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Utilization of genomic retrotransposons as cladistic markers

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Abstract

Genomic retrotransposons are major genomic components in most eukaryotic organisms. Their abundance in the genome is generally correlated with genome size. These elements spread throughout the genome by a process termed retro-transposition consisting of transcription, reverse transcription and reinsertion of the copied element into a new genomic location. The target sites are relatively unspecific and independent, thus, integration of the same element into specific site in different taxa is negligible over evolutionary time scales. However, utilization of retrotransposons as cladistic molecular markers represents a particularly interesting complement to other molecular and morphological data. These markers can differentiate between ancestral and derived character state at a respective locus. Retrotransposon content of a given species is strongly influenced by the host evolutionary history, with periods of rapid turnover of retrotransposons sequences. Thus, retrotransposon integration markers are an ideal tool for determining the common ancestry of taxa by a shared derived transpositional event.

Key words**:** Retrotransposon, molecular marker, IRAP, REMAP, SSAP.

Genomik retrotranspozonların taksonomik markör olarak kullanımı

Özet

Genomik retrotranspozonlar birçok ökaryotik organizmada ana genomik komponentlerdendir. Genomdaki miktarları genellikle genom büyüklüğüyle ilişkilidir. Bu elementler genom boyunca transkripsiyon, ters transkripsiyon ve kopyalanmış elementin yeni bir genomik lokasyona tekrar insersyonundan oluşan ve retrotranspozisyon olarak adlandırılan bir yöntemle yayılırlar. Hedef bölgeler göreceli olarak spesifik değildir ve bağımsızdır. Böylece, aynı elementin farklı sınıflarda spesifik bir bölgeye entegrasyonu evrimsel zaman ölçeklerinde ihmal edilebilir. Bununla birlikte, retrotranspozonların taksonomik markör olarak kullanımı diğer moleküler ve morfolojik verileri ilginç bir şekilde tamamlar. Bu markörler ilgili bir lokusta soysal ve türevlenmiş karakter durumlarını ayırdedebilir. Belli bir türün retrotranspozon içeriği konağın evrimsel hikayesinden, retrotranspozon dizilerinin hızlı yenilenme periyodlarıyla kuvvetli bir şekilde etkilenir. Dolayısıyla, retrotranspozon entegrasyon markörleri paylaşılan bir türevlenmiş transpozisyonel olayla sınıfların ortak atalarının belirlenmesinde ideal araçlardır.

Anahtar Sözcükler: Retrotranspozon, moleküler markör, IRAP, REMAP, SSAP.

Introduction

Retrotransposons and their genomic impact

Genomic retrotransposons are the most abundant class of transposable elements and they outnumber the genes in the eukaryotic genomes (Sabot and Schulman, 2006; Feschotte *et al* 2002; IHGSC, 2001). Their copy number and genomic locations are plastic. Plant genomes contain hundreds of thousands of these elements, together forming the vast majority of the total DNA (Schulman and Kalendar, 2005). Because of their copy-and-paste mode of transposition, these elements tend to increase their copy number while they are active (Mansour, 2007). The differences in genome size observed in the plant kingdom are accompanied by variations in retrotransposon content, suggesting that retrotransposons might be important players in the evolution of genome size (Vitte and Panaud, 2005). Mammalian genomes also contain an abundance of retrotransposons, however, the majority are not LTR retrotransposons but long interspersed elements (LINEs) and short interspersed elements (SINEs), which replicate by a different copy-and-paste mechanism (Hedges and Batzer, 2005; Ostertag and Kazazian, 2005). These unique properties of retrotransposons have been exploited as genetic tools for plant genome analysis. Many applications such as phylogeny, genetic diversity and the functional analyses of genes using retrotransposons were developed (Kalendar and Schulman, 1999; Flavell et al., 1998; Waugh et al., 1997).

