorphisms in the ITS sequences. Under the optimized condition, ITS heteroduplexes caused by even a 1 bp indel could be well identified in DHPLC analysis. This sensitivity is thus sufficient for the subsequent experimental analysis.

Rapid Intrachromosomal Recombination Drives Homogenization of rDNA Arrays

Based on the results that two-peak individuals with equal peak area occupied the highest proportion (57.5%) in natural population and their haploid genome contained only one type of ITS sequence, we concluded that homogenization of rDNA arrays in the population of *C. farreri* was mainly driven by rapid intrachromosomal recombination. This conclusion is consistent with previous theoretical and experimental studies (Smith 1976; Schlotterer and Tautz 1994; Copenhaver and Pikaard 1996; Liao et al. 1997). Further, Copenhaver and Pikaard (1996) pointed out that homogenization of the rDNA family should be a consequence of local spreading of new rDNA variants.

Specific mechanisms responsible for intrachromosomal homogenization are unknown. Unequal sister–chromatid exchange (USCE) and/or intrachromosomal gene conversion may play important roles in intrachromosomal recombination (Liao 1999). Although there are many pieces of evidence to support both of these, gene conversion has become more popular in recent theoretical and experimental studies (Hughes and Petersen 2001; Kovarik et al. 2004; Parkin and Butlin 2004; Santoyo and Romero 2005). In this study, all polymorphic sites and their potential relevant bases coexisted in a form of tandem $(SA_n)_n$. Further, we found that this motif might be conservative among scallops, because it was also identified in abundance in ITS1 and ITS2 sequences of other scallops which were available in GenBank database. Extensive and frequent regions of $(CT)_n$, $(CTTT)_n$, $(TG)_n$, and G- and GC-rich segments in rDNA sequences are known to exist at hot spots for recombination (Stringer 1985; Treco and Arnheim 1986; Steinmetz et al. 1987; Htun and Dahlberg 1989; Katzenberg et al. 1989; Wahls et al. 1990). These motifs may function as a pairing or initiation sites for repeated rounds of recombination and/or gene conversion to facilitate concerted evolution (Liao and Weiner 1995). Therefore, we speculated that the $(SA_n)_n$ motif identified in this study might play an important role in intrachromosomal or/and interchromosomal recombination.

Interchromosomal Recombination in the Homogenization Process of the rDNA Family

In this study, one-peak individuals can be viewed as the ones in which all repeated units have been homogenized, while two-peak individuals with significantly unequal peak areas can be viewed as being in a transient state from the two-peak type to the one-peak type. Based on the general opinion on concerted evolution of multigene families, they should occur with high frequency in natural population. However, both of them occur at low frequencies (7.5% and 5.0% respectively). Therefore, although interchromosomal recombination is involved in the homogenization process of the rDNA family, it should be much less frequent than intrachromosomal recombination in the homogenization process.

Besides the function of homogenization, interchromosomal recombination has some unusual characteristics in the homogenization process of the *C. farreri* population:

(i) Formation of new variants. New variants can be generated not only by mutation but also by interchromosomal recombination. Although recombinant variants have been identified in a three-peak individual (C.F5), there still exists the possibility that recombinant variants are artifacts generated by PCR amplification (Bradley and Hillis 1997). In order to exclude this possibility, PCR products of single sperms of C.F5 were cloned and sequenced (data not shown). It was found that recombinant sequences and only one type of parental sequence coexisted in a sperm, while the other type existed uniquely in a sperm. Therefore,

