DETECTION OF ANCIENT GENOME DUPLICATIONS IN SEVERAL FLOWERING PLANT LINEAGES AND SYNTE-MOLECULAR COMPARISON OF HOMOLOGOUS REGIONS

by

JINGPING LI

(Under the Direction of Andrew H. Paterson)

ABSTRACT

Flowering plants have evolved through repeated ancient genome duplications (or paleo-polyploidies) for ~200 million years, a character distinct from other eukaryotic lineages. Paleo-duplicated genomes returned to diploid heredity by means of extensive sequence loss and rearrangement. Therefore repeated paleo-polyploidies have left multiple homeologs (paralogs produced in genome duplication) and complex networks of homeology in modern flowering plant genomes. In the first part of my research three paleo-polyploidy events are discussed. The Solanaceae "T" event was a hexaploidy making extant Solanaceae species rare as having derived from two successive paleo-hexaploidies. The *Gossypium* "C" event was the first paleo-(do)decaploidy identified. The sacred lotus (*Nelumbo nucifera*) was the first sequenced basal eudicot, which has a lineage-specific "λ" paleo-tetraploidy and one of the slowest lineage evolutionary rates. In the second part I describe a generalized method, GeDupMap, to simultaneously infer multiple paleo-polyploidy events on a phylogeny of multiple lineages. Based on such inferences the program systematically organizes homeologous regions between pairs of genomes into groups of orthologous regions, enabling synte-molecular analyses (molecular comparison on synteny backbone) and graph representation. Using 8 selected eudicot and monocot genomes I showed this framework of multiple paleo-polyploidy detection and synte-molecular network facilitates genomic comparisons among flowering plants and reveals deep correspondences among their genome structure.

INDEX WORDS: Ancient genome duplication, Paleo-polyploidy, Synteny, Synte-molecular comparison, Genome evolution, Flowering plants

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DEDICATION

To my parents.

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CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

Flowering plants, or angiosperms, the Earth's most successful vegetation, dominate most terrestrial and semiaquatic habitats, and inhabit many aquatic habitats. They form the most abundant group of all plants, including more than 80% of known plant species. In total there are more than 270,000 recorded species of angiosperms (for example compared to about 75,000 species of chordates). The enormous number and ubiquitous distribution of flowering plants are equated with their astonishing diversity and adaptation, such as those exhibited in ranges from *Colobanthus quitensis* (Antarctic pearlwort) to *Lecythis ampla* (rainforest emergent), from cactus (desert specialist) to *Zostera* (marine eelgrass), from *Wolffia* (flower < 0.5mm in diameter) to *Rafflesia* (flower one meter in diameter), from *Spartina alterniflora* (salt mashes specialist) to *Saxifraga oppositifolia* (blooming in highest mountains), from the "all-healing" *Panax ginseng* to the carnivorous *Nepenthes* (pitcher plants). Diverse characters of flowering plants have been associated with human society from its birth, and are now indispensable ingredients in almost all aspects of human society. It is of great interest and efforts to study flowering plants.

Despite their tremendous diversity, the majority of research so far indicated that all modern angiosperm species evolved from a crown group common ancestor, which likely lived in the Jurassic period (Bell, Soltis, & Soltis, 2010; D. E. Soltis *et al.*, 2009; Wikstrom, Savolainen, & Chase, 2001) about 200~145 MYA (million years ago). While evolutionary rates of different angiosperm lineages are variable and generally more rapid than animals, this is nonetheless recent enough that comparative approaches are especially useful to reveal homologous and unique characters across the flowering plant phylogeny.

An early and fundamental observation about plant genome structure is that most angiosperm genomes are paleo-polyploid (V. Grant, 1981; Masterson, 1994; G. L. Stebbins, 1966). While having disomic inheritance now, these genomes possess multiple homologous loci retained from one or more paleo-polyploidy, or ancient genome duplication, events. In fact, many angiosperm genomes experienced

repeated paleo-polyploidies, followed by extensive gene loss and genome rearrangement in the "diploidization" processes that restored them to disomy and generally low chromosome numbers. As a result, there are high and variable levels of genomic redundancy and complex 'networks' of gene synteny/colinearity among duplicated regions, making alignment of angiosperm genomes surprisingly difficult. Accordingly, it is essential to know the paleo-polyploidy history as the prerequisite to angiosperm genome comparisons and downstream analyses, for example, to study the evolution of specific gene families, genomic architecture of some traits, or to transfer knowledge from model to non-model organisms. These goals fully rely on accurate and sensitive paleo-polyploidy inference and homologous loci mapping.

1.1 Dissertation structure and related publications

My dissertation describes two main results: 1. Identification and characterization of three paleo-polyploidy (ancient genome duplication) events, including the first characterized paleo-polyploidy and paleo-hexaploidy in asterid plants (Chapter 2), the first identified paleo-(do)decaploidy (Chapter 4), and the first identified paleo-polyploidy in basal eudicots (Chapter 3), and circumscription of two paleo-tetraploidies in monocots (Chapter 5); 2. The GeDupMap program (Chapter 6) for simultaneous detection of multiple paleo-polyploidies in multiple lineages, and a synte-molecular framework for detailed comparison of those genomes.

The dissertation starts with this introduction chapter to lay out the necessary background for my research, and lead to the questions I aimed to answer. Then Chapters 2, 3, 4 discuss three newly identified paleo-polyploidy events, which I contributed initially as part of their reference genome projects: the Solanaceae "T" event (in the tomato genome sequencing project (Tomato Genome Consortium, 2012)), the *Gossypium* "C" event (in the cotton genome sequencing project (Paterson *et al.*, 2012)), and the *Nelumbo* "λ" event (in the sacred lotus genome sequencing project (Ming *et al.*, 2013)). These analyses were done in collaboration with former and current lab members Haibao Tang (tomato, cotton, lotus), Xiyin Wang (tomato, cotton), and John E. Bowers (lotus). It should be noted that Haibao Tang is

responsible for the initial discovery that the Solanaceae paleo-polyploidy is a hexaploidy rather than a tetraploidy. Chapter 5 describes circumscription of two monocot paleo-tetraploidies using synteny patterns of four monocot genomes (rice, sorghum, oil palm, banana) and two outgroup eudicot genomes (grape, lotus). Chapter 6 is about a Python program GeDupMap I wrote which for the first time enables simultaneous detection of multiple paleo-polyploidy events in multiple lineages. The algorithm is based on mapping of syntenic regions between the input genomes, and also sorts out related regions across the input genomes, which can then be represented in novel graph formats such as the A Bruijn graph. This synte-molecular framework facilitates alignments of local regions, genes, and nucleotide sequences among those genomes. Using this framework I also identified genomic regions that are 'deletion-resistant' or 'duplication-resistant' across repeated paleo-polyploidies. Finally Chapter 7 summarizes major results of the dissertation and gives a short perspective.

Of this dissertation Chapters 1, 3, 5, 7 contain partial contents from my publications listed below. In those papers only my sections were included here while those from other co-authors were excluded. In many cases the contents have been re-organized to better fit in this dissertation. Chapter 2 is a re-print of a to-be-published book chapter (J. Li, Tang, Wang, & Paterson), except the preface. Copyright permissions have been requested from the respective journals. Chapters 4 and 6 are two manuscripts soon to be submitted.

List of my publications related to this dissertation:

- Li, J., Tang, H., Wang, X., & Paterson, A. H. Two paleo-hexaploidies underlie formation of modern Solanaceae genome structure. In M. Causse, J. Giovannoni, M. Bouzayen & M. Zouine (Eds.), *Compendium of Plant Genomes: The Tomato Genome*: Springer. (in print)
- Li, J., Tang, H., Bowers, J. E., Ming, R., & Paterson, A. H. (2014). Insights into the Common Ancestor of Eudicots. In A. H. Paterson (Ed.), *Genomes of Herbaceous Land Plants* (Vol. 69, pp. 137-174): Elsevier.
- Paterson, A. H., Wang, X., Li, J., & Tang, H. (2012). Ancient and Recent Polyploidy in

Monocots. In P. S. Soltis & D. E. Soltis (Eds.), *Polyploidy and Genome Evolution* (pp. 93-108): Springer Berlin Heidelberg.

- Jiao, Y., Li, J., Tang, H., & Paterson, A. H. (2014). Integrated syntenic and phylogenomic analyses reveal an ancient genome duplication in monocots. *Plant Cell, 26*(7), 2792-2802.
- Ming, R., Vanburen, R., Liu, Y., Yang, M., Han, Y., *et al.* (2013). Genome of the long-living sacred lotus (*Nelumbo nucifera* Gaertn.). *Genome Biol, 14*(5), R41.
- Tomato Genome Consortium. (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature, 485*(7400), 635-641.
- Paterson, A. H., Wendel, J. F., Gundlach, H., Guo, H., Jenkins, J., *et al.* (2012). Repeated polyploidization of *Gossypium* genomes and the evolution of spinnable cotton fibres. *Nature, 492*(7429), 423-427.
- Wang, X., Wang, H., Wang, J., Sun, R., Wu, J., *et al.* (2011). The genome of the mesopolyploid crop species *Brassica rapa*. *Nat Genet, 43*(10), 1035-1039.

1.2 Brief overview of flowering plant phylogeny

Flowering plants, or angiosperms, can be classified into six major clades including dicotyledons (~198,000 species in ~336 families), monocotyledons (~62,000 species in ~93 families), Ceratophyllaceae, magnoliids, Chloranthaceae, and basal angiosperms (Hedges & Kumar, 2009; Stevens, 2012; The Angiosperm Phylogeny Group, 2009). Eudicots and monocots together comprise ~96% of living angiosperm species. Eudicot plants typically have two embryonic cotyledons (thus the name "eudicotyledons"), most of which also have tricolpate pollen grains. In contrast, monocot plants typically have one embryonic cotyledon (thus the name "monocotyledons"), most of which also have trimerous flowers. Most sequenced plant genomes come from the two clades.

Many studies supported that living angiosperm lineages form a monophyletic clade, having evolved from a common ancestor that diverged from other seed plants in the Triassic to Jurassic periods

about 240~145 MYA (Bell *et al.*, 2010; Clarke, Warnock, & Donoghue, 2011; James A. Doyle, 2012; S. A. Smith, Beaulieu, & Donoghue, 2010; D. E. Soltis *et al.*, 2009; Wikstrom *et al.*, 2001). Being the most abundant and diverse group of all plants, angiosperms are also a young division: gymnosperms first emerged at least ~340 MYA, other vascular ~420 MYA and non-vascular land plants ~450 MYA, and the streptophyte algae ~725 MYA. Nonetheless, through several phases of morphological and functional diversification, by the late Cretaceous angiosperms had successfully dominated many habitats across the Earth (Crane & Lidgard, 1989; J. A. Doyle & Donoghue, 1993; Friis, Pedersen, & Crane, 2006). The majority of extant angiosperm lineages emerged so suddenly in the evolutionary history that Darwin in 1879 once referred to it as "an abominable mystery".