Long Terminal Repeat retrotransposons

Long Terminal Repeat (LTR) retrotransposons are the most abundant class of retrotransposons (Schulman and Kalendar, 2005) (Figure 1a and 1b). LTR-retrotransposons contribute substantially to the structural diversity of plant genomes (Vitte and Panaud, 2005). In plant genomes, huge numbers of LTR retrotransposon insertions are found in many species and constitute more than half the entire genome in some cases (Kumar and Bennetzen, 1999; Sanmiguel et al. 1996).

LTR retrotransposons, belongs to Type I transposable elements, replicates by a process of reverse transcription resembling that of the lentiviruses (such as HIV) (Feschotte et al 2002). As their name indicates, the LTR retrotransposons are flanked by LTRs and have a great impact in shaping their host genomes through insertional mutagenesis. They encode most of the proteins needed for their own replication and integration back into the genome (Sabot and Schulman, 2006). Different stresses have been shown to influence many plant LTR retrotransposons (Mansour, 2007). For instance, various biotic and abiotic stresses shown to increase the expression of various transcriptionally active LTR retrotransposons including chilling, infection, mechanical damage, in vitro regeneration, hybridization and generation of doubled haploids (Grandbastien et al., 2005; Hirochika, 1995). LTR retrotransposons are classified into two main groups, the copia group and the gypsy group, on the basis of conserved sequence features and gene order (Xiong and Eickbush, 1990) (Figures 1a and 1b); although more recent findings show this to be an oversimplification (Jurka et al, 2007; Havecker et al. 2004).

Non LTR retrotransposon

Approximately one-quarter of the human genome is composed of short and long nuclear interspersed elements (IHGSC, 2001) (Figure 1c and 1d). The LINE-1(L1) family of LINE elements and the Alu family of SINE elements together comprise roughly 30% of human genomic DNA and nearly 2 million copies (Jurka, 2004). The mechanism of LINE retrotransposition and integration into the genome is well studied and is viewed as a coupled process called target-primed reverse transcription (TPRT). In the TPRT model, reverse transcription is primed by the free 3' hydroxyl group at the target DNA nick introduced by EN (Eickbush and Malik, 2002). The model was recently enhanced by the finding that initiation of the L1 reverse transcription does not require base pairing between the

primer and template (Kulpa and Moran, 2006). Many non-LTR retrotransposons have been reported to be activated by stress. It was reported that many cellular stress signals can activate LINE-1(L1). For instance, exposing human cells to reactive carcinogenic intermediates, increases L1 retrotransposition in HeLa cells (Stribinskis et al., 2006). Moreover, recombination events involving these elements, including novel insertions into active genes, have been associated with developing number of human diseases (Hagan and Rudin, 2002). On the other hand, the short interspersed nucleic elements (SINE) retrotransposons is a nonautonomous non-LTR retrotransposons. SINEs do not encode a functional reverse transcriptase protein and rely on other mobile elements for transposition (King and Stansfield, 1997). Their mosaic structures are derived from transfer RNA (tRNA) or 7SL or 5S ribosomal RNA, and typically contain 5' internal pol III promoters involved in transcription (Figure 1d). However, they may share common structural constraints with LINE elements and their retrotransposition is catalyzed by RT/EN encoded by such autonomous non-LTR retrotransposons (Sun et al, 2007). With about 1 million copies, SINEs make up about 13% of the human genome (Pierce, 2005). While previously believed to be "junk DNA", recent research suggests that both LINEs and SINEs have a significant role in gene evolution, structure and transcription levels (Santangelo et al, 2007).