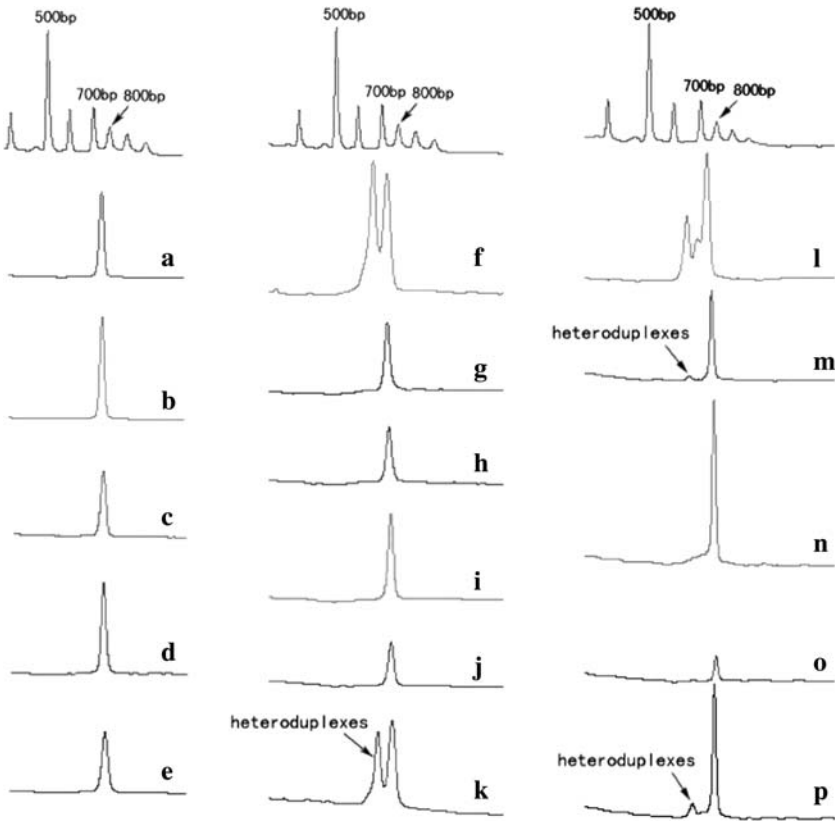

Fig. 3 Indel polymorphism in the ITS sequences of single sperms of individuals belonging to different peak types. (a) PCR product of a one-peak individual (C.F1). (b)–(d) PCR products of single sperms of C.F1. (e) Denaturing and renaturing mixtures of PCR products of multiple sperms of C.F1. (f) PCR product of a two-peak individual (C.F3). (g)–(i) PCR products of single sperms of C.F3. (j)–(k) Denaturing and renaturing mixtures of g, h and g, i respectively. (l) PCR product of a three-peak individual (C.F5). (m)–(o) PCR products of single sperms of C.F5. (p) Denaturing and renaturing mixture of n and o

recombinant variants found in this study are not artifacts generated by PCR amplification. Variants resulting from recombination among divergent sequence variants were also found in ITS sequences of some plants (van Houten et al. 1993; Baldwin et al. 1995; Wendel et al. 1995; Buckler et al. 1997). By this way, new mutations can occasionally spread to other variants, and join with other mutations. This process can accelerate the evolutionary opportunities of the rDNA family by combining favorable mutations.

(ii) The rate of interchromosomal recombination may not be rare. The evolving rate of the ITS region is between those of genes and nontranscribed region (Lopez-Pinon et al. 2002). In *Drosophila*, at least half of ITS sequences evolved with a rate close to the neutral rate of nucleotide substitution (Schlotterer et al. 1994). Therefore, occasional mutation can not account for the existence of a high proportion of three-peak individuals in this study. On the other hand, frequent interchromosomal recombination may provide a reasonable explanation (Ganley and Scott 1998; Ganley and Scott 2002). In general, exchange rates in the heterochromatin were expected to be rather low and interchromosomal recombination between rDNA arrays was thus thought to occur rarely (Schlotterer and Tautz 1994). However, Williams et al. (1989) found that recombination between the rDNA arrays occurred at a much higher rate than the rest regions of the heterochromatin in *Drosophila melanogaster*. It has been suggested that gathering of rDNA in/at the nucleolus and its exposure to nucleolin play an important role in interchromosomal recombination (Hanakahi et al. 1997; Borggrefe et al. 1998; Thyagarajan et al. 1998). Therefore, with frequent interchromosomal recombination, new variants can be generated in two-peak individuals, which can cause the emergence of a high frequency of three-peak individuals.

