Chromosome painting of telomeric repeats reveals new evidence for genome evolution in peanut

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Abstract
Interspecific hybridization is an important approach to improve cultivated peanut varieties. Cytological markers such as tandem repeats will facilitate alien gene introgression in peanut. Telomeric repeats have also been frequently used in chromosome research. Most plant telomeric repeats are (TTTAGGG)n that are mainly distributed at the chromosome ends, although interstitial telomeric repeats (ITRs) are also commonly identified. In this study, the telomeric repeat was chromosomally localized in 10 Arachis species through sequential GISH (genomic in situ hybridization) and FISH (fluorescence in situ hybridization) combined with 4',6-diamidino-2-phenylindole (DAPI) staining. Six ITRs were identified such as in the centromeric region of chromosome B5 in Arachis ipaënsis, pericentromeric regions of chromosomes A5 in A. stenop sperma, B7 in A. hoehnei and A5 in A. villosa, nucleolar organizer regions of chromosomes A3 in A. stenosperma and A3 in A. diogoi, subtelomeric regions of chromosomes B7 in A. hoehnei and A2 in A. duranensis, and telomeric region of chromosome E7 in A. stenophylla. The distributions of the telomeric repeat, 5S rDNA, 45S rDNA and DAPI staining pattern provided not only ways of distinguishing different chromosomes, but also karyotypes with a higher resolution that could be used in evolutionary genome research. The distribution of telomeric repeats, 5S rDNA and 45S rDNA sites in this study, along with inversions detected on the long arms of chromosomes K10 and B7, indicated frequent chromosomal rearrangements during evolution of Arachis species.

Keywords: Arachis species, inversion, interstitial telomeric repeats, karyotype

1. Introduction
The telomere is a specific DNA-protein structure with functions of regulating cell-senescence and carcinogenesis (Au bert and Lansdorp 2008). It also protects chromosomes from exonuclease digestion (Blackburn 1991). In both animals and plants, telomere repeats generally comprise tandemly repeated DNA sequences of (TTAGGG)n and (TTTAGGG)n (Burr et al. 1992; Fajkus et al. 2005).
Telomeric repeats are generally presented at chromosome ends. Nevertheless, interstitial telomeric repeats (ITRs) occur in plants (Regad et al. 1994) and animals (Nergadze et al. 2004; Mattos et al. 2014; Scacchetti et al. 2015), including human (Park et al. 1992). In plants, ITRs are found at different chromosomes locations, including centromeric, rDNA, subtelomeric and telomeric regions (Gortner et al. 1998; Tek and Jiang 2004; Presting et al. 1996). Further analysis revealed that ITRs involved chromosome breakage, recombination and amplification (Lin 1996). Further analysis revealed that ITRs involved chromosome breakage, recombination and amplification (Lin and Yan 2008; Bolzan 2012).

Peanut (Arachis hypogaea, 2n=4x=40, genome AABB) is one of the most important food and forage crops in the world. Previous studies identified 80 annual and perennial species of Arachis in nine taxonomic sections, including Arachis (A, B and D), Caulorrhizae (C), Erectoides (E), Extranervosae (Ex), Heteranthae (H), Procumbentes (P), Trirectoides (Trie), Triseminatae (Tris) and Rhizomatoseae (R) (Fernández and Krapovickas 1994; Krapovickas and Gregory 1994; Valls and Simpson 2005). However, the classification and phylogeny of many Arachis species are still in a state of flux. For example, FISH (fluorescence in situ hybridization) analysis using 45S and 5S rDNA and heterochromatin as probes led to re-designation of the A-genome due to its smallest “chromosome A” and similar patterns of 45S and 5S rDNA and heterochromatin as shown in other A-genome species (Robledo et al. 2009).