Early angiosperms diversified extensively and very rapidly in the early Cretaceous (Crane, Friis, & Pedersen, 1995; Hickey & Doyle, 1977), perhaps within about 5 million years (Moore, Bell, Soltis, & Soltis, 2007; D. E. Soltis, Bell, Kim, & Soltis, 2008). Molecular estimations suggested that basal angiosperm lineages diverged from mesangiospermae around 170~140 MYA, after which the five clades of mesangiospermae, Chloranthales, magnoliids, monocots, eudicots and Ceratophyllales, rapidly separated, with the exact order of origins remaining uncertain (Bell *et al.*, 2010; Magallón, 2009; Moore *et al.*, 2007). The two major angiosperm clades, eudicots and monocots, are estimated to have diverged some time between 160~125 MYA (Bell *et al.*, 2010; Clarke *et al.*, 2011; Crane *et al.*, 1995; S. A. Smith *et al.*, 2010; D. E. Soltis *et al.*, 2008; K. H. Wolfe, Gouy, Yang, Sharp, & Li, 1989), in general accordance with current fossil records (Friis, Pedersen, & Crane, 2010; Sun, Dilcher, Wang, & Chen, 2011). This is a time period overlapping with the Gondwanaland break-up (Ezcurra & Agnolin, 2012), emergence of bees and a major radiation period of insects (Cardinal & Danforth, 2013; Grimaldi, 1999), which may be important environmental factors driving rapid diversification of early angiosperms. The first major diversification in the dicotyledon clade was estimated to have occurred in early to mid-Cretaceous, involving many aspects of the organisms' physiology such as floral structure, pollen structure, leaves, and pollination type (Crane *et al.*, 1995; Friis *et al.*, 2006; Hickey & Doyle, 1977). Evidence also revealed extensive diversifications of core eudicots and monocots respectively, beginning

in late Cretaceous (Crane *et al.*, 1995; Friis *et al.*, 2006). Episodic rapid diversification is a theme throughout angiosperm evolution (Moore, Soltis, Bell, Burleigh, & Soltis, 2010; S. A. Smith *et al.*, 2010; D. E. Soltis *et al.*, 2008).

This brief overview of the angiosperm phylogeny is intended as necessary background for my research subject. For detailed discussions of morphological, phylogenetic characters and evolution of angiosperm lineages the readers are referred to comprehensive review articles and books such as (Bell *et al.*, 2010; Crane *et al.*, 1995; James A. Doyle, 2012; Friis, Crane, & Pedersen, 2011; Magallón, 2009; D. E. Soltis *et al.*, 2008; D. E. Soltis, Soltis, Endress, & Chase, 2005), and the Angiosperm Phylogeny Website (http://www.mobot.org/MOBOT/research/APweb/welcome.html).

Many genes in floral development pathways appear to have been duplicated in parallel time frames around early angiosperm and eudicot diversification events, implying that they may have been produced in polyploidy events rather than individual gene duplications (reviewed in (D. E. Soltis *et al.*, 2008)). It is now known that all angiosperms are paleo-polyploids, having experienced one or more whole genome duplications (WGDs) at some point(s) during their evolutionary histories (Blanc & Wolfe, 2004; Bowers, Chapman, Rong, & Paterson, 2003; Jiao *et al.*, 2011; Tang, Bowers, *et al.*, 2008). Widespread paleo-polyploidy events in angiosperms, and their trend of coincident occurrence with major species radiations support the hypothesis that paleo-polyploidies were a major driving force in angiosperm evolution and diversification (J. J. Doyle *et al.*, 2008; Fawcett, Maere, & Van de Peer, 2009; Lynch & Conery, 2000; Otto & Whitton, 2000; Paterson, Bowers, & Chapman, 2004; D. E. Soltis *et al.*, 2009), while the ultimate radiation of species and diversity in the affected lineages likely depends on many post-WGD factors such as migration, environmental changes, and differential extinction rates (Schranz, Mohammadin, & Edger, 2012).

1.3 Repeated ancient genome duplications prevalent in flowering plant evolution

Several seminal cytological and genetic studies revealed duplications of genes and chromosome segments that were thought to be possibly ancient and of evolutionary importance (McClintock, 1941; Metz, 1947;

Rhoades, 1951; Stadler, 1929). On the other hand, plants with high haploid chromosome numbers were hypothesized to have possibly originated via polyploidy, a major mechanism of plant speciation (V. Grant, 1981; George Ledyard Stebbins, 1950, 1971). As genetic maps became available for some plant genomes, regions of colinear markers were identified between different linkage groups, providing more concrete support than ever before for possible paleo-polyploidies in several lineages (Ahn & Tanksley, 1993; Chittenden, Schertz, Lin, Wing, & Paterson, 1994; Helentjaris, Weber, & Wright, 1988; K. M. Song, Suzuki, Slocum, Williams, & Osborn, 1991; Whitkus, Doebley, & Lee, 1992), and in some cases multiple incidences in a single lineage (Kowalski, Lan, Feldmann, & Paterson, 1994; Shoemaker *et al.*, 1996). The first plant genome sequence (Arabidopsis Genome Initiative, 2000) confirmed that even the compact 125 Mb genome of thale cress was indeed a paleo-polyploid, and further revealed surprising multiple rounds of paleo-polyploidies (Blanc, Barakat, Guyot, Cooke, & Delseny, 2000; Blanc, Hokamp, & Wolfe, 2003; Bowers *et al.*, 2003; D. Grant, Cregan, & Shoemaker, 2000; Ku, Vision, Liu, & Tanksley, 2000; Paterson *et al.*, 2000; Simillion, Vandepoele, Van Montagu, Zabeau, & Van de Peer, 2002; Vision, Brown, & Tanksley, 2000). Since then, rapidly growing scale of genome sequencing has nurtured paleo-polyploidy studies in expanding clades of the angiosperm phylogeny, opening the 'big data' era of comparative studies in plant genome structure and evolution.

But for now let's step back and look at the genetic basis of polyploid formation. Polyploid plant cells, having more than one set of diploid chromosomes, are formed in two ways. Autopolyploids acquire multiple sets of a single parent genome mainly through unreduced gametes, or less frequently through somatic doubling. Examples of neo-autopolyploid plants include sugar cane in monocots and potato in eudicots. On the other hand, allopolyploids are derived from fusion of different genomes usually through hybridization of related species. Examples of neo-allopolyploid plants include bread wheat in monocots and cultivated cotton in eudicots.

Polyploidies that involve more than doubling of a genome, for example hexaploidy (genome triplication), can occur in several different ways (**Figure 1.1**). In panel 1 the auto-hexaploid (AAAAAA, 2n=6x) is formed by joining three identical diploid genomes (2n=2x). Two natural hexaploids, the 'marsh

pea', *Lathyrus palustris* (Khawaja, Ellis, & Sybenga, 1995) and the grass 'timothy', *Phleum pretense* (NordenskiÖLd, 1953), were formed in this way. Panel 2 illustrates one-step allo-hexaploid formation. In panel 3 the hexaploid organism (AAAABB, 2n=6x) is formed by a combination of an auto-tetraploidization (resulting in AAAA, 2n=4x) and a subsequent allo-tetraploidization with a related diploid (BB, 2n=2x) organism. The recent hexaploid wheat *Triticum zhukovskyi* and some synthetic hexaploid cotton species (Brown & Menzel, 1952) were formed in this way. In panel 4 the hexaploid (AABBCC, 2n=6x) is formed by two successive allo-tetraploidies. The bread wheat (*Triticum aestivum*) (Matsuoka, 2011) and some synthetic hexaploid cottons (Brown & Menzel, 1952) were formed in this way. In theory, the hexaploid organisms in panel 3 and 4 can also be formed in one step, as described in panel 2, likely via processes similar to double fertilization.

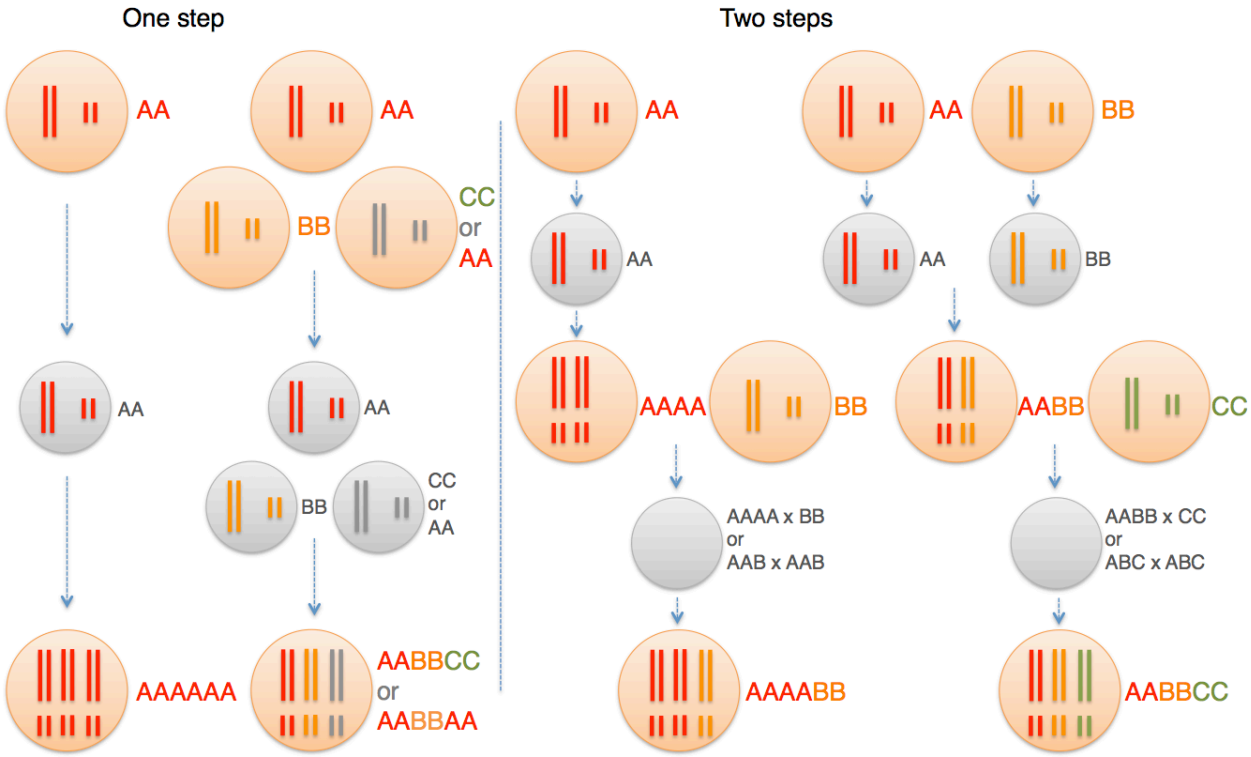


Figure 1.1 Different models to form (paleo)hexaploidy. Panel 1 illustrates one-step auto-hexaploid formation. Panel 2 illustrates one-step allo-hexaploid formation. Panel 3 illustrates a two-step auto-tetraploidy and allo-tetraploidy hybrid model to form a hexaploidy organism. Panel 4 illustrates two-step

formation of an allo-hexaploid via two successive allo-tetraploidizations. The big dark circles represent normal diploid cells (germline cells and embryos). The small light circles represent gametes.

Although having more than two genetic paths to form, from a phylogenetic point of view autopolyploids arise from within a single species, while allopolyploids arise from hybridization of more than one species. It should be noted that in terms of general post-duplication genomic adaptation and long-term evolution there is no absolute boundary between auto- and allo- polyploidies (Ramsey & Schemske, 1998; Douglas E. Soltis, Buggs, Doyle, & Soltis, 2010).