An Expanded Model for Concerted Evolution of the rDNA Family in a Natural Population of *C. farreri*

A couple of classic models for the concerted evolution of rDNA or U2 snRNA have been proposed (Schlotterer and Tautz 1994; Liao et al. 1997). These models are basically similar. In their models (see Fig. 4I), when a repeat unit in a particular array acquires a mutation (X), the mutation is then fixed rapidly within this original array by intrachromosomal homogenization. Once the mutation is spread to the homologous array by interallelic genetic exchange, the mutation is finally fixed throughout the second array by additional rounds of intrachromosomal homogenization. However, these models are not sufficient enough to explain our experimental results in *C. farreri*. Therefore, we proposed an expanded model for the

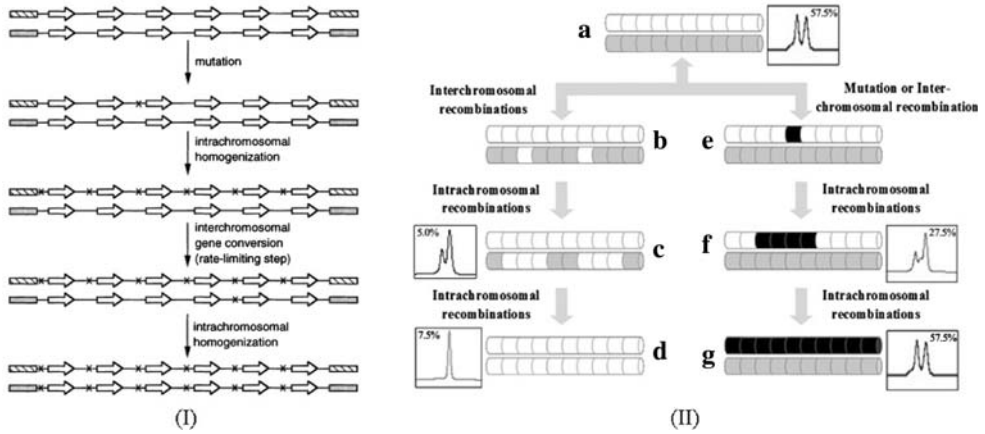

Fig. 4 (I) A model for concerted evolution of U2 snRNA in humans and primates (Liao et al. 1997). U2 snRNA coding regions are shown as hollow arrows, spacer sequences as lines, and flanking chromosomal DNA as rectangles (crosshatched and shaded). (II) A new model for concerted evolution of a rDNA family in a natural population of *C. farreri*. Three possible pathways: (i) homogenization of all repeated units (a→b→c→d); (ii) homogenization of a variant unit (a→e→f→g); (iii) elimination of ingoing units (a→b→a) or variant units (a→e→a). Different repeat units are shown by different colors

concerted evolution of the rDNA family in a natural population of *C. farreri*. As shown in Fig. 4II, there are three possible pathways in the homogenization process: (i) homogenization of all repeated units (a→b→c→d); (ii) homogenization of a variant unit (a→e→f→g); (iii) elimination of ingoing units (a→b→a) or variant units (a→e→a). In the new model, pathway (i) results in homogenization of all repeated units which is consistent with the classic model. Therefore, the classic model can be viewed as a submodel of our proposed model. However, because of the low frequency of one-peak individuals and two-peak individuals with significantly unequal peak areas, pathway (i) seems to be infrequent. Pathway (ii) does not result in homogenization of all repeated units, whereas it homogenizes a variant unit, and begins and ends with two-peak type individuals. This pathway is a larruping pathway in the natural population of *C. farreri*, which can not be explained by the classic model. Because of high frequency of three-peak individuals and two-peak individuals with equal peak areas, pathway (ii) seems to be more frequently used than pathway (i). Therefore, as the highest proportion in the natural population, two-peak individuals with equal peak areas can be viewed as being in a steady and balanced state which is maintained by rapid intrachromosomal recombination. It may be more complex that four-peak individuals can be viewed as being in the state of occasionally homogenizing two or more variants.

Acknowledgments The authors thank Prof. Guanpin Yang and two anonymous reviewers for their helpful comments on this manuscript. This work was supported by grants from The National High Technology Research and Development Program of China (2006AA10A408; 2006AA10A402), a specialized research fund for the Doctoral Program of Higher Education (20060423015), and Program for New Century Excellent Talents in University under contract No. NCET-06-0594.

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