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# Retro transposon-markers: an overview of the recent progress in Citrus germplasm

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**Key words:** Retro transposon marker, SSAP, IRAP, REMAP, RBIP, Citrus germplasm.

**Abstract**

Citrus is a commercially important genus of the family Rutaceae and widely cultivated fruit crop in the world. Retro transposons are the most abundant class of transposable elements and they outnumber the genes in the eukaryotic genomes. Repetitive sequences make up a large part of the genome, up to 80% in certain species. Dozens or even hundreds of copies of members of some transposon families can be present in a single genome. Transposons can serve as a very rich source of identifiable polymorphisms. In this revised, several retro transposon-based marker systems such as SSAP, IRAP, REMAP and RBIP have been developed and discuss their use to visualize the genetic diversity generated by retro transposon in Citrus germplasm. Sequencing of the recent draft genome represents a valuable resource for understanding and improving of retro-transposons regions in Citrus germplasm. Therefore, sequences information of retro-transposons regions should exhibit more phylogenetic informative sites, which must be received more attention in future research in Citrus germplasm. Consequently, retro transposons integration markers are ideal tools for rapid characterization of Citrus and its related genera. This approach could be efficiently employed for conservation and management of Citrus germplasm genetic resource.

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## Introduction

Citrus is one of the most widespread fruit crops globally, which is widely grown in most areas with suitable climates tropical, subtropical between latitude 35°N~35°S. The total global production reported to be 7.4 million metric tons in 2009–2010 (FAOSTAT, 2010). Leading Citrus producing countries are China, Brazil, the USA and Spain and its native origins are China, eastern India, and Southeast Asia (Gmitter, 1995).

The genus Citrus L. belongs to the sub tribe *Citrineae*, the tribe *Citreae* within the subfamily *Aurantioideae* of the *Rutaceae* family (Webber, 1967). The *Aurantioideae* is one of seven subfamilies of *Rutaceae* which consists of two tribes and 33 genera (Ulubelde, 1985).

Citrus species have small genomes. Most species in these genera are diploid (2n=2x=18), with relatively small genomes; for instance, the size of the *Citrus sinensis* haploid genome is estimated to be 372 Mb (Arumuganathan and Earle, 1991). The largest and smallest genomes were respectively *Citrus medica L.* (the citron, with an average value of 398 Mb/ haploid genome) and Citrus reticulata Blanco (the mandarin, with an average value of 360 Mb/haploid genome). Citrus maxima (Burm) Merrill, the pummelo, was intermediate with an average 383 Mb/haploid genome (Gmitter *et al* ., 2012).

The taxonomy of Citrus and phylogeny is difficult and controversial due to various taxonomic systems, such as Swingle & Reece (1967), and Tanaka (1977), recognized 16 and 162 species, respectively. Also the relationship among taxa are complicated by several factors such as asexual seed production, high frequency of bud mutation, the long history of cultivation and the paucity of remaining wild Citrus species, wide dispersion and widely sexual compatibility between Citrus and related genera (*Poncirus* and *Fortunella*) ( Pang *et al*., 2007). *Poncirus* and *Fortunella* are the closely related genera of Citrus, which are sexually compatible with Citrus species (Froelicher *et al*., 2011). According to recent research there were originally only three different kinds of Citrus (Citron, *Pummelo* and Mandarin) and all other Citrus fruit are late hybrids of these three (http://users.kymp.net/citruspages/classification.html#history). Consequently, improvement of Citrus cultivar through conventional methods is quite difficult, inefficient, and costly and time consuming due to its prolonging juvenility, unusual sexual behavior and complex genetic background.

Citrus taxonomy and phylogeny, based on morphology and geography are very complicated, controversial and confusing (Jannati *et al*., 2009). Therefore, use of molecular markers has more advantages than that of morphologically based phenotypic characterization, because molecular markers are generally unaffected by external impact (Uzun and Yesiloglu, 2012).