Once happened, polyploidy is arguably the most dramatic mutation experienced by a genome. In fact most polyploids simply reach a dead end because of failure in reproduction or ecological establishment. Surviving ancient polyploids experienced a subsequent process called 'diploidization', during which the sister paleo-subgenomes extensively restructured and the species was 'diploidized', i.e. restored diploid heredity. Genomic studies in the past decade have revealed at least six mechanisms through which the evolutionary effects of ancient genome duplication operate. Firstly, increased intragenomic homology promotes structural shuffling and rearrangements, resulting in re-organization of genomic regions. Secondly, massive non-random gene deletion, also known as 'fractionation' (Langham *et al.*, 2004; Thomas, Pedersen, & Freeling, 2006), can result in subgenomic dominance (J. C. Schnable, Springer, & Freeling, 2011; Tang *et al.*, 2012), altered biochemical pathways and rewired connections in the cellular interaction network (Arabidopsis Interactome Mapping Consortium, 2011; Bekaert, Edger, Pires, & Conant, 2011). Thirdly, the newly created "redundant" copies are often relieved of selective pressure and sometimes experience functional modifications via subfunctionalization or neofunctionalization (Kellis, Birren, & Lander, 2004; M. Lynch & A. Force, 2000; Ohno, 1970). Fourthly, the gene balance (or gene dosage) theory constrains changes in some duplicated genes coding for interacting products to fulfill stoichiometric balance (Birchler, Bhadra, Bhadra, & Auger, 2001; Papp, Pal, & Hurst, 2003; Thomas *et al.*, 2006), possibly driving them to different post-WGD evolutionary paths. Moreover, the cohort of whole genome duplicates greatly increases the "buffer capacity" of a

genome, perhaps making it more genetically robust (Chapman, Bowers, Feltus, & Paterson, 2006; Gu *et al.*, 2003; Paterson *et al.*, 2006). Finally, polyploids often have increased regulatory and morphological complexity (Freeling & Thomas, 2006), and a higher chance of obtaining new gene combinations and hybrid vigor (De Bodt, Maere, & Van de Peer, 2005; Rieseberg *et al.*, 2003). These changes, some of which quickly follow WGDs, are believed to often underlie emergence of derived or novel phenotypes and diversification of plant species (Otto & Whitton, 2000; Paterson *et al.*, 2000; P. S. Soltis & Soltis, 2000).

When multiple incidences of paleo-polyploidies have occurred in a lineage, as is often the case in flowering plants, some portions of the genome can acquire high copy numbers by compounding the effects of individual duplications. For example, the *TEOSINTE-LIKE1, CYCLOIDEA, and PROLIFERATING CELL FACTOR1* (*TCP*) gene family, encoding a group of plant-specific transcription factors, has 40 copies in *B. rapa* (4 paleo-polyploidies); 24 in *A. thaliana* (3 paleo-polyploidies); 19 in *V. vinifera* and 21 in *C. papaya* (1 paleo-polyploidy), indicating that recursive WGDs have continually contributed to this family's expansion in *B. rapa* (X. Wang, Wang, *et al.*, 2011). It has been observed that some gene families repeatedly delete or retain new gene copies through multiple rounds of WGDs, and some gene families can 'prefer' different directions in different lineages (Buggs *et al.*, 2012; De Smet *et al.*, 2013; Paterson *et al.*, 2006).

Genome structural mutations include insertions, deletions, inversions, translocations, recombinations, chromosome fissions and fusions, all of which are often elevated after polyploidization events (Leitch & Leitch, 2008; Mandakova, Joly, Krzywinski, Mummenhoff, & Lysak, 2010; Pecinka, Fang, Rehmsmeier, Levy, & Mittelsten Scheid, 2011; Xiong, Gaeta, & Pires, 2011). Synteny detection software developed in mammalian studies was typically designed to identify single best matching or orthologous regions (Bray & Pachter, 2004; Brudno *et al.*, 2003; Dubchak, Poliakov, Kislyuk, & Brudno, 2009; Kent, Baertsch, Hinrichs, Miller, & Haussler, 2003; Miller *et al.*, 2007), and is not suitable for comparing plant genomes. This is because mammalian genomes have been free of polyploidization for over 500 million years (Nakatani, Takeda, Kohara, & Morishita, 2007; J. J. Smith *et al.*, 2013), and have

much more conserved genome structure than in plants. In plants, for example, synteny conservation patterns across eudicots and monocots, which are separated by 240~140 million years, are extremely deteriorated (Salse *et al.*, 2009) due in large part to repeated paleo-polyploidies and associated genome restructuring. Even when aligning a plant genome which experienced a lineage specific paleopolyploidy with the genome of its closely related sister species lacking this event, such as *Arabidopsis thaliana* versus *Brassica rapa*, an additional paleo-hexaploidy in *B. rapa* has made their synteny map quite complicated for two lineages separated only 17~13 MYA (X. Wang, Wang, *et al.*, 2011). Therefore plant genome structure comparisons demand methods appropriate to deal with recurring genome duplications.

1.4 Methodological development for homeologous region detection and ancient genome duplication inference.

Early WGD studies at the dawning of the whole genome sequencing era mainly adopted two methods: distribution of nucleotide substitution rate at synonymous sites (Ks) between paralogous gene pairs (Blanc & Wolfe, 2004; Cui *et al.*, 2006; Lynch & Conery, 2000), or topology of homologous gene family phylogenetic trees (Bowers *et al.*, 2003). Both methods work well in many cases, but also have different limitations. Paralogous genes descendant from a single WGD are expected to have similar Ks values, forming a single peak when genomic Ks distribution is plotted. WGD peaks are distinctive from the background of continuous single gene duplications with exponential shape of loss. “Age grouping” of Ks values for homologous genes can be calculated without prior information about gene order, and is the only method for dating WGDs using transcriptome data alone. A major limitation of this method is that a paleo-polyploidy event has to be of moderate antiquity for the paralogous Ks values to form a well-behaved peak of distribution. For very recent WGD, Ks signals are usually masked by noise from continuous single gene duplications and occasional artificial gene homologs from mis-annotation. For very ancient WGD, base substitution can approach saturation and sequence-based methods become less useful (Blanc & Wolfe, 2004). In addition, blending of gene families with different modes of Ks divergence can create artificial separation of genes from single events, or blurring boundaries of Ks peaks

from different events. The original phylogenetic method for dating WGD (Bowers *et al.*, 2003) determines the coalescence order of speciation and polyploidy events by distinguishing tree topologies as duplication-first ("external" topology) or speciation-first ("internal" topology) scenarios (Bowers *et al.*, 2003). Although tree classification avoided the heterogeneous sequence divergence limitation of Ks methods, it still often suffers from taxon sampling limitation in early studies and lineage evolutionary rate variation (Tang, Wang, *et al.*, 2008).

Different from the often-confusing evolutionary signatures in individual gene sequences, patterns of paleo-polyploidies are better preserved as conservation of local gene order, or 'synteny', between the homeologous regions. Synteny-based WGD detection has powered the so far most reliable methods (Paterson, Freeling, Tang, & Wang, 2010). Synteny comparison can be carried out in two directions. The "bottom-up" approach iteratively interleaves gene positions on paralogous genomic segments to compensate for loss of ancestral genes on either segment. The merged pre-duplicated segments can then be used to look for additional duplication (Bowers *et al.*, 2003). Automatic pairwise detection of synteny blocks can be achieved through seed-and-extend algorithms such as LineUp (Hampson, McLysaght, Gaut, & Baldi, 2003), map-based algorithms such as ADHoRe (Vandepoele, Saeys, Simillion, Raes, & Van de Peer, 2002), DiagHunter (Cannon, Kozik, Chan, Michelmore, & Young, 2003), ColinearScan (X. Wang *et al.*, 2006), or graph algorithms such as DAGchainer (Haas, Delcher, Wortman, & Salzberg, 2004). A "top-down" approach implemented in MCscan (Tang, Bowers, *et al.*, 2008; Tang, Wang, *et al.*, 2008) is able to align multiple gene orders (for example, A-B-C instead of A-B, B-C, A-C) in one pass by taking advantage of the transitive property of synteny. A complementary PAR (putative ancestral region) algorithm from the same author exhaustively identifies and groups homeologous regions in two genomes by hierarchically clustering (Tang, Bowers, Wang, & Paterson, 2010). Some of the software has been incorporated into user-friendly interfaces of online comparative genomics platforms such as PGDD (T. H. Lee, Tang, Wang, & Paterson, 2013), PLAZA (Proost *et al.*, 2009), and CoGe (Lyons & Freeling, 2008).

Although it has become routine to identify and align synteny blocks between angiosperm genomes thanks to ingenious software developed in the last decade, it is so far not possible to: 1.

simultaneously infer multiple (often nested) paleo-polyploidy events in multiple lineages; 2. based on such inferences systematically extract and align homologous regions in the studied genomes. These are two greatly needed tasks, especially with growing sequencing efforts in wide samples of plant taxa. Chapter 6 of this dissertation describes the GeDupMap program addressing these needs.

1.5 Ancient genome duplications identified in sequenced flowering plant lineages

After being first discussed by a few pioneering scientists as early as the 1920s~1970s, paleo-polyploidy studies have greatly benefitted from advances in genome technology and rapid data growth in this century. In the past decade about 15 paleo-tetraploidies, 3 paleo-hexaploidies, and 1 paleo-(do)decaploidy have been identified in sequenced eudicot genomes, and 12 paleo-tetraploidies in sequenced monocot genomes. These events are summarized in **Table 1.1** below. Sequencing capacity has continued to increase while cost decreases, promising that genome data from many more plant taxa will soon be released, many of which are already under way. There are exciting opportunities and pressing challenges to solve automatic paleo-polyploidy detection and systematic plant genome comparisons needed for both basic and applied research.

Table 1.1 Paleo-polyploidy events identified in sequenced angiosperm genomes.

Species	Common name	Family	1x	Paleo-polyploidy	Genome size (Mb)	Protein coding genes	TE (%)	Assembly level	Scaffold N50 (Kb)	Release publication
Eurosids I										
Cucumis sativus	cucumber	Cucurbitaceae	7	γ	360	26,682	24	chromosome	1144.7	(Huang *et al.*, 2009)
Cucumis melo	melon	Cucurbitaceae	12	γ	450	27,427	19.7	chromosome	4677.8	(Garcia-Mas *et al.*, 2012)
Citrullus lanatus	watermelon	Cucurbitaceae	11	γ	425	23,440	45.2	chromosome	2380	(Guo *et al.*, 2013)
Malus x domestica	apple	Rosaceae	17	M,γ	742	57,386	42.4	chromosome	1542.7	(Velasco *et al.*, 2010)
Pyrus bretschneideri	pear	Rosaceae	17	M,γ	527	42,812	53.1	chromosome	540.8	(J. Wu *et al.*, 2013)
Prunus persica	peach	Rosaceae	8	γ	265	27,852	37.1	chromosome	26800	(International Peach Genome *et al.*, 2013)
Prunus mume	mei	Rosaceae	8	γ	280	31,390	45	chromosome	577.8	(Zhang *et al.*, 2012)
Fragaria vesca	woodland strawberry	Rosaceae	7	γ	240	34,809	22.8	chromosome	1361	(Shulaev *et al.*, 2011)
Cannabis sativa	hemp	Cannabaceae	10	γ	820	30,074	NA	scaffold	16.2	(van Bakel *et al.*, 2011)
Medicago truncatula	medicago	Fabaceae	8	L,γ	480	47,845	30.5	chromosome	1270	(Young *et al.*, 2011)
Cicer arietinum	chickpea	Fabaceae	8	L,γ	740	28,255	58.1	chromosome	39990	(Varshney *et al.*, 2013)
						27,571	40.4	chromosome	77.3	(Jain *et al.*, 2013)
Lotus japonicus		Fabaceae	6	L,γ	472	30,799	33	chromosome	92.3	(Sato *et al.*, 2008)
Glycine max	soybean	Fabaceae	20	S,L,γ	1115	46,430	59	chromosome	47800	(Schmutz *et al.*, 2010)
Phaseolus vulgaris	common bean	Fabaceae	11	L,γ		27,197	45.42	chromosome	50400	Schmutz *et al.*, 2014
Cajanus cajan	pigeonpea	Fabaceae	11	L,γ	833	48,680	51.7	chromosome	516.06	(Varshney *et al.*, 2012)
Malpighiales										
Populus trichocarpa	black cottonwood	Salicaceae	19	P,γ	475~550	45,555	42	chromosome	3100	(Tuskan *et al.*, 2006)
Linum usitatissimum	flax	Linaceae	15	F,γ	373	43,384	24.4	scaffold	693.5	(Z. Wang *et al.*, 2012)
Ricinus communis	castor bean	Euphorbiaceae	10	γ	320	31,237	50.3	scaffold	496.5	(Chan *et al.*, 2010)
Manihot esculenta	cassava	Euphorbiaceae	18	γ	770	30,666	37.5	scaffold	258.1	(Prochnik *et al.*, 2012)
Hevea brasiliensis	rubber tree	Euphorbiaceae	18	NA	2150	68,955	~78	chromosome	NA	(Rahman *et al.*, 2013)
Eurosids II										
Carica papaya	papaya	Caricaceae	9	γ	372	24,746	51.9	scaffold	1000	(Ming *et al.*, 2008)
Arabidopsis thaliana	thale cress	Brassicaceae	5	α,β,γ	135	27,416	14	chromosome	NA	(AGI, 2000; Swarbreck *et al.*, 2008)
Arabidopsis lyrata		Brassicaceae	8	α,β,γ	207	32,670	29.7	chromosome	24500	(Hu *et al.*, 2011)
Capsella rubella		Brassicaceae	8	α,β,γ	219	26,521	~50	chromosome	15100	(Slotte *et al.*, 2013)