Telomeric repeats were identified through FISH in many species (Okazaki et al. 1993; Meyne et al. 1995). To our knowledge, the only ITR analysis using FISH analysis in peanut was conducted by Zhang (2013) in the species Arachis hypogaea, A. ipaënsis, and A. duranensis, with six ITRs being identified in the species A. hypogaea and A. ipaënsis, and none in A. duranensis. In the current study, ITRs from 10 wild Arachis species were analyzed by FISH using telomeric repeat probes combining with GISH (genomic in situ hybridization), 5S and 45S rDNA FISH and DAPI staining to: 1) characterize telomeric repeat distributions in Arachis species; 2) develop karyotypes with a higher resolution in these species; and 3) reveal new genomic evidence for evolution of Arachis species.

2. Materials and methods

2.1. Plant materials

Ten Arachis species, A. hypogaea, A. ipaënsis, A. duranensis, A. chiquitana, A. stenosperma, A. diogoi, A. villosa, A. batizocoi, A. hoehnei and A. stenophylla, were used in the study. The accession number, chromosome number and genome constitutions of the 10 Arachis species were provided in Table 1.

2.2. Chromosome preparation

Seeds of the 10 Arachis species were germinated on moist filter paper at 25°C for 7 d. Healthy lateral root tips were excised and pretreated with 2 mmol L⁻¹ 8-hydroxyquinoline for 3 h at 25°C, and fixed in absolute ethanol (3):glacial acetic acid (1) for 12 h at 4°C. Then, 0.3–0.5 mm of root tips meristem was excised and squashed in 45% glacial acetic acid and freezed at –80°C for 12 h. Thus, the spread chromosomes were dehydrated in 100% ethanol and dried in air after the cover slips were removed.

2.3. Cytogenetic analysis

Total genomic DNA was extracted from young fresh leaves of A. ipaënsis (2n=2x=20, BB) (Wang et al. 2002). The

| Table 1 Plant materials used in this study and their chromosome constitution |
|--------------------------|--------------------------|--------------------------|--------------------------|
| Species                  | Accession no.            | Plant introduction (PI) no. | Chromosome no.          | Genome (references)     |
| A. chiquitana            | 36027                    | PI 476006                  | 2n=2x=20                | AA (Robledo et al. 2009) |
| A. stenosperma           | 410                      | PI 338280                  | 2n=2x=20                | AA (Robledo et al. 2009) |
| A. diogoi                | 20029                    | PI 276235                  | 2n=2x=20                | AA (Robledo et al. 2009) |
| A. villosa               | 35885                    | PI 298636                  | 2n=2x=20                | A (Robledo et al. 2009)  |
| A. duranensis            | 7988                     | PI 219823                  | 2n=2x=20                | AA (Robledo et al. 2009) |
| A. hypogaea              | Z5297                    | PI 319768                  | 2n=4x=40                | AABB (Seijo et al. 2004) |
| A. ipaënsis              | 30076                    | PI 469322                  | 2n=2x=20                | BB (Robledo and Seijo 2010) |
| A. batizocoi             | 30076                    | PI 298639                  | 2n=2x=20                | KK (Robledo and Seijo 2010) |
| A. hoehnei               | 30006                    | PI 469150                  | 2n=2x=20                | BB (Krapovickas and Gregory 1994; Holbrook and Stalker 2003) |
| A. stenophylla           | 30136                    | PI 468178                  | 2n=2x=20                | EE (Krapovickas and Gregory 1994; Holbrook and Stalker 2003) |
primer pair, F-5’-TTTAGGGTTTAGGGTTTAGGGTTTAGGGTTTAGGGTTTAGGG-3’ and R-5’-AAATCCCAAATCCCAAATCCCAAATCCCAAATCCC-3’), modified from the primers using in amplifying human telomeric repeats (Ijdo et al. 1991), were used for specific amplification of plant telomeric repeats (TTTAGGG). Two clones of 5S and 45S rDNA of wheat (Triticum aestivum L.) used by Du et al. (2015), were provided by Dr. Bikram S Gill, Kansas State University, USA. The 5S rDNA and (TTTAGGG), were labeled with biotin-16-dUTP (Roche, Mannheim, Germany) by nick translation and detected with fluorescein anti-biotin (Roche, Mannheim, Germany). And 45S rDNA and total genomic DNA of A. ipaënsis were labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany) and detected with anti-digoxigenin-rhodamine (Roche, Mannheim, Germany).