Molecular markers have undergone great development in the last decade and are now routinely used in the management of *germplasm* collections (Krueger and Roose, 2003). The use of molecular markers for characterization and management of Citrus *germplasm* is currently in its early stages. There are many reports on the development or use of molecular markers in Citrus breeding, phylogenetic studies, etc,. Several attempts to revisit the intra and inter-species relationships in *Aurantioideae* (Nicolosi *et al*., 2000; Abkenar *et al*., 2004; Pang *et al*., 2007) have been constrained by restricted taxon representation, dependence on a few inferred sequences (RFLPs), or reliance on potentially unsound genetic markers for phylogenetic analysis such as isozymes, RAPDs, ISSRs, or SSR .Yet another marker system has emerged during the last few years, when it becomes evident that another class of highly abundant sequences dominates in genomes. Presently sequencing of the recent draft genome in Citrus *germplasm* (Xu *et al*., 2013) represents a valuable

resource for understanding and improving of numerous repeated sequences methods like microsatellites and retro-transposons regions. Retro transposons are the most abundant class of transposable elements and they outnumber the genes in the eukaryotic genomes. Their copy number and genomic locations are plastic (Mansour, 2008). 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 retro transposon content, suggesting that retro transposons might be important players in the evolution of genome size (Panaud and Vitte, 2005). Retro transposons (RTNs), together with transposons, represent a ubiquitous class of repetitive elements in all eukaryotic genomes (Smykal, 2006). They can constitute a majority of the genome, e.g. in grasses making up to 60–90% (Kumar and Bennetzen, 1999). Both their ubiquitous nature and activity in creating genomic diversity (Kazazian, 2004) by integrating stable DNA fragments into dispersed chromosomal loci make these elements ideal for use as molecular markers. Unlike transposons, the retro transposons do not excise, but are transcribed and by reverse transcription.

These retro-element groups can be distinguished on the basis of their structure, organization, and the amino acid sequences of the enzymes encoded, especially in the coding region of the reverse transcriptase (RT) (Bernet and Asins, 2003).
Retro transposons are one of the two major groups of transposable elements in eukaryotic genomes and are defined according to their mode of propagation (Kalendar, 2011). Retro transposons belong to class I TEs and transpose via RNA intermediate in contrast to other transposons (class II) that do not have RNA intermediate (Finnegan, 1989). Retro transposons are separated in two major subclasses that differ in their structure and transposition cycle. These are the LTR retro transposons and the non-LTR retro transposons (long interspersed repetitive elements (LINE) and short interspersed nuclear elements (SINE), are distinguished by the respective presence or absence of long terminal repeats (LTRs) at their ends (Kalendar, 2011). All groups are complemented by their respective no autonomous forms which lack one or more of the genes essential for transposition: MITEs (Miniature Inverted-Repeat Tandem Elements), for Class II, SINEs for non-LTR retro transposons, and TRIMs (Terminal-Repeat Retro transposons in Miniature) and LARDs (Large Retro transposon Derivatives) for LTR retro transposons (Kalendar *et al.*, 2004). LTR retro transposons are transcribed from one LTR of an integrated element to produce a nearly full length RNA copy containing a single copy of the LTR split between its two ends. This RNA is then reverse-transcribed into an extra chromosomal cDNA, reconstituting the full length element that is ultimately integrated back into the genome. Immediately internal to the LTRs are the priming sites for reverse transcription (Kalendar, 2011).The ubiquitous presence of LTR RTNs in plant genomes suggests that the use of these techniques in combination would allow breeders to obtain markers close to virtually any important agronomical trait and that the hyper variable nature of these elements should make them excellent sources of polymorphic markers (Mandoulakani *et al.,* 2012)..

*Retro transposons as Molecular Markers in citrus germplasm*

Retro transposon marker systems generally rely on PCR to generate fingerprints (Kalendar and Schulman, 2006). The marker systems generate fingerprints, or multilocus profiles, for the members of given families of retro transposons (Antonius-Klemola *et al.,* 2006). Hence, retro transposons provide an excellent opportunity to develop molecular marker system (Kalendar *et al.*, 1999) due to their long, defined, conserved sequences and new insertional polymorphisms produced by replication

ally active members. In the context, retro transposon-based molecular analysis relies on amplification using a primer corresponding to the retro transposon and a primer matching a section of the neighboring genome (Agarwal *et al.*, 2008).