Brassica rapa	Chinese cabbage	Brassicaceae	10	B,α,β,γ	284	41,174	39.5	chromosome	1971.1	(Wang *et al.*, 2011)
Thellungiella parvula	salt cress	Brassicaceae	7	α,β,γ	160	28,901	7.5	chromosome	5290	(Dassanayake *et al.*, 2011)
Thellungiella salsuginea	salt cress	Brassicaceae	7	α,β,γ	260	28,457	52	chromosome	403.5	(H. J. Wu *et al.*, 2012)
Gossypium raimondii	cotton	Malvaceae	13	C,γ	630~880	37,505	61	chromosome	18800	(Paterson *et al.*, 2012)
						40,976	57	chromosome	2284	(K. Wang *et al.*, 2012)
Theobroma cacao	cacao	Malvaceae	10	γ	430	28,798	24	chromosome	473.8	(Argout *et al.*, 2011)
Azadirachta indica	neem	Meliaceae	14	NA	383	20,169	13	scaffold	452	(Krishnan *et al.*, 2012)
Citrus clementina	clementine	Rutaceae	9	γ	~300	24,533	45	chromosome	6800	Wu *et al.*, 2014
Citrus sinensis	sweet orange	Rutaceae	9	γ	367	29,445	20.5	chromosome	1690	(Xu *et al.*, 2013)
Eucalyptus grandis	rose gum	Myrtaceae	11	EG, γ	640	36,376	50.1	chromosome	53900	Myburg *et al.*, 2014
Basal rosids										
Vitis vinifera	grape	Vitaceae	19	γ	475	26,346	41.4	chromosome	2065	(Jaillon *et al.*, 2007)
Asterids - Euasterids I										
Solanum lycopersicum	tomato	Solanaceae	12	T,γ	900	34,727	63.3	chromosome	16467.8	(Tomato Genome Consortium, 2012)
Solanum tuberosum	potato	Solanaceae	12	T,γ	844	39,031	62.2	chromosome	1318	(P. G. S. Consortium, 2011)
Coffea canephora	coffee	Rubiaceae	11	γ	710	25,574	~50	chromosome	1261	Denoeud *et al.*, 2014
Mimulus guttatus	monkey flower	Scrophulariaceae	14	MGa,γ	430	26,718-28,140	49.8	scaffold	1100	Hellsten *et al.*, 2013
Utricularia gibba	bladderwort	Lentibulariaceae	14	Ua,Ub,Uc,γ	77	28,494	3	scaffold	80.8	(Ibarra-Laclette *et al.*, 2013)
Basal asterids										
Actinidia chinensis	kiwifruit	Actinidiaceae	29	Ka,Kb,γ	758	39,040	36	chromosome	646.786	Huang *et al.*, 2013
Basal core-eudicots										
Beta vulgaris	sugar beet	Amaranthaceae	9	γ	714~758	27,421	63	chromosome	2010	Dohm *et al.*, 2013
Basal eudicots										
Nelumbo nucifera	sacred lotus	Nelumbonaceae	8	λ	929	26,685	57	megascaffold	3435	(Ming *et al.*, 2013)
Commelinids										
Phoenix dactylifera	date palm	Arecaceae	18	P,τ	658(381)	28,890	~4.5	scaffold	30.48	Al-Dous *et al.*, 2011
					671	41,660	38.41	scaffold	329.9	Al-Mssallem *et al.*, 2013
Elaeis guineensis	oil palm	Arecaceae	16	P,τ	1800	34,802	50	chromosome	1270	Singh *et al.*, 2013
Musa acuminata	banana	Musaceae	11	Mα,Mβ,Mγ,τ	523	36,542	43.7	chromosome	1311.1	D'Hont *et al.*, 2012
Sorghum bicolor	sorghum	Poaceae	10	ρ,σ,τ	818	33,032	62	chromosome	62,400	Paterson *et al.*, 2009

Oryza sativa	japonica rice	Poaceae	12	ρ,σ,τ	420	32,000~ 50,000		chromosome		Goff *et al.*, 2002
Oryza sativa	indica rice	Poaceae	12	ρ,σ,τ	466	~53,398	16	chromosome	11.76	Yu *et al.*, 2002
Brachypodium distachyon	purple false brome	Poaceae	5	ρ,σ,τ	~272	25,532	21.4	chromosome	59,300	International Brachypodium Initiative, 2010
Zea mays	maize	Poaceae	10	Z,ρ,σ,τ	2300	39,656	85	chromosome	76	Schnable *et al.*, 2009
Setaria italica	foxtail millet	Poaceae	9	ρ,σ,τ	510	24,000	40	chromosome	47,300	Bennetzen *et al.*, 2012
					490	38,801	46	chromosome	1000	Zhang *et al.*, 2012
Aegilops tauschii	wheat D genome	Poaceae	7	ρ,σ,τ	4360	43,150	65.9	chromosome	57.6	Jia *et al.*, 2013
Triticum urartu	einkorn wheat (A genome)	Poaceae	7	ρ,σ,τ	4940	34,879	66.9	chromosome	63.69	Ling *et al.*, 2013
Phyllostachys heterocycla	moso bamboo	Poaceae	24	Bm,ρ,σ,τ	2075	31,987	59	scaffold	328.698	Peng *et al.*, 2013
other monocots										
Spirodela polyrhiza	greater duckweed	Araceae	20	αSP,βSP	158	19,623	NA	superscaffold	3,759	Wang *et al.*, 2014
Basal angiosperms										
Amborella trichopoda	amborella	Amborellaceae	13		748~870	26,846	~57	scaffold	4900	AGC 2013

1.6 Framework of plant genome comparison

Abrupt origins, dynamic and often fast diversifications, prevailing paleo-polyploidies, and divergent lineage evolutionary rates are four key factors shaping the paths to modern flowering plants. In particular, compared to two rounds of WGDs (2R) followed by ~500 million years of quiescence in mammals (Dehal & Boore, 2005; J. J. Smith *et al.*, 2013), flowering plant lineages exhibit continuous propensity for polyploidies in the past ~200 million years. As a result, modern flowering plant genomes are highly variable in genome size, content and structure (Kejnovsky, Leitch, & Leitch, 2009; Salse *et al.*, 2009; D. E. Soltis, Soltis, Bennett, & Leitch, 2003). A direct multiple genome alignment, like the 28-way vertebrate alignment (Miller *et al.*, 2007), is not feasible in plants because the number of common anchors soon diminishes with taxa from a few families added to the alignment, and numbers of homeologous regions vary both within the same genome and across different genomes. Therefore, a framework of systematic genome comparisons is key to accurately and effectively identifying the locations of functional conservations and innovations among numerous flowering plant genomes.

Indeed, synteny-based molecular evolutionary analyses have started to show great power in dissecting genome functions. For example, past inference of relationships among members of a gene family based on sequence alignment can be complicated by many factors such as gene loss, different mutation rates across taxa and domains, tandem duplication, gene conversion, and horizontal gene transfer. Alternatively, evolutionary inferences based on genome-wide synteny patterns, making use of additional position information, were shown to be less confusing and more accurate in both animal (Dehal & Boore, 2005) and plant (Tang, Bowers, *et al.*, 2008) studies. In addition, compared to sequence evolution, synteny divergence is slower and seldom suffers from issues such as saturation and homoplasy, thus it often provides more reliable phylogenetic signals (Rokas & Holland, 2000).

Therefore, as genome technology provides us with increasingly greater data sets to study, it is not only beneficial but also necessary to establish the framework of plant genome comparison that will power the best use of synte-molecular comparisons of homologous regions.

CHAPTER 2 TWO PALEO-HEXAPLOIDIES UNDERLIE FORMATION OF MODERN SOLANACEAE GENOME STRUCTURE [1]

[1] Li, J., Tang, H., Wang, X., & Paterson, A. H. Accepted by *Compendium of Plant Genomes: The Tomato Genome*. Reprinted here with permission of the publisher.

2.1 Preface

Before the release of the tomato and potato genomes in 2011, there had been more than a dozen genomes published of rosid plants, but zero from asteroid plants. Although previous research based on genetic maps and ESTs indicated several possible paleo-polyloidy events in asterids, absence of complete genome sequences precluded their confirmation and detailed studies. Therefore publishing of high quality tomato and potato reference sequences was a milestone in plant paleo-polyploidy research. I fortunately had the opportunity to participate in the international tomato genome sequencing project led by Drs. Dani Zamir and Giovanni Giuliano. When tomato genome assembly was available to collaborators in 2011, Haibao Tang immediately discovered syntenic patterns indicative of paleo-hexaploidy on the dot plot between tomato and grape genomes. However, it was also clear on the dot plot that many regions of the tomato genome only shows 1-to-1 or 2-to-1 correspondence to orthologous grape regions, rather than the expected 3-to-1. Although high level of homeolog loss is not untypical for ancient polyploidy events, detailed analysis of the genome structure is necessary to draw a conclusion. Meanwhile the potato paper that just came out at that time only briefly noted a whole genome duplication in ancient potato that far predated potato-tomato divergence. We needed clear dissection of the nature of this paleo-polyploidy, which was my first task in the project. In order to prove that the event was a hexaploidy rather than a tetraploidy I was able to divide the extant tomato genome into three subgenomes originated from this ancient hexaploidy event (**Figure 2.1**, reprinted from (Tomato Genome Consortium, 2012)). Not only does the comparable size of the three subgenomes strongly favors a paleo-hexaploidy versus a paleo-tetraploidy (which typically leaves only two fractionated subgenomes in the present genome), but the genome-wide distribution of the three subgenomes also indicated that they are much more likely to have be produced in a polyploidy event rather than a large number of synchronous individual duplications. Furthermore, same analyses were also performed on the potato genome, which clearly confirmed that this shared event was a paleo-hexaploidy. Thus we confirmed the first case of paleo-hexaploidy in asterids.