Sequential GISH/FISH was performed as described by Sepsi et al. (2008). Briefly, the Arachis chromosomes were identified by FISH and GISH. Where, FISH using 5S and 45S rDNA as probes combined with 4',6-diamidino-2-phenylindole (DAPI) staining; GISH using total genomic DNA of A. ipaënsis as probe, was used to distinguish A- and B-genome chromosomes. And FISH with the telomeric repeats as probes were used to characterize the distribution of (TTTAGGG) in Arachis chromosomes.

2.4. Imaging analysis

Chromosomes were photographed with a DMRX fluorescence microscope (Leica, Germany). The merged images were further processed using Photoshop 6.0 for brightness, contrast, and intensity. To distinguish the signals produced by different probes in each chromosome after sequential FISH/GISH, the original green signal of 5S rDNA was converted to pseudo-color white, and the red signal of GISH converted to fuchsia, while the red signal of 45S rDNA and the green of telomeric repeat (TTTAGGG) remained.

2.5. Karyotypic analysis

Chromosome size was measured in at least five metaphase cells of each species using the Image-Pro Plus 6.0 system. The centromeric index (\(i=\frac{\text{Short arm length} \times 100}{\text{Chromosome length}}\)) was calculated and used to classify the chromosomes as described by Levan et al. (1964). Then chromosomes were grouped into metacentric (m, \(i=50–37.51\)), submetacentric (sm, \(i=37.50–25.01\)) and subtelocentric (st, \(i=25–12.51\)) based on the index. Mean karyotype value of each species was represented in ideograms. Chromosomes of the 10 Arachis species were distinguished according to Seijo et al. (2004) combined with the characterised distributions of telomeric repeats.

3. Results

3.1. Preliminary karyotype analysis in 10 Arachis species

Preliminary karyotypes of the tested Arachis species in this study were based on the distributions of 5S rDNA and 45S rDNA and the staining patterns of GISH and DAPI through sequential GISH/FISH (Fig. 1 and Appendixes A, B, C and D). The karyotypes were then used for chromosome mapping of telomeric repeats. Six Arachis karyotypes including A. batizocoi, A. diogoi, A. duranensis, A. hoehnei, A. hypogaeae and A. ipaënsis agreed well with previous reports (Seijo et al. 2004; Robledo et al. 2009, 2010; Custódio et al. 2013). The karyotypes of A. batizocoi, A. diogoi, A. duranensis and A. hoehnei were same as previous reports.
(Fig. 2 and Appendix E-a, b, c and d), whereas karyotypes of *A. hypogaea* cultivar Z5297 and *A. ipaënsis* were similar to previous reports, and the only difference was shown by the DAPI staining patterns. Although DAPI bands that were previously observed only in A-genome chromosomes (Seijo et al. 2004; Robledo and Seijo 2010), clear DAPI bands were also observed on seven B-genome chromosomes (Fig. 2 and Appendix E-e and f).

Almost all chromosomes of the E-genome of *A. steno-phylla*, showed clear DAPI bands; E^4, E^10 and E^9 had submetacentric and E^8 had subtelomeric bands. Chromosome E^4 showed signals of 45S rDNA and E^10 had signals of 5S and 45S rDNA (Fig. 2 and Appendix E-e and f).

Compared to findings in Robledo et al. (2009), differences were seen on chromosomes 4 and 7 in *A. chiquitana*, *A. villosa* and *A. stenosperma*. In *A. chiquitana* and *A. villosa*, heterogeneous distributions of 45S rDNA sites were presented on chromosome 7. For example, the 45S rDNA site on one chromosome A^c^7 was much larger than the site on its homologous partner in *A. chiquitana*, while in *A. villosa*, only one homolog chromosome A^v^7 carried a 45S rDNA site (Fig. 2 and Appendix E-h and i). In *A. stenosperma*, a new but weak 45S rDNA site was observed on chromosome A^s^4 in addition to sites on chromosomes A^s^2, A^s^7 and A^s^10 reported by Robledo et al. (2009) (Fig. 2 and Appendix E-j).

### 3.2. Chromosome painting of telomeric repeats in the 10 *Arachis* species

Based on the above karyotypes, the chromosome positions of the telomeric repeat (TTTAGGG) were determined for the 10 species through sequential GISH/FISH. This repeat was located in many chromosomal regions including the chromosome ends. Among the chromosomal regions with telomeric repeats in the 10 species, six kinds of ITRs were observed in centromeric region (centromeric ITR), pericentromeric regions (pericentromeric ITR), intercalary region of long or short arms (long or short arm ITR), nucleolar organizer region (rDNA ITR), subtelomeric regions (subtelomeric ITR), and telomeric regions (telomeric ITR), respectively (Fig. 2).

In *A. hypogaea* cultivar Z5297, ITRs were observed on three chromosomes, with one centromeric ITR on chromosome B^hy^5, one subtelomeric ITR on chromosome A^hy^7 and one small ITR on the long arm of chromosome A^hy^5. *A. ipaënsis* and *A. hypogaea* had the same ITR distributions on B-genome chromosomes. The ITR distributions in *A. duranensis* and *A. hypogaea* showed differences on chromosomes 4 and 5 of the A-genome. Chromosome A^hy^4 carried an ITR on the short arm that was not obvious in the chro-

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**Fig. 2** New karyotypes of ten *Arachis* species. The first column indicates chromosomes captured in white and black after DAPI counterstaining to show the characteristic bands of DAPI (gray white). Other color signals in the chromosomes were produced by different probes. White color, the original green signal of 5S rDNA converted to pseudo color white; red color, 45S rDNA; green color, telomeric repeats (TTTAGGG); fuchsia, original red signal of total genomic DNA converted to pseudo fuchsia color, shows GISH (genomic in situ hybridization) signal with total genomic DNA of *Arachis ipaënsis* labeled with digoxigenin-11-dUTP and detected with anti-digoxigenin-rhodamine; blue, chromosomes counterstained with DAPI. The same as below.
mosome A^h4, and chromosome A^h5 had an ITR on the long arm which was larger than the one on the chromosome A^h5 (Fig. 2). The different ITR distributions (number and size) on the three A-genome chromosomes were also observed in the other four species (A. villosa, A. diogoi, A. stenosperma and A. chiquitana). A small rDNA ITR was presented in chromosomes A^h2 and A^v2, but was not seen in chromosomes A^h2 and A^v2. Another small rDNA ITR observed in chromosomes A^v3, A^h3 and A^v3 was not detected on chromosome A^h3. In addition, one ITR observed only on the long arms of chromosomes A^h5 and A^v5 and a small pericentromeric ITR was detected only on the short arm of chromosomes A^h5 and A^v5 (Figs. 3 and 4).

ITRs in A. batizocoi, A. hoehnei and A. stenophylla were observed on chromosomes 5, 7, 8 and 9. The ITR distributions on chromosomes 5 and 9 were similar across the three species with a small pericentromeric ITR in chromosome 5 and the largest subtelomeric ITR covering almost entire short arm of chromosomes 9. As for chromosomes 7 and 8, a small pericentric ITR was presented in chromosomes K^h7 and B^h7, a large telomeric ITR presented in chromosome E^h7, and an rDNA ITR was presented only in chromosome K^h8 (Fig. 2; Table 2).

3.3. Genomic analysis of the ten Arachis species

According to the chromosomal locations of telomeric repeats above and 5S rDNA, 45S rDNA and DAPI bands, karyotypes with higher resolution were developed for the 10 species. Among them, six A-genome karyotypes (A^v, A^h, A^v, A^d, A^h and A^v) had similar karyotypic formulae and distributions of 5S rDNA, 45S rDNA and DAPI bands including the smallest “chromosome A” (Table 2). However, chromosomes 2, 3, 4, 5 and 7 showed different ITRs, which indicated genetic diversity among the A-genome chromosomes in these species (Fig. 3).

The two B-genomes had the same karyotype formulas (B^h=B^v=10m), which included the similar distribution of 5S and 45S rDNA on chromosomes 3, 7, and 10, DAPI bands on seven chromosomes, and a large centromeric ITR on chromosome 5 (Fig. 3).

The karyotype formulas of the remaining genomes K^o, B^h and E^h were K^o=7m+3sm, B^h=9m+1sm and E^h=6m+3sm+1st, respectively, they were quite different. Among them, the whole chromosome 9 had a large subtelomeric ITRs, the whole chromosome 4 had a 45S rDNA site, and the whole chromosome 10 had both 5S and 45S rDNA sites. However, chromosomes K^h3 and K^h8 both had a 5S rDNA site but B^h3, B^h8, E^h3 and E^h8 did not have that; E^h7 had a large telomeric ITR in the short arm but K^h7 and B^h7 each had a small pericentromeric ITR in the long arm (Fig. 3). All of these indicated the variation among these chromosomes.

4. Discussion

45S and 5S rDNA as probes combined with DAPI staining method had been used by previous chromosome painting studies, and the karyotypes of most Arachis species were

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**Fig. 3** Idiogram karyotypes of 10 Arachis species. sm, submetacentric; st, subtelocentric.
revealed six ITRs in important chromosome painting using the telomeric repeat chromosome E. resolution were developed for chromosome identification in peanut. After chromosome painting using the telomeric repeat combined with sequential GISH/FISH and DAPI staining, almost all chromosomes of the 10 species were clearly distinguished and karyotypes with higher resolution were developed for chromosome identification in peanut.  

**Table 2** New karyotypes of 10 Arachis species

| Species          | Karyotype formula | Karyotype length (μm) | Chromosome rDNA site | No. of 5S rDNA sites (chromosome) | No. of 45S rDNA sites (chromosome) | No. of ITRs sites (chromosome) | No. of DAPI bands3) (intensity) | Chromosomes with higher accuracy of multicolor GISH using total genomic DNAs from A- and B-genome donor species were stained by DAPI (She et al. 2012; Du et al. 2015). Moreover, some ITRs showed variation in size (Fig. 4-A); for example, chromosomes K9, B9 and E9 had the largest ITRs which almost covering the entire short arms. Our results thus provide new cytogenetic markers for chromosome identification in peanut. After chromosome painting using the telomeric repeat combined with sequential GISH/FISH and DAPI staining, almost all chromosomes of the 10 species were clearly distinguished and karyotypes with higher resolution were developed for chromosome identification in peanut.  

**Fig. 4** Organizations of telomeric repeats (A): 45S rDNA (B), 45S (B) and 5S rDNAs (C) in individual chromosomes of different Arachis genomes.

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Values are means±SE.
and evolutionary research in *Arachis*. To our knowledge, this is the first time to report the karyotype of *A. stenophylla*.

The locations of ITRs generally indicate that chromosome fusion (Azzalin et al. 2001), and chromosome breakage or rearrangement (Lin and Yan 2008; Bolzan 2012). Our research indicated that various chromosome rearrangements had occurred and the telomeric repeat might be a useful tool for studying genomic evolution in *Arachis* species. Previous studies also indicated that ITRs or telo-box elements could be presented in the promoter regions of some genes in *Arabidopsis thaliana* (Manevski et al. 2000), and telo-box might have function in regulating eukaryotic elongation factor 1A (EF1A) or ribosomal protein gene expression (Tremousaygue et al. 1999). Further analysis of various ITRs in peanut might reveal their possible roles.

In addition to various ITRs found in *Arachis*, various rDNA distributions were also established in this study. For example, homologous chromosomes of A7 and A7 showed different rDNA sizes, and the 45S rDNA signal of one of the A7 homologs was missing (Fig. 4-B). Heterogeneous ITRs previously reported in *Solanum* (He et al. 2013) were attributed to unequal crossing over. Our results further indicated that chromosomal rearrangements might have occurred relatively frequently in *Arachis*. And the typical example was one obvious inversion detected in chromosomes K10 and B10 (Fig. 4-C). Further analysis of the detailed structural variation will be important for genetic research and chromosome engineering in *Arachis*.

Previous reports concluded that *A. duranensis* and *A. ipaënsis* were likely donors of the A- and B-genomes in cultivated peanut (Robledo et al. 2009, 2010; Koppolu et al. 2010). In this study, the diversity between the A-genomes of *A. duranensis* (Aa) and cultivated peanut (AaAbBb) on chromosomes 4 and 5 was obvious. Also, both the diversity between Aa and B genomes in *A. duranensis* and *A. ipaënsis* on chromosomes 2, 3, 4, 5, 7 and 10, and diversity between Aa and B genomes in cultivated peanut on chromosomes 2, 3, 5, 7 and 10 were more obvious. These results indicated that chromosomal rearrangements had occurred in these six chromosomes. Recent genome sequencing also indicated that six chromosomes of the Aa and B genome had frequent rearrangements, including inversions and translocations, whereas the other four chromosomes maintained high collinearity (Bertioli et al. 2016).

The genomes in *A. hoehnei* and *A. batizocoi* were previously grouped in B-genome, and *A. stenophylla* was grouped into E-genome species in section *Erectoides* (Krapovickas and Gregory 1994). However, Robledo and Seijo (2010) suggested that *A. batizocoi* should be allocated to a new genome named “K” based on rDNA FISH. Our study showed different distributions of telomeric repeats on chromosomes 5, 7, 8 and 9 in *A. batizocoi* and *A. ipaënsis*, which provided evidence for the new K designation.

The genomic origin of *A. hoehnei* (Bns) was controversial (Tallury et al. 2005; Friend et al. 2010; Custódio et al. 2013; Moretzsohn et al. 2013). The present characteristic patterns of the telomeric repeat suggested that the genome Bns of *A. hoehnei* might be similar to E-genome and different from A- and B-genomes (Fig. 2). However, due to the genetic complexity, it is necessary to use additional landmarks or whole genome sequencing to determine the real origin and differentiation of genome Bns.

5. Conclusion

Chromosome painting reveals various distributions of the telomeric repeat and rDNA in *Arachis*. These markers combining with sequential GISH/FISH and DAPI staining allowed the development of higher resolution karyotypes for the 10 *Arachis* species. These analyses not only distinguished most chromosomes but also revealed many chromosomal rearrangements in *Arachis* species. The markers could be extended to other *Arachis* species and should facilitate both evolutionary genomic research and introgression of alien genes into cultivated peanut via interspecific hybridization.

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

References


Robledo G, Lavia G I, Seijo G. 2009. Species relations among wild Arachis species with the A genome as revealed by FISH mapping of rDNA loci and heterochromatin detection. Theoretical and Applied Genetics, 118, 1295–1307.

Robledo G, Seijo G. 2010. Species relationships among the wild B genome of Arachis species (section Arachis) based on FISH mapping of rDNA loci and heterochromatin detection, a new proposal for genome arrangement. Theoretical and Applied Genetics, 121, 1033–1046.


Silvestri M C, Ortiz A M, Lavia G I. 2014. rDNA loci and heterochromatin positions support a distinct genome type for ‘x = 9 species’ of section *Arachis* (Arachis, Leguminosae).


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