Retro transposons insertions behave as Mendelian loci (Huo *et al.*, 2009). Hence, retro transposon-based markers would be expected to be co-dominant and involve a different level of genetic variability, i.e. transposition events, than arbitrary markers systems such as RAPD or AFLPs, which detect polymorphism from simple nucleotide changes to genomic rearrangements (Kalendar, 2011). Consequently, retro transposon markers are more informative in a variety of crops including Citrus germplasm. Unfortunately retro transposon markers are still less explored in Citrus research comparing to other plant species. Asins *et al.*, (1999), investigated the presence of copia-like retro transposons in Citrus genome and found that these elements were quite abundant throughout the Citrus genome and very heterogeneous for the RT domain. Additionally, polymorphisms based on copia-like elements have been found distinguishing groups of varieties within Citrus sinensis (Asins *et al.,* 1999), Citrus clementina (Breto *et al.,* 2001) and Citrus limon (Bernet *et al.*, 2004). Moreover, polymorphisms based on these elements are more abundant than those based on primers of random sequence or simple sequence repeats (Breto *et al.,* 2001).

Several retro transposon-based marker systems have been developed in Citrus and below we present an overview of the various retro transposon-based molecular marker methods that have been developed and discuss their use to visualize the genetic diversity in Citrus germplasm generated by retro transposon.

*Sequence-specific amplified polymorphism (SSAP)*

Sequence-specific amplified polymorphism (S-SAP or SSAP) analysis is one of the first retro transposon methods described (Waugh *et al.*, 1997). SSAP can be used mainly to measure the distribution and structure of specific retro element populations in an organism (Kalendar, 2011).

**Fig. 1.** Schematic representation of Sequence Specific Amplified Polymorphisms technique (SSAP).

The SSAP procedure was modified utilizing universal retro transposon primers instead of sequence-specific primers (Wegscheider, 2006). By using universal primers it was expected to target a wider spectrum of retro transposon sequences or remnants thereof in the plant genome. However, one primer matched the end of retro transposon and the other matched an AFLP-like restriction site adaptor (Fig. 1).Hence, 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 (Mansour, 2008). Usually, SSAP shows more polymorphism, more co-dominance and more chromosomal distribution than AFLP (Kalendar, 2011).Indeed, SSAP has been optimized for multiple plant species and protocols for rapidly obtaining retro transposon sequence information for SSAP primer design have been developed (Kalendar *et al.*, 2010). SSAP are used extensively ever since in many plant species, for instance, in barley (Leigh *et al.*, 2003), wheat (Queen *et al.*, 2004), lettuce (Syed *et al.*, 2006), pepper and tomato (Tam *et al.*, 2005), sweet potato (Tahara *et al.,* 2004), apple (Venturi *et al.*, 2006) and in Citrus germplasm (Biswas *et al.*, 2011). The recent investigation by Biswas *et al.*, (2011), suggested that SSAP and SAMPL are more effective for Citrus species identification and certification than the

AFLP. He explains that SSAP and SAMPL had a higher discrimination capacity in Citrus germplasm and could simultaneously detect several polymorphic markers per reaction.

In principle, All SSAP systems described to date utilize LTRs (Long Terminal Repeats) of Ty1-copia or Ty3-gypsy retro transposons. They were all proved to be as efficient as or even more efficient than the original AFLP technique (Grzebelus, 2006). There is clear evidence that retro-elements in Citrus genome are heterogeneous due to their variable number and size (Asins *et al.*, 1999) Ty-1 copia-like-retro transposon are ubiquitous with variable size and number in Citrus genome, it is present in Citrus genome around 1.8–7.2 molecules and accounted for almost 17% of whole genome. Which lead to find polymorphism among the tested Citrus genotypes? It is confirmatory to the earlier reports regarding the presence of Ty-1 copies elements and their abundance in Citrus genome (Wei, 2007). Unfortunately, to date, the information about SSAP transposons in Citrus germplasm is very limited.