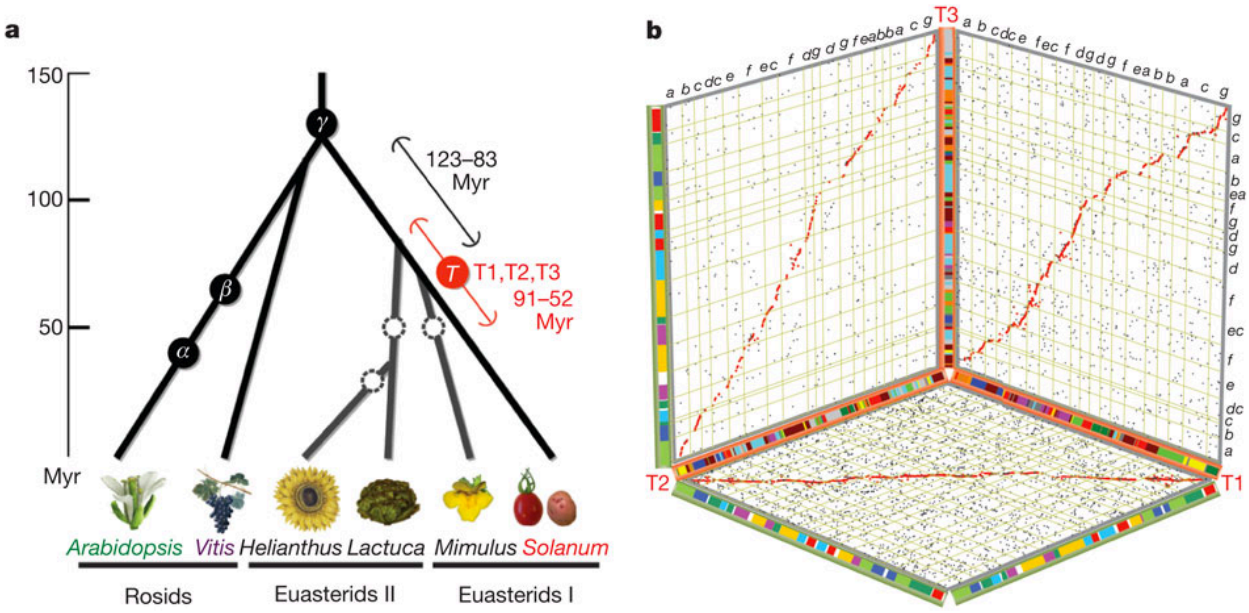


Figure 2.1 The *Solanum* whole genome triplication. (a) Speciation and polyploidization in eudicot lineages. Confirmed whole-genome duplications and triplications are shown with annotated circles, including 'T' (this paper) and previously discovered events α, β, γ. Dashed circles represent one or more suspected polyploidies reported in previous publications that need further support from genome assemblies. Grey branches indicate unpublished genomes. Black and red error bars bracket indicate the likely timings of divergence of major asterid lineages and of 'T', respectively. The post-'T' subgenomes are designated T1, T2, and T3. (b) On the basis of alignments of multiple tomato genome segments to single grape genome segments, the tomato genome is partitioned into three non-overlapping 'subgenomes' (T1, T2, T3), each represented by one axis in the three-dimensional plot. The ancestral gene order of each subgenome is inferred according to orthologous grape regions, with tomato chromosomal affinities shown by red (inner) bars. Segments tracing to pan-eudicot triplication (γ) are shown by green (outer) bars with colours representing the seven putative pre-γ eudicot ancestral chromosomes10, also coded a–g.

2.2 Abstract

Polyploidy, multiplication of whole genome content, is an important evolutionary force. Paleo-polyploidies (ancient genome duplications) have been identified in early lineages of animals, yeasts, and ciliates, but are particularly widespread in plants, with more than 32 events described. Deep impacts of paleo-polyploidies on plant evolution and diversity are a research focus in recent years. There are three unequivocally known paleo-hexaploidy (ancient genome triplication) events: one predated divergence of core eudicots ('γ'), one predated divergence of Solanaceae lineages ('T'), and one predated divergence of *Brassica* species. Two of the three events, γ and T, have affected the ancestors of all modern Solanaceae species, which includes tomato (*Solanum lycopersicum*). Signatures of the paleo-hexaploidy T were first described in the tomato genome, and confirmed in the potato (*Solanum tuberosum*) genome. Comparison among several asterid genomes revealed that T likely occurred in the Solanaceae lineage, and may have been chronologically close to the Solanaceae-Rubiaceae divergence. The successive γ and T paleo-hexaploidies produced 9 theoretical copies of each ancestral locus in a modern Solanaceae haploid genome, although only a fraction of these were retained. Following triplication, the paleo-genomes underwent massive non-random gene loss and extensive structural rearrangement, resulting in adaptive genetic changes and evolutionary novelties. In this chapter we will review recent research on the timing and formation of the γ and T paleo-hexaploidies, and their evolutionary effects on the shaping of modern Solanaceae genomes.

2.3 Introduction

The first two asterid plant genomes, those of tomato and potato from the Solanaceae (nightshade) family, were sequenced about a decade after the first plant genome was published, that of *Arabidopsis thaliana* (a rosid) (Arabidopsis Genome Initiative, 2000). They greatly expanded our knowledge of angiosperms (flowering plants), the Earth's dominant vegetation, which contain about 80% of known plant species. Todays' angiosperms consist of about 250,000 recorded species in about 450 families, of which about 75% or 198,000 species in about 336 families are eudicots (Hedges & Kumar, 2009; Stevens, 2012; The

Angiosperm Phylogeny Group, 2009). Eudicots, characterized by two embryonic cotyledons and tricolpate pollen grains, contain two major crown clades of taxa, the rosids (~70,000 species) and the asterids (~80,000 species), which diverged about 125~93 million years ago (MYA) in early- to mid-Cretaceous (Bell *et al.*, 2010; Bremer, Friis, & Bremer, 2004; Moore *et al.*, 2010; H. Wang *et al.*, 2009). The asterid plants consist of ~102 families, many of which are very closely associated with humans, such as tomatoes, potatoes, blueberries (Ericaceae family), coffee (Rubiaceae family), lavender (Lamiaceae family), olives (Oleaceae family), elderberries (Adoxaceae family), dogwoods (Cornaceae family), and sunflower (Asteraceae family).

One question that benefits greatly from whole-genome sequencing is the effects of paleo-polyploidies, or ancient whole genome duplications (WGDs), on the evolution of plant genome structure (see section 13.2). Paleo-polyploidy refers to ancient polyploidy (whole genome multiplication) events that have subsequently been diploidized (returning to disomic inheritance), resulting in the present-day haploid genome content containing more than one set of the ancestral genome. For example, a paleo-tetraploid genome has 2 sets of haploid genomes each containing 2 sets of the pre-duplication ancestral haploid genomes. Paleo-polyploidies have been reported in the eukaryotic kingdoms of Animalia (Dehal & Boore, 2005; Ohno, 1970), Fungi (Kellis *et al.*, 2004; K. H. Wolfe & Shields, 1997), and Chromalveolata (Aury *et al.*, 2006), but are most widespread in Plantae. All angiosperms are paleo-polyploids, having experienced at least one, and usually more, WGDs in their lineage histories (Blanc & Wolfe, 2004; Cui *et al.*, 2006; Jiao *et al.*, 2011; Masterson, 1994; D. E. Soltis *et al.*, 2009; G. L. Stebbins, 1966; Tang, Bowers, *et al.*, 2008). More than 32 paleo-polyploidy events have been identified in sequenced angiosperm genomes.

Even before any plant genome was sequenced, comparative mapping of molecular markers suggested that the small genome of *Arabidopsis thaliana* actually contains many paralogous regions, which may be descended from paleo-polyploidy events (Kowalski *et al.*, 1994; Paterson *et al.*, 1996). This inference was supported by later studies using sequence from the first plant genome of *A. thaliana* (Bowers *et al.*, 2003; D. Grant *et al.*, 2000; Ku *et al.*, 2000; Paterson *et al.*, 2000; Simillion *et al.*, 2002;

Vision *et al.*, 2000). One of the key findings from the first sequenced plant genomes was the pan-core eudicot paleo-hexaploidy (2n=6x) 'γ' (discussed in section 13.5). Paleo-hexaploidy (ancient genome triplication) occurs or survives much less frequent than paleo-tetraploidy (ancient genome duplication, or doubling). Before the sequencing of the tomato genome, the only two other paleo-hexaploidies identified were one in the *Brassica* lineage estimated to have occurred 13~17 MYA (X. Wang, Wang, *et al.*, 2011), and γ. The tomato genome revealed the third case of paleo-hexaploidy (also the first case in asterids), the T event (Tomato Genome Consortium, 2012), discussed in detail in sections 13.3 and 13.4 of this chapter.

This chapter focuses on the two paleo-hexaploidies experienced by Solanaceae ancestors. We will start by a very brief methodological overview. Then we will first discuss the pan-Solanaceae T event because it was the terminal WGD event in this lineage and therefore easier to study than the more ancient γ event that was nested inside T. After that we will discuss the pan-core eudicot γ event by first profiling it using the grape (rosids) genome where γ is a terminal WGD (grape genome experienced no re-duplication following γ), and then prove that it was also shared by ancestral asterids. In the end we will discuss the evolutionary effects of γ and T on the tomato genome structure, and raise a few questions for future studies on these two and more paleo-hexaploidy events.

2.4 Methods to identify paleo-polyploidy

Paleo-polyploidy events are difficult to identify because they occurred in the ancient past, during which time conservation of sequence and synteny between paralogous regions has been severely eroded. Typically more than 70~80% of the genes duplicated in a paleo-polyploidy are subsequently lost. The remaining loci are further shuffled by post-WGD genome rearrangements. Therefore it is necessary to collect genome-wide signals for detection of WGDs. Because a paleo-polyploidy event duplicates all loci in the progenitor genome at the same time, the histogram of their paralogous genes Ks (nucleotide substitutions per synonymous site) values forms a peak corresponding to the event (Lynch & Conery, 2000). Those distributions can therefore be used to identify paleo-polyploidies, with the limitations that

Ks divergence cannot be resolved when it is either too small or too large, and that the rate of accumulation of mutations varies among gene families.

When genome sequence is available, the most sensitive and accurate paleo-polyploidy detection methods are synteny-based, which have been used in studies in yeasts (Kellis *et al.*, 2004), vertebrates (Dehal & Boore, 2005; J. J. Smith *et al.*, 2013) and plants (Bowers *et al.*, 2003; Tang, Bowers, *et al.*, 2008). In addition, synteny conservation is preserved across very long evolutionary distances, for example across eudicot-monocot comparison, and is unaffected by DNA substitution rate variation. Two synteny detection programs that are capable of aligning multiple genomes are MCscan (Tang, Bowers, *et al.*, 2008; Tang, Wang, *et al.*, 2008; Y. Wang *et al.*, 2012) and ADHoRe (Proost *et al.*, 2012; Simillion, Janssens, Sterck, & Van de Peer, 2008). On the other hand, because paralogous regions from a paleo-polyploidy event usually undergo reciprocal gene loss, having a reference genome that did not experience the paleo-polyploidy (and subsequent gene loss) under study is very helpful in recovering maximum syntenic mapping between the regions. For example, in rosids some genomes have not experienced additional WGDs after γ, such as grape (Jaillon *et al.*, 2007), papaya (Ming *et al.*, 2008), and peach (Verde *et al.*, 2013). These often serve as outgroups when studying more recent WGDs in other rosid lineages. For more comprehensive reviews of the methods used in paleo-polyploidy identification, readers are referred to (Paterson *et al.*, 2010) and Chapter 8 in (Paterson, 2014).

2.5 The paleo-hexaploidy T: triplication of the Solanaceae ancestral genome

Paleo-polyploidy in Solanaceae was first detected from studies of genetic map data, and supported by EST data. Early comparison of a 293 loci potato genetic map with the *A. thaliana* genome suggested possible ancient segmental duplications (Gebhardt *et al.*, 2003). Based on patterns of paralogous genes synonymous (third codon position) substitutions (Ks) in tomato and potato EST sequences this event was inferred to be a genome-wide duplication, and estimated to predate tomato-potato divergence (Blanc & Wolfe, 2004; Cui *et al.*, 2006; Schlueter *et al.*, 2004). Using 1,392 duplicated gene families shared by 8 plant species Schlueter *et al.* (Schlueter *et al.*, 2004) modeled a log normal Ks component (median 0.632)

in tomato corresponding to an inferred WGD ~52 MYA. Independent study by Blanc *et al.* (Blanc & Wolfe, 2004) analyzed 7,963 tomato and 6,597 potato paralogs, and estimated a modal Ks peak of ~0.60. Using constant-rate birth-death process as a null model Cui *et al.* (Cui *et al.*, 2006) identified a significant Ks peak (median ~0.79) in tomato paralogous genes from 10,028 EST and 5,303 Unigene sequences, further supporting this paleo-polyploidy event.

Analysis of the tomato genome sequence revealed this WGD event to be a paleo-hexaploidy (triplication) (Tomato Genome Consortium, 2012), which was called 'T' for easy reference. Distribution of Ks values between syntenic tomato paralogs confirmed previous inferences of the paleo-polyploidy. To dissect the patterns of homeology, syntenic regions, i.e. with matching gene content and order, were aligned between the genomes of tomato and the rosid plant grape (*Vitis vinifera*) that has been free of additional WGDs after the pan-core eudicot γ event (Jaillon *et al.*, 2007; Tang, Bowers, *et al.*, 2008; Tang, Wang, *et al.*, 2008), and is therefore a valuable reference in plant genome comparisons. This comparison clearly showed the shared γ event between the two lineages and the unshared T event in tomato (Tomato Genome Consortium, 2012). Because of massive gene loss following paleo-polyploidy, most (~95.8%) T triplicates in tomato have lost 1~2 homeologs. However across the entire genome signals of synteny are strong enough to allow detection of the triplication patterns. Genome-wide, 73% of tomato gene loci are in blocks that are each orthologous to one grape region, collectively covering 84% of the grape gene space. Among those grape regions, 26.8% map to one orthologous region in tomato, 47.4% to two, and 25.7% to three, a pattern most parsimoniously explained by a historical triplication in tomato. By aligning against single orthologous grape genomic regions, the present-day tomato genome can be partitioned into three nearly non-overlapping T 'subgenomes' (Figure 2 in (Tomato Genome Consortium, 2012), also as **Figure 2.1** in this chapter). Each of the three subgenomes now spans all 12 tomato chromosomes, indicating extensive genome rearrangement since the triplication. After polyploidization, there is sometimes noticeable difference in the evolution of the subgenomes, known as biased fractionation or subgenome dominance (Sankoff & Zheng, 2012; J. C. Schnable *et al.*, 2011; Tang *et al.*, 2012; Thomas *et al.*, 2006). The three paleo-subgenomes in the present-day tomato genome cover

45.5%, 21.5%, and 9.9% of gene loci, respectively, possibly reflecting this phenomenon.

The potato, another species in the genus *Solanum* that diverged from tomato ~7.3 MYA, was sequenced at about the same time (Potato Genome Sequencing *et al.*, 2011), and shared the T event. The potato and tomato genomes are highly colinear (**Figure 2.7**). There is relatively small ~8.7% nucleotide divergence and 9 major inversions between the two genomes (Tomato Genome Consortium, 2012). Comparison of potato and grape genomes showed single grape regions corresponding to 1~3 potato regions. Overall 27.8% of grape genes are in regions orthologous to one region in potato, 38.1% to two regions, and 14.5% to three regions, collectively spanning 68% of the gene space in potato and 80% in grape, consistent with the results between tomato and grape. Patterns of Ks distribution among triplicated potato paralogs closely resemble those of tomato as well, and are clearly distinct from those of γ paralogs (Tomato Genome Consortium, 2012). The only discrepancy lied in that the potato genome paper (Potato Genome Sequencing *et al.*, 2011) reported this event as a duplication instead of a triplication. However, careful re-examination of Supplementary Figure 6b of the paper, which aligned syntenic regions between grape, *Arabidopsis*, poplar, and potato, revealed that the figure missed the third T region on potato chromosome 8. Therefore, both independent analysis of the potato genome and re-examination of previous results support that T was a triplication that predated potato-tomato divergence.

2.6 Further circumscribing the T event using additional asterid genomes

Based on Ks distributions of paralogous tomato genes the triplication T was estimated to have occurred 90.4~51.6 MYA (**Figure 2.2** and (Tomato Genome Consortium, 2012)). The divergence of ancestral Euasterid I and II lineages is around 123~85 MYA (Hedges, Dudley, & Kumar, 2006), making it possible that T was shared by those lineages. In order to evaluate these possibilities, newly published genomes of asterid species monkey flower (*Mimulus guttatus*, Scrophulariaceae family), bladderwort (*Utricularia gibba*, Lentibulariaceae family), kiwifruit (*Actinidia chinensis*, Actinidiaceae family), and 6 BACs from coffee (*Coffea Arabica*, Rubiaceae family) were analyzed and compared to the tomato genome. The circumscription of WGD events in these lineages is summarized in **Figure 2.3**.

Kiwifruit (*Actinidia chinensis*) belongs to the basal asterid order Ericales. The kiwifruit genome experienced the γ triplication, after which it experienced two lineage-specific WGDs that were not shared with the Euasterid I and II lineages (Huang *et al.*, 2013). Comparing the kiwifruit genome to the tomato genome revealed a synteny pattern of 4-to-3 correspondence (**Figure 2.4**), indicating that the T triplication event was not shared by kiwifruit, as otherwise a 1-to-4 synteny correspondence would be observed. This inference is consistent with dating of the relative WGD and speciation events on the two lineages based on molecular data (not shown), and inferences from the kiwifruit genome paper (Huang *et al.*, 2013).

The recently published genomes of monkey flower (*Mimulus guttatus*) and bladderwort (*Utricularia gibba*) helped confine the timing of T within the Euasterids. Bladderwort has one of the smallest genomes among flowering plants (~82 Mb). However it has experienced the γ triplication as well as three more WGDs in its lineage (Ibarra-Laclette *et al.*, 2013) that were close in time (**Figure 2.2**). Detailed synteny analysis revealed that the first of these three WGDs was shared with its sister lineage *Mimulus* of the Lamiales (Ibarra-Laclette *et al.*, 2013), which is also the only lineage-specific WGD in *Mimulus* (**Figure 2.3**). Since ancestral linkages are preserved better in the monkey flower genome which experienced fewer WGDs than bladderwort, the former is compared to the tomato genome (**Figure 2.5**). Each set of T paralogous regions in tomato (up to 3 regions retained in the present-day genome) correspond to up to two paralogous regions in monkey flower (**Figure 2.5**), collectively spanning 88.4% of the monkey flower genome and 82.0% of the tomato genome. Distribution of the synteny blocks' anchor gene pairs median Ks values (an approximation of evolutionary distance between the syntenic regions) forms a single population, again suggesting that the tomato-monkey flower split predated their lineage-specific WGDs. Therefore T is likely not shared with the Lamiales, an inference also supported in the bladderwort genome paper (Ibarra-Laclette *et al.*, 2013).

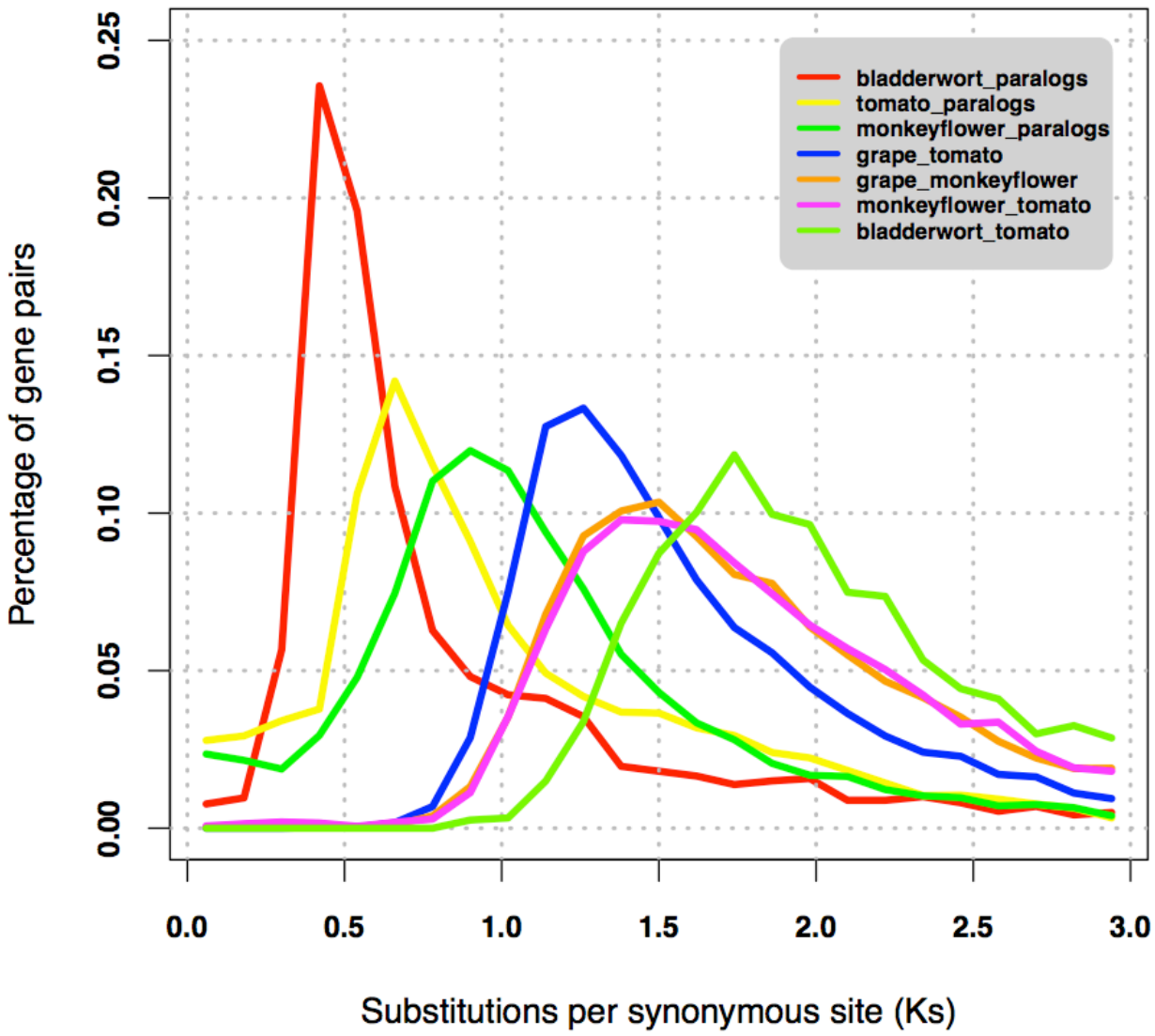


Figure 2.2 Histograms of Ks (nucleotide substitutions per synonymous site) between paralogous and orthologous gene pairs in tomato, monkey flower, bladderwort, and grape. The x-axis is Ks values filtered as [0, 3] since Ks < 0 reflects invalid calculation in PAML and Ks > 3 exceeds empirical threshold for saturation of nucleotide divergence. The y-axis is percentage of gene pairs. The curves are plotted with different colors, but also labeled by their peak order from left to right. Comparison among the Ks distributions indicated that tomato has average nucleotide substitution rate slower than that of monkey flower, while bladderwort has the highest rate.

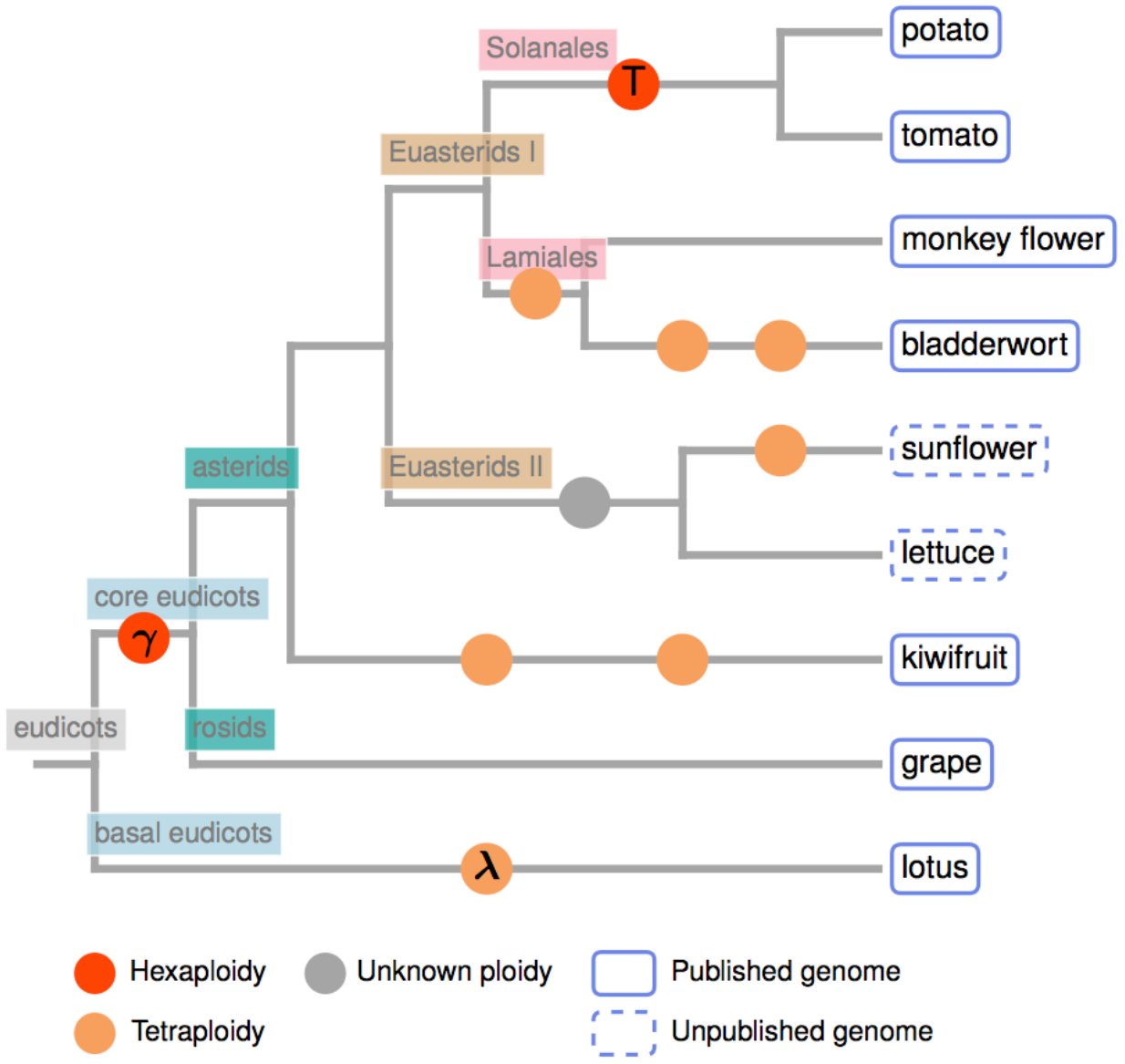


Figure 2.3 Simplified cladogram of some representative asterid and outgroup lineages. The phylogenetic relationships are according to APG III (The Angiosperm Phylogeny Group, 2009) and to our current best knowledge are unambiguous. Branch length has no meaning. Paleo-polyploidy events identified in those lineages are represented by circles, labeled with their names if given. The WGD event in the ancestor of sunflower and lettuce may be a triplication (Truco *et al.*, 2013). The main references for the paleo-polyploidy events are: (Barker *et al.*, 2008; Hellsten *et al.*, 2013; Huang *et al.*, 2013; Ibarra-Laclette *et al.*, 2013; Jaillon *et al.*, 2007; Ming *et al.*, 2013; Tang, Wang, *et al.*, 2008; Tomato Genome Consortium, 2012; Truco *et al.*, 2013).

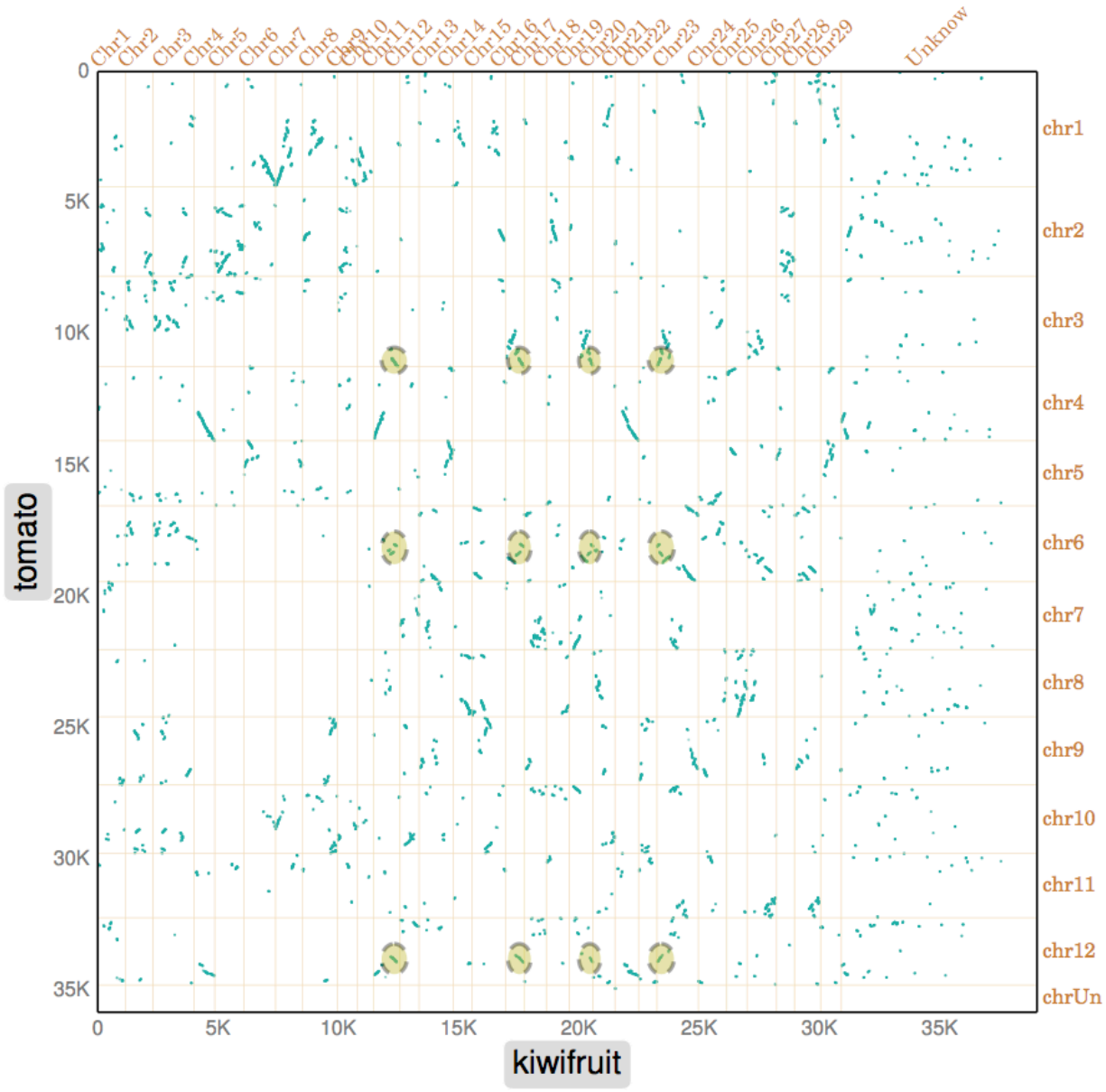


Figure 2.4 Alignment of tomato and kiwifruit genomes. The segments labeled as "Unknown" and "chrUn" are un-anchored scaffolds in the genome assemblies. Each dot represents a pair of syntenic genes. Continuous stretches of synteny matching are broken down by gene loss and rearrangement. Yellow circles with dashed borders highlight an exemplary set of syntenic regions with multiple-to-multiple (in this case 3 tomato - 4 kiwifruit) correspondences, reflecting lineage-specific triplication T (3x) in tomato and 2 duplications (4x) in kiwifruit.

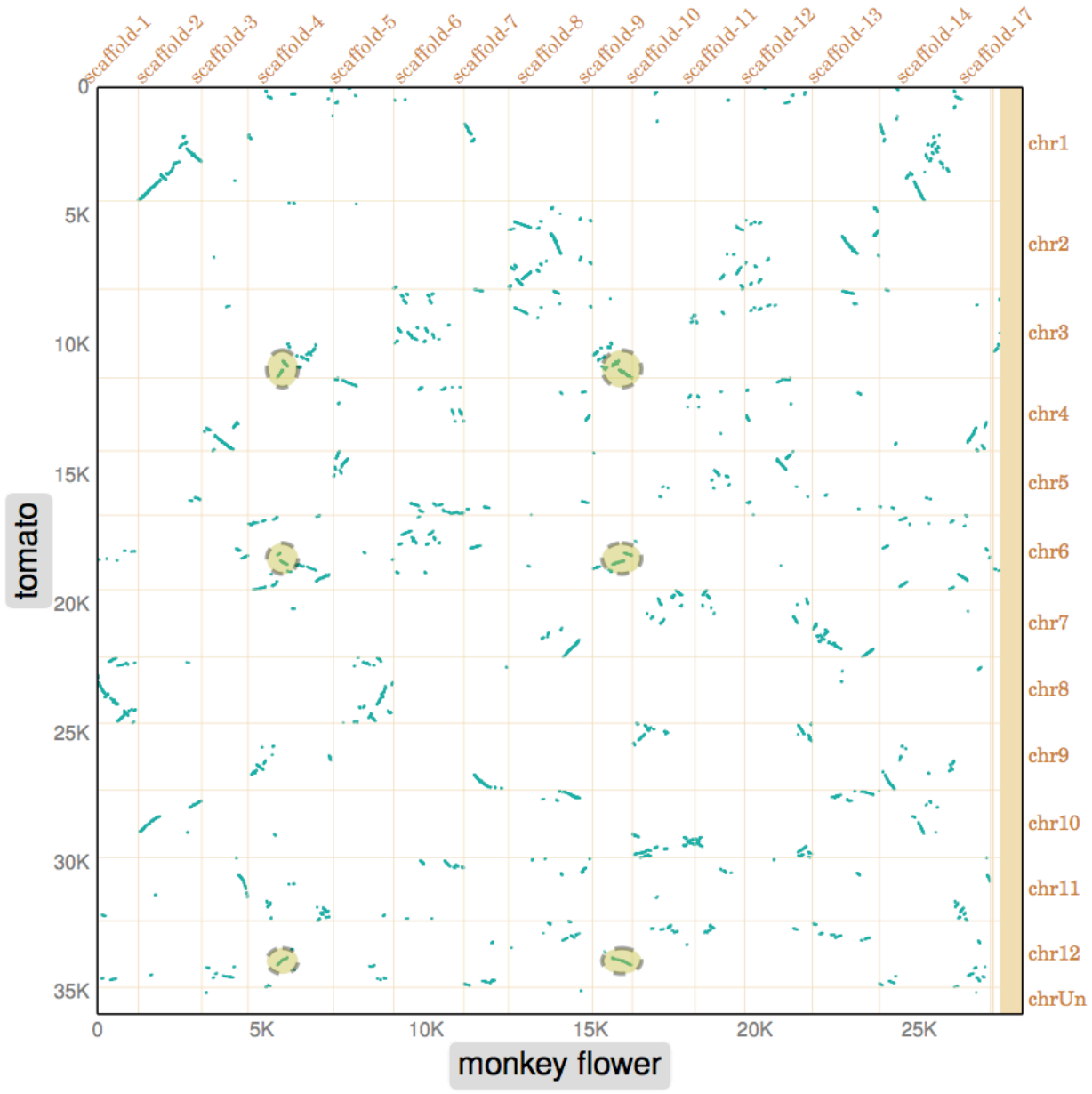


Figure 2.5 Alignment of tomato and monkey flower genomes. Segment labeled as "chrUn" in tomato genome (y-axis) contains un-anchored scaffolds in the genome assembly. Each dot represents a pair of syntenic genes. Continuous stretches of syntenic matching are broken down by gene loss and rearrangement. Yellow circles with dashed borders highlight an exemplary set of syntenic regions with multiple-to-multiple (in this case 3 tomato - 2 monkey flower) correspondences, reflecting lineage-specific triplication T (3x) in tomato and duplication (2x) in monkey flower.

The coffee plant *Coffea arabica* belongs to the asterid order Gentianales, which is thought to have separated with the Solanales after their common ancestor diverged from the Lamiales (Moore *et al.*, 2010; D. E. Soltis *et al.*, 2011). As of this writing, there is no published genome sequence in Gentianales, but there are 6 coffee BACs in NCBI (GU123894 ~ GU123899) coming from a contiguous region of ~900 Kb. Sequence alignment and colinearity analysis revealed that this region is syntenic to three tomato regions triplicated in T: Chr3:0.13-0.35Mb, Chr6:33.0-33.4Mb, Chr9:63.7-64.7Mb (**Figure 2.6**). The region on tomato Chr9 has significantly more hits to the coffee region than those on chr6 or chr3 (198, 86, 108 respectively, Chi-square test *P*=1.79e-11), favoring the `WGD shared` model, i.e. tomato-coffee divergence postdated triplication T. Analysis of two additional BACs (MA29G21 and MA17P03) from a pair of orthologous regions in a recent allo-tetraploid *Coffea arabica* strain also supported the model of triplication shared, with both of the BACs showing differentiated distance to the tomato triplets, and synteny between at least one pair of the homeologous regions lost or diminished beyond detection. Although biased fractionation of the T paleo-subgenomes could be an alternative explanation, such levels of difference in synteny retention as seen in the coffee-tomato comparisons are not usually seen among orthologous regions, but often seen between orthologous and out-paralogous regions, hence favoring the hypothesis that T was shared by ancestors of tomato and coffee. On the other hand, percentage identity of hits is not significantly different among the three alignments (**Figure 2.6**, pairwise Wilcoxon rank sum test P-values are: Chr3 hits and Chr6 hits: 0.277; Chr3 hits and Chr9 hits: 0.008; Chr6 hits and Chr9 hits: 0.212), supporting the alternative hypotheses that coffee did not share T, or that tomato and coffee diverged shortly after sharing T. A definitive inference will be possible when the genome sequences of coffee or other Gentianales become available.

In summary our current best inference is that the T event likely occurred near the Gentianales-Solanales split, a rough estimation of which is 108~71 MYA (Hedges *et al.*, 2006). The exact distribution of asterid lineages that have experienced the paleo-hexaploidy T will become clear when more genomes are sequenced from this clade.

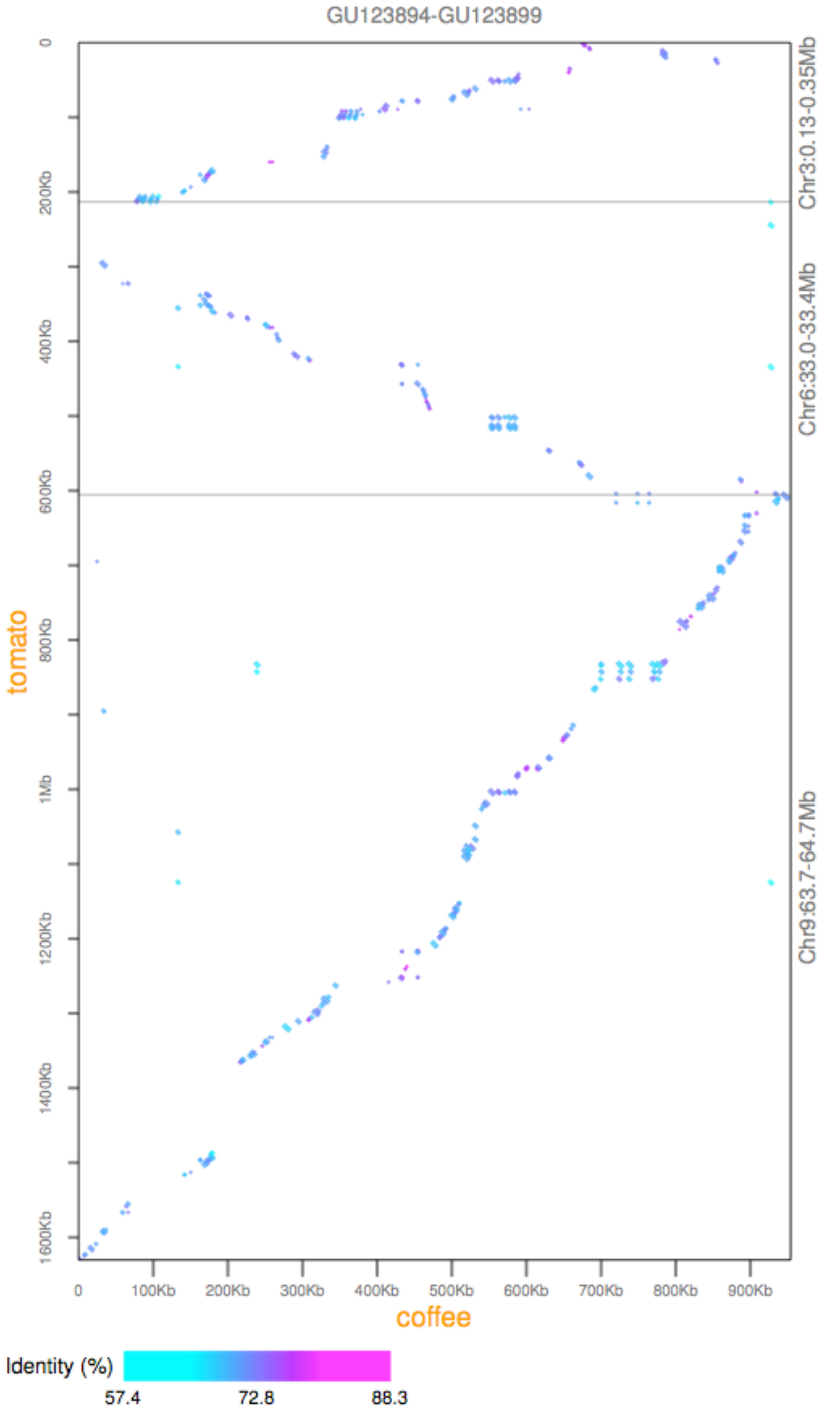


Figure 2.6 LASTZ alignment between the coffee BAC region and tomato genome. The ~900 kb coffee BAC sequenced region is from (Cenci, Combes, & Lashermes, 2010). It is aligned to three syntenic regions on tomato chromosomes 3, 6, 9 (triplet from the paleo-hexaploidy T). The hits are represented by stretches of lines on the plot, with colors coded by percent identity, and line width proportional to the logarithm of hit length.

2.7 A more ancient hexaploidy γ predated divergence of rosid and asterid plants

When comparing the first plant genome of *A. thaliana* with a soybean genetic map (D. Grant *et al.*, 2000) and a 105 Kb tomato BAC region (Ku *et al.*, 2000) it was suggested that the compact *A. thaliana* genome may nonetheless contain more than two paleo-subgenomes, possibly resulting from two or more paleo-polyploidies (Ku *et al.*, 2000). Indeed, using a sensitive phylogenomic approach 34 paralogous regions covering a total of 89% of the *A. thaliana* genome were circumscribed into three WGD events, named 'γ', 'β', and 'α' (Bowers *et al.*, 2003), the first of which turned out to be a hexaploidy (Jaillon *et al.*, 2007; Tang, Wang, *et al.*, 2008). Through several studies in recent years, the γ event has been found to be shared by most or all core eudicot lineages.

Synteny comparison between tomato and grape revealed that γ predated the asterid-rosid divergence. In an analysis of 72 tomato BACs and the sequenced grape genome, each individual tomato BAC has primary association to only one of the triplicate regions rather than showing equal matches to each of the three γ regions in grape, suggesting that γ likely predated tomato-grape divergence (Tang, Wang, *et al.*, 2008). This inference was later supported by analysis of the tomato genome, in which individual regions correspond most closely to only one of the triplicated regions in grape, and no grape region is orthologous to more than one set of re-triplicated regions in tomato (Tomato Genome Consortium, 2012).

On the other hand, the genome of the first sequenced basal eudicots, Sacred lotus (*Nelumbo nucifera*) of the order Proteales, did not share γ (but rather had a lineage-specific paleo-tetraploidy event 'λ') (Ming *et al.*, 2013), placing γ somewhere on the basal eudicot branches after the Proteales lineage branched off. Two recent studies have further confined the timing of the γ paleo-hexaploidy to a narrow window shortly predating the divergence of the earliest core eudicot lineages. Phylogenetic analysis of 769 gene families from a large collection of angiosperm species dated γ after the divergence of the Ranunculales (a basal eudicot) and core eudicots (Jiao *et al.*, 2012). Phylogenetic analysis of subfamilies of MADS-box genes and transcriptomes from several basal eudicot species further placed γ after the

divergence of two basal eudicot orders (Buxales and Trochodendrales) and the rest of eudicots, but before the branching of the Gunnerales (basal core eudicots) (Vekemans *et al.*, 2012).

2.8 The nature and consequences of the γ and T paleo-hexaploidy events

Subgenomes joined in a polyploidization event are typically 'diploidized', i.e. gradually restoring diploid inheritance through