Utilization of Retrotransposons in phylogenetic studies

Retroelements are an important source of plant genetic diversity (Kumar and Bennetzen, 1999). Marker systems based on transposable elements, in contrast to other methods, detect large changes in the genome. Retrotransposons can be used as

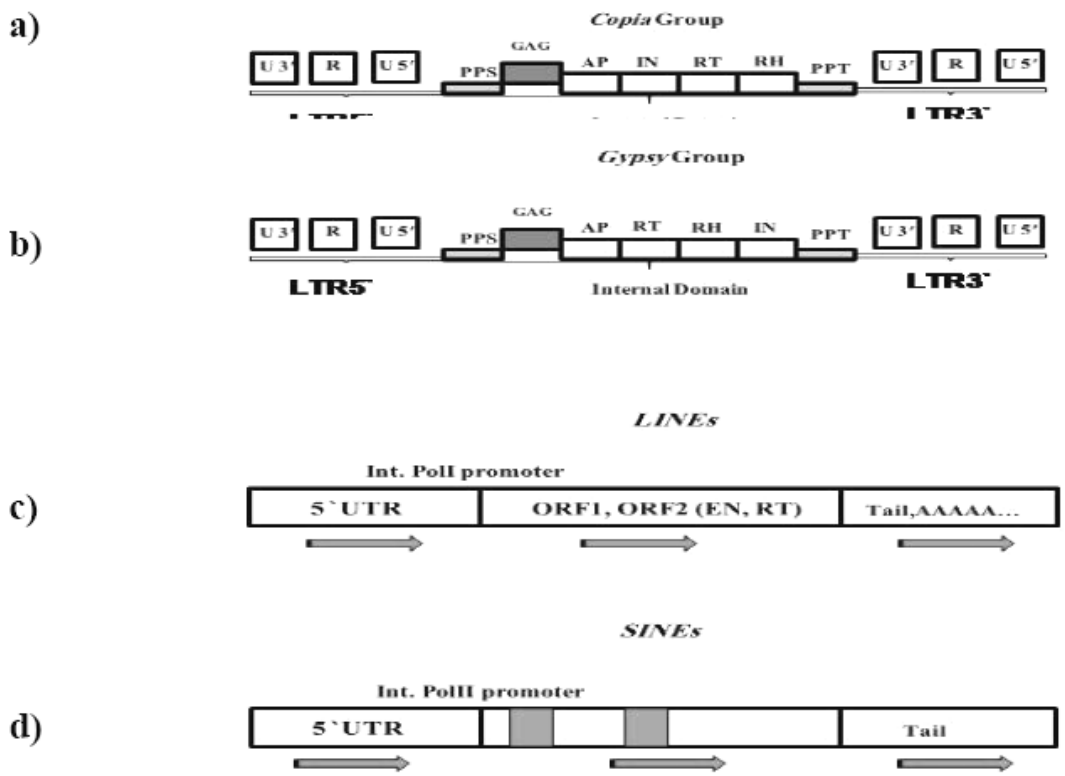

Figure 1. LTR and Non LTR retrotransposons a, b) The structural differences between two major classes of LTR-retrotransposons, Copia and Gypsy respectively c, d) LINEs and SINEs, Non LTR retrotransposons, are autonomous and non autonomous respectively.

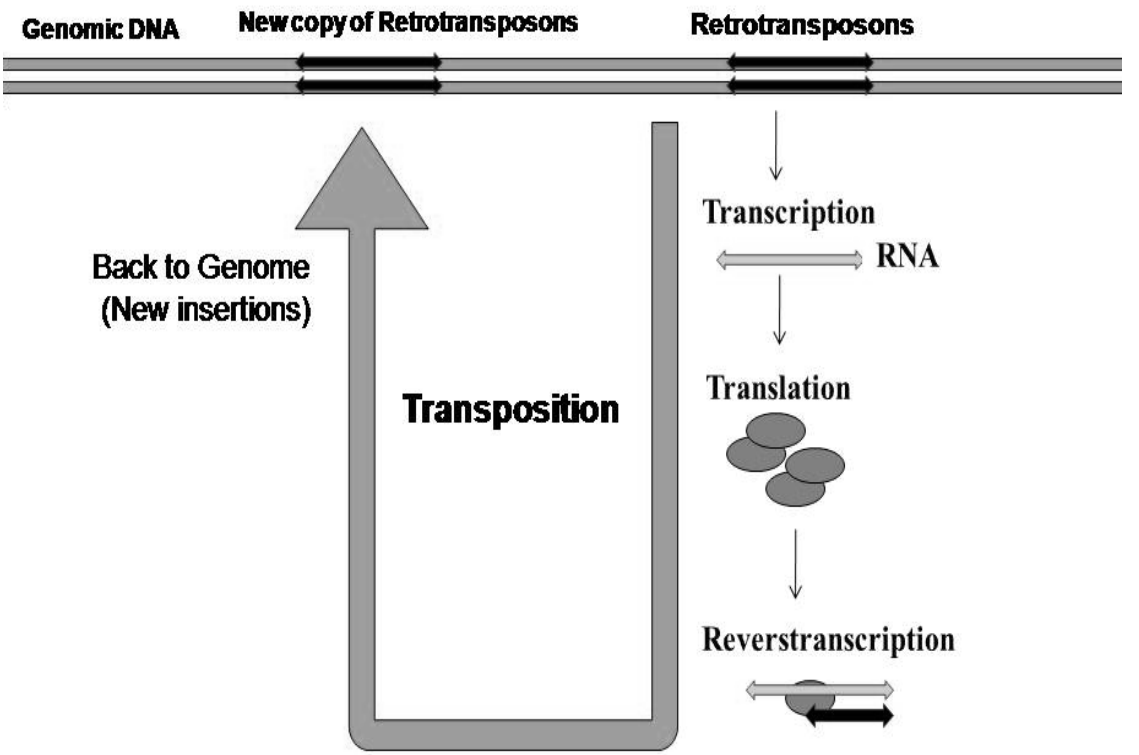

Figure 2. Scheme of retrotransposition event inside the genome. Retrotransposons can be used as molecular markers because their integration creates new joints between genomic DNA and their conserved ends.

molecular markers because their integration creates new joints between genomic DNA and their conserved ends. Most retrotransposon-based marker systems use PCR to amplify a segment of genomic DNA at this joint (Kalendar and Schulman, 2006). Hence, genomes diversify occurs through the insertion of new copies, but old copies persist. The differences between ancestral and derived specific locus becomes possible as the absence of the introduced sequence can be, with high confidence, considered ancestral. Simply, the presence of a given retrotransposon in related taxa suggests their orthologues integration while the absence of particular elements indicates the plesiomorphic condition prior to integration in more distant taxa. This presence/absence analyses can construct phylogenetic tree of species based on the availability of retrotransposons distribution and its irreversible events during evolution. This is why retrotransposons are assumed to represent powerful noise-poor synapomorphies (Shedlock and Okada, 2000).

Sequence-specific amplified polymorphism (SSAP)

Sequence-specific amplified polymorphism (S-SAP or SSAP) analysis is one of the first retrotransposon methods described (Waugh et al., 1997). In this method, one primer matched the end of retrotransposon and the other matched an AFLP-like restriction site adaptor (Figure 3). This method can be used mainly to measure the distribution and structure of specific retroelement populations in an organism. For SSAP analysis, primers corresponding to a region immediately upstream of the 3'LTR of the retroelement should be chosen and an adjacent restriction site in the flanking genomic DNA. The methods are used extensively ever since in

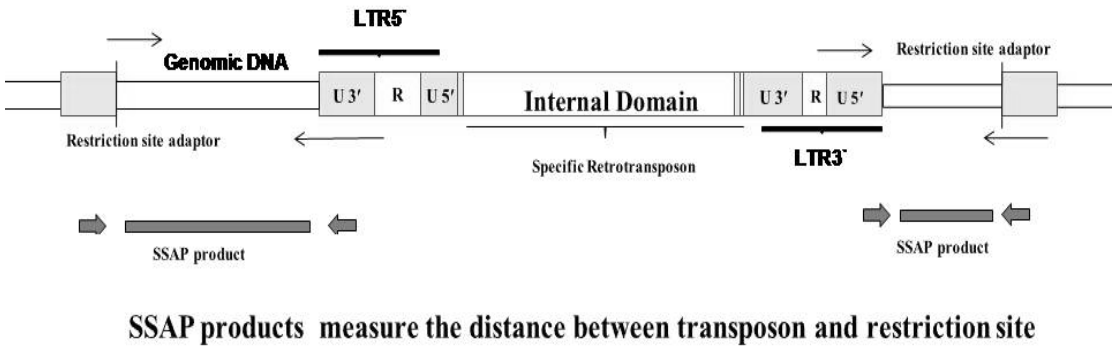

Figure 3. SSAP (Sequence Specific Amplified Polymorphism) measure the distance from the transposon to the restriction site. Amplification is carried out between primers matching an LTR and a restriction site adapter ligated to genomic DNA digested with a restriction enzyme.

many plant species; for instance, in barley (*Hordeum vulgare*) (Leigh et al., 2003), wheat (Queen et al, 2004) and Aegilops species (Nagy et al, 2006), oat (*Avena sativa L.*) (Yu and Wise, 2000), apple (Venturi et al, 2006), artichoke (Lanteri et al, 2006), lettuce (Syed et al, 2006), pea (*Pisum sativum*).and other legumes (Jing et al, 2005; Porceddu et al, 2002; Ellis et al; 1998), pepper and tomato (Tam et al, 2005) and sweet potato (Tahara et al, 2004).

SSAP analysis is also used as cladistic molecular markers to show evolutionary history in a given species. In this regard it was used in tobacco (*Nicotiana tabacum*) (Petit et al, 2007), Vicia species (Sanz et al., 2007), Rice (*Oryza sativa*) (Gao et al, 2004), wheat (Qween et al., 2004) and Zea (García-Martínez and Martínez-Izquierdo, 2003). For instance, it was used recently to show high number of species-specific subgroups of SIRE-1 retrotransposon in annual and perennial glycine species. Based on SSAP results, it was concluded that SIRE-1 retrotransposon has been active and evolving independently in each species during the course of glycine evolution (Chesnay et al, 2007).

Technical design of SSAP experiment.

SSAP marker systems are based on amplification between primers matching an LTR and a restriction site adapter ligated to genomic DNA digested with a restriction enzyme. LTR sequences of specific retrotransposons can be obtained by database searches or from published sequences. Different SSAP primers are usually cross-compared for SSAP marker quality by genotyping some genomic samples. The most promising primers, giving highly polymorphic, reproducible and clear SSAP banding patterns, are used subsequently. The SSAP procedure is usually performed exactly as described by (Waugh et al., 1997), with some modification. Selective amplifications are usually performed with primer pairs containing two or three selective nucleotides on MseI or PstI (or any other restriction enzyme) adapter primers and one selective nucleotide on the ^{33}P-labeled retrotransposon-based primers. Adaptor primers with selective bases are usually uses different combinations with retrotransposon primers. The amplified fragments are commonly separated on 6% polyacrylamide sequencing gels and are visualized by autoradiography.

Retrotransposons- based insertion polymorphism (PBRIP)

RBIP was primarly reported developed in studying the PDR1 retrotransposon in the pea (*Pisum sativum)* (Flavell *et al*., 1998). This method is more expensive and technically complicated than other methods for detecting transposon insertions. However, RBIP generates codominant markers, providing sufficient number of polymorphic retrotransposon insertion sites in the species under investigation. RBIP produces less data per experiment than do multiplex approaches but is more accurate for

studies of deeper phylogeny in wide germplasm (Jing et al., 2005).

For instance, it can detect both the presence and absence of the insertion, whereas other multiplex approaches detect onlythe presence of insertion, while absence is inferred by band absence. RBIP analysis was used as cladistic molecular markers to show evolutionary history in some given species such as Pea (*Pisum sativum*) (Vershinin et al., 2003); Pisum (*Pisum sp.*) (Jing et al., 2005).

Technical design of RBIP experiment.

Retrotransposons based insertion polymorphism (RBIP) is different from other methods. This is because it requires the sequence of the 5`and 3`regions flanking the retrotransposons insertions to be completely known. RBIP can detect the presence and absence of retrotransposon by PCR. Full sites containing a retrotransposon are generally scored by an amplification reaction with an LTR primer and a primer in flanking region (Figure 4). In this regard, a primer specific to retrotransposon LTR is used together with a primer designed to anneal to the flanking region to generate a product from template DNA containing the insertion. Meanwhile, primers specific to both flanking regions amplify a product if the insertion is absent. In other words, empty sites are usually scored by amplification between the left and right flanks of the presumptive integration site. Genomic polymorphisms can be identified by using standard agarose gel electrophoresis, or by hybridization to detect amplified PCR fragment. For automated and high throughput analysis, hybridization is more useful.

Inter-Retrotransposons Amplified Polymorphisms (IRAPs).

Inter-Retrotransposons Amplified Polymorphisms (IRAPs) is a valuable retrotransposon-based marker. It is based on the fact that retrotransposons generally tend to cluster together in 'repeat seas' surrounding 'genome islands', and may even nest within each other (Kalendar et al., 1999). The IRAP method detects retrotransposon insertional polymorphisms by amplifying the portion of DNA between two retroelements. This method requires neither restriction enzyme digestion nor ligation to generate the marker bands (Kalendar and Schulman, 2006). Variation in retrotransposon insertions into the genome means that the number of sites amplified and sizes of inter-retroelement fragments can be used as markers to detect genotypes polymorphisms, which in turn, could be used to measure diversity or reconstruct phylogeny (Kumar & Hirochika 2001). For instance, IRAP technique was used widely with BARE-1 primers in Barley (*Hordeum vulgare*) genome to measure the diversity between genotypes (Manninen et al., 2006; Brick et

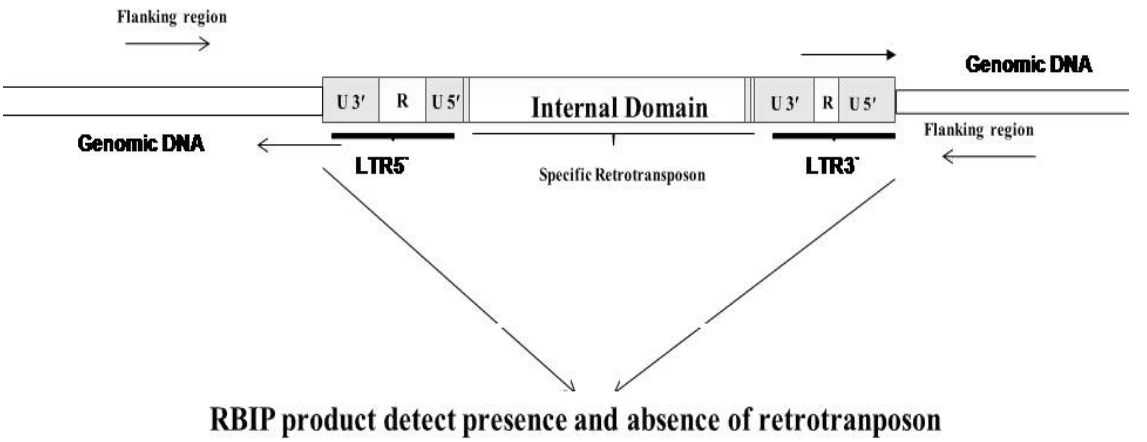

Figure 4. Retrotransposon based insertion polymorphism (RBIP). This technique can detect the presence and absence of retrotransposon.

al 2006; Manninen et al., 2000; Kalendar et al., 1999). This technique is used ever since to measure diversity, similarity and cladistic relationships in many genotypes such as rice (*Oryza sativa*) (Branco et al., 2007), orphan crop banana (Ashalatha et al., 2005; Teo et al., 2005), brassica (*Brassica sp.*) (Tatout et al., 1999), spartina (*Spartina sp.*) (Baumel et al., 2002), pisum (*Pisum sp.*) (Pearce et al., 2000), musa (*Musa sp.*) (Teo et al., 2002), and triticum (*Triticum sp.*) (Boyko et al., 2002)

Technical design of IRAP experiment

In this method, one or two PCR primers pointing outwards from the LTR of retrotransposons are made to amplify between two retroelements. The two primers may be from the same retrotransposon element family or from different families. The complexity of the resulting fingerprinting pattern is influenced by the retrotransposon copy number, by the insertion pattern and by the size of the retrotransposon families chosen for analysis. IRAP pattern represents the result of competition between the targets and products in the reaction. Therefore, the patterns obtained with the two primers do not represent the simple sum of the products obtained with each primer individually. In the case of retrotransposons dispersed within the genome, IRAP would either produce products too large to give good resolution on gels or target amplification sites too far apart to produce products. IRAP method overcomes the drawbacks of other techniques. For instance, proper use of the SSAP technique requires either radioactivity or fluorescent labeling of primers and product detection while IRAP does not.

Retrotransposon-microsatellite amplified polymorphism (REMAP)

REMAP differs from IRAP in the sense that REMAP primers are combined with locus-specific simple sequence repeat (SSR) primers to identify polymorphic products of the amplification of a segment between the retrotransposon and a SSR (Schulman and KalendarSchulman, 2006 and 1999) (Figure 6). This technique is based on the fact that the SSR site, of the form $(NN)^n$, $(NNN)^n$ or $(NNNN)^n$, are normally found throughout plant and animal genomes (Tsumura et al. 1996), Likewise, the retrotransposons. In most cases, it could be found close to retrotransposable elements. For instance, in cereals they appear to be associated with retrotransposons (Ramsay et al., 1999). This technique is used ever since to measure diversity, similarity and cladistic relationships in many genotypes such as (Manninen et al., 2000 and 2006; Brick et al., 2006; Kalendar et al., 1999), rice (*Oryza sativa.*) *(Branco et al 2007)*, Rice blast pathogen *(Magnaporthe grisea SP.*) (Chadha and Gopalakrishna, 2005), spartina *(Spartina sp.)* (Baumel et al., 2002) and oat *(Avena sativa L.)*. (Tanhuanpää et al., 2007).

Technical design of REMAP experiment

In REMAP, amplification between retrotransposons proximal to simple sequence repeats (microsatellites) produces the marker bands. This method also requires neither digestion with restriction enzymes nor ligation to generate the marker bands. However, produced bands are resulted from the amplification between retrotransposons close to simple sequence repeats (microsatellites). PCR

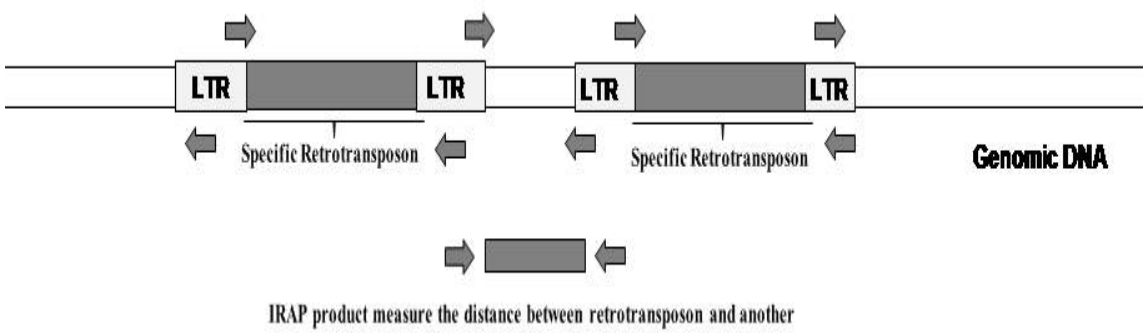

Figure 5. Inter-Retrotransposons Amplified Polymorphisms (IRAPs). IRAP product measures the distance between retrotransposon and another.

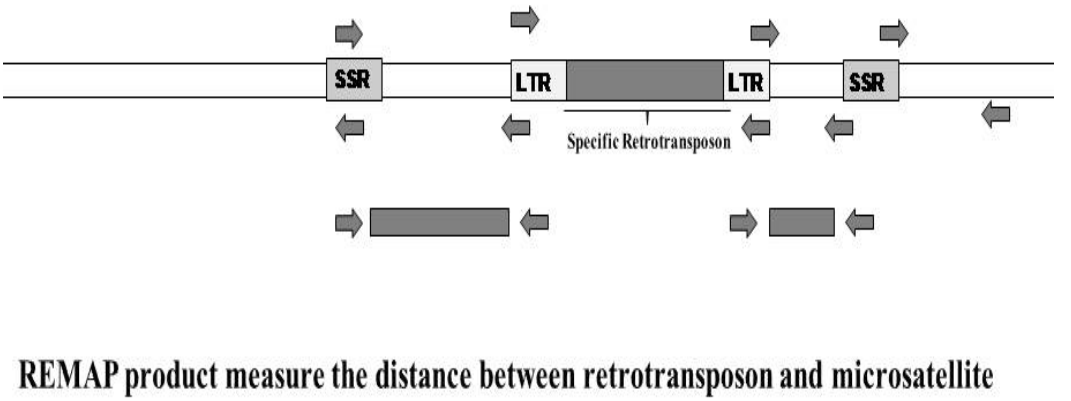

Figure 6. REMAP (Retrotransposon-microsatellite amplified polymorphism). Amplification is carried out between primers matching an LTR and a microsatellite domain (SSRs).

amplification can be performed using single primer or with two primers. Gel electrophoresis of the product is performed using optimal electrophoresis buffers and conditions. This protocol can be completed in 1-2 days. For samples with many or large (41000 bp) bands, gel electrophoresis is performed at a constant voltage of 70 V overnight (20 h). A useful guide for gel architectures differing from 20-20 cm is to first calculate the total volt-hours required for 500-bp fragments to migrate to the bottom of the gel and then adjust the voltage for subsequent runs so that the run time to achieve that total is at least 12 h (Kalendar and Schulman, 2006)

Conclusion

To detect polymorphisms for retrotransposon insertion, marker systems generally rely on PCR amplification between retrotransposon conserved ends and some component of flanking genomic DNA (Kalendar and Schulman, 2006). Based on the above mentioned facts, retrotransposon integration markers are ideal tools for determining the common ancestry of taxa by a shared derived transpositional event (Shedlock and Okada, 2000; Hamdi et al., 1999). Integration activity, persistence, dispersion, conserved structure and sequence motifs, and high copy number features together suggest that retrotransposons are appropriate genomic features on which to build molecular marker systems (Kalendar and Schulman, 2006). In this regard, retrotransposon insertional polymorphisms can be detected by a variety of PCR-based techniques in which outward-facing primers are designed to conserved domains such as LTRs within an elements such as SSAP (Waugh et al., 1997), RBIP (Flavell et al., 1998) and IRAP and REMAP (Kalendar et al., 1999).

Many phylogenetic studies were conducted based on retrotransposon presence/absence data. For instance, the definition of whales as members of the order Cetartiodactyla with hippos being their closest living relatives (Nikaido et al., 1999), hominoid relationships (Salem et al. 2003), the Strepsirrhine tree (Roos et al., 2004) and the placental mammalian evolution (Kriegs et al., 2006). Thus, we conclude that the utilization genomic retrotransposon as cladistic markers will enrich our understanding and provide us with new insight of evolutionary time scales of different organisms.

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