*Inter-Retro transposons Amplified Polymorphisms (IRAP)*

Inter-Retro transposons Amplified Polymorphisms (IRAP) is a valuable retro transposon-based marker. In this method, PCR oligonucleotide primers facing outwards from the LTR or other regions of retro transposons are made and amplify between two retro elements inserted into the genome (Flavell *et al.*, 1998; Kumar and Hirochika, 2001). This method requires neither restriction enzyme digestion nor ligation to generate the marker bands (Kalendar and Schulman, 2006). The IRAP amplification primers (Fig. 2) are commonly designed to match segments of LTRs conserved within element families (Antonius-Klemola *et al.,* 2006). The variation in retro transposon insertions into the genome means that the number of sites amplified and sizes of inter-retro element fragments can be used as markers to detect genotypes polymorphisms, which in turn, could be used to measure diversity or reconstruct phylogeny (Kumar and Hirohiko, 2001). Polymorphisms based on copia and gypsy have been used distinguishing groups of varieties within Citrus sinensis (Asins *et al.*, 1999), Citrus clementina (Breto *et al.*, 2001), Citrus limon (Bernet *et al.*, 2004).Moreover, Gypsy like retro transposon was identified in Citrus and Poncirus genome and studied their distribution on the genome (Bernet and Asins, 2003). Linkage analysis of IRAP has shown that gypsy elements are less abundant than copia elements in the Citrus genome (Ruiz *et al.*, 2003). Whereas, Bernet and Asins (2003), reported that gypsy based IRAPs present a different genomic distribution compared to that of copia-based IRAPs, they constitute a new, complementary set of molecular markers that are available to study cultivar diversity and follow the variation of agronomic traits in segregate progenies derived from Citrus. Therefore, Ty1-copia retro transposons possess several important characteristics that make them suitable for studying the structure and organization of plant genomes (Khaliq, 2009). In subsequence study, copia-like retro transposons were isolated and characterized from 12 Sweet Orange (Citrus sinensis Osbeck) Cultivars, and found that high heterogeneity among the cloned sequences, frame shifting, termination, deletion, and substitution accounted for the heterogeneity of these retro transposons sequences. Different copy number also reported in this study among the tested sweet orange cultivars. This study suggested that retro transposon might be an effective molecular marker to detect Citrus evolution events and to reveal its relationship with bud mutation (Tao *et al.*, 2005). In a related approach, retro-transposon based genetic similarity among the 48 Citrus and its relative's species was investigated by Biswas *et al.*, (2010b). The genetic similarities results of this investigation agreed with the previously reported phylogenetic relationship of Citrus and suggested that retro transposon based fingerprinting methods are useful tool for rapid characterization of Citrus and its related genera. This approach might be efficiently

employed for conservation and management of Citrus germplasm genetic resource.

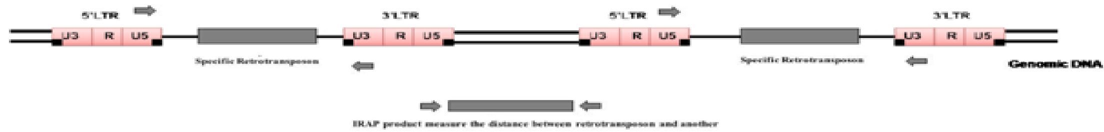


**Fig. 2.** Schematic representation of Inter-Retrotransposons Amplified Polymorphisms technique (IRAPs).

*Retrotransposon-microsatellite amplified polymorphism (REMAP)*

The REMAP method is similar to IRAP, but one of the two primers matches a microsatellite motif (Fig. 3) (Kalendar *et al.*, 1999; Kalendar and Schulman, 2006).

The REMAP method relies on one outward-facing LTR primer and a second primer from a microsatellite (Kalendar *et al.*, 1999). Recently, kalendar *et al.,* (2011), reported that banding patterns are completely different if REMAP primers are used individually or in combination, indicating that the majority of bands are derived from sequences bordered by a microsatellite on one side, and by an LTR on the other. Furthermore, REMAP, anchor nucleotides are used at the 3' end of the SSR primer to avoid slippage of the primer within the SSR. It also prevents the detection of variation in repeat numbers within the microsatellite (kalendar, 2011). As discussed above, produced bands are resulted from the amplification between retro transposons close to simple sequence repeats (microsatellites) (Mansour, 2008). In addition to the above, PCR amplification can be performed using single primer or with two primers. Therefore, they may show much variation at individual loci within a species (kalendar, 2011). This technique is used ever since to measure diversity, similarity and cladistics relationships in many genotypes such as rice (Oryza sativa) (Branco *et al.*, 2007), oat (Avena sativa L.) (Tanhuanpaa *et al.*, 2007) and in Citrus germplasm (Biswas *et al.*, (2010 a,b).The IRAP and REMAP techniques can be used separately or combined for a more complete genome survey, and they are excellent sources of polymorphic markers (Yuan *et al.*, 2010). The ubiquitous presence of LTR retro transposons in plant genomes suggests that the use of these techniques (either isolated or in combination) would allow breeders to obtain markers close to virtually any important agronomical trait and that the hyper variable nature of these repeat elements should make them excellent sources of polymorphic markers (Branco *et al.*, 2007). As stated above, IRAP and REMAP retro transposon based fingerprinting methods are useful tool for rapid characterization of Citrus and its related genera. This approach could be efficiently employed for conservation and management of Citrus germplasm genetic resource.

*Retro transposons-based insertion polymorphism (RBIP)*

Among the classes of retro transposon-based markers, RBIP markers can detect polymorphism caused by the integration of an element at a particular locus and supply an accurate DNA profile (Kim *et al.*, 2012). RBIP (Retro transposons-based insertion polymorphism) was described as a simple PCR based detection of retro transposon insertions using PCR between primers flanking the insertion site and primers from the insertion itself (Kalendar, 2011).This technique was primary reported developed using the PDR1 retro transposon in the pea (Pisum sativum) (Flavell *et al.*, 1998). It requires the sequence information of the 50 and 30 regions flanking the transposon (Agarwal *et al.*, 2008). In fact, RBIP is the sole retro transposon method designed to detect polymorphism for the integration of an element at a particular locus (Fig. 4) (Kalendar *et al.*, 2011). The RBIP method uses primers flanking retro transposon insertions and

scores the presence and absence of insertions at individual sites (Kim *et al.*, 2012).This method is more expensive and technically complicated than other methods for detecting transposon insertions (Mansour, 2007). Whereas, it generates co-dominant markers, which can be at least as useful as SSR markers, provided that a sufficient number of polymorphic transposon insertion sites are identified in the species under investigation (Grzebelus *et al.,* 2006). 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). Therefore, RBIP is the only retro transposon-based method designed to detect polymorphism for the integration of an element at a particular locus (Kalendar *et al.*, 2011). RBIP has been used in rice (Vitte *et al.*, 2004) to address the issue of the evolution of Indica and Japonica rice varieties. In a related approach, Queen *et al.,* (2004), confirmed that RBIP markers are distributed widely enough to support genetic mapping projects within the generally narrower germplasm of cultivars. Unfortunately, to date, no such studies have been reported yet about retro transposons-based insertion polymorphism and their effectiveness and mapping ability in Citrus germplasm.
Through the discussion above, we can declare that retro transposon markers have been demonstrated to be powerful tools for investigating linkage, evolution and genetic diversity in plants (Antonius-Klemola *et al.*, 2006). Indeed, retro transposons markers can be used to detect genotype variation among species or interspecies in Citrus and its relative species.

This article maybe will provide new information and facilitate about retro transposons markers for advancing breeding techniques and developing better conservation strategies, evolutionary, phylogenetic and population genetic studies in the genus Citrus and its relative species.

**Conclusion**

Molecular marker techniques allow the analysis of variation at the genomic level and permit detection of genetic variation at the genomic level. Therefore, information obtained from the molecular level could be used to assess genetic relationships among the major germplasm groups, genetic diversity, fingerprinting, hybrid identification, linkage map construction and marker assisted selection etc. in Citrus germplasm. Retro transposons are the largest class of transposable elements and occur in all plant genomes. The elements transpose via an RNA intermediate, which leads to an increase in the element copy number and, consequently, plant genome expansion (Sun *et al.*, 2012).

Retro-transposons are common components of plant genomes, functional at transcription, translation and integration levels. Their abundance and ability to transpose render them good potential markers. Markers based on LTR retro transposons, often generically referred to as ‘transposon display,’ have come of age since their introduction over 13 years ago. At least 99 studies using these marker systems had been published by the end of 2009. A major disadvantage of SSAP, IRAP, REMAP and RBIP all the methods described above is the need for retro transposon sequence information to design family-specific primers. However, related species have similar TE sequences (retro elements or transposons), meaning that primers for the anonymous marker methods described above (SSAP, IRAP, REMAP and RBIP) from one species can be used in another species. In this article, TE markers can be readily used across species lines, among closely related genera of Citrus germplasm. In this case, primers designed to conserved TE sequences are advantageous. Moreover, TEs are dispersed throughout the genome and often interspersed with other elements and repeats. By combining PCR primers from different classes of repeats and families of LTRs, PCR fingerprints can be improved. Sequencing of the recent draft genome represents a valuable resource for understanding and improving of retro-transposons regions in Citrus germplasm. Therefore, sequences information of retro-transposons regions should exhibit more

phylogenetic informative sites, which must be received more attention in future research in Citrus germplasm. Consequently, retro transposons integration markers are ideal tools for rapid characterization of Citrus and its related genera. This approach could be efficiently employed for conservation and management of Citrus germplasm genetic resource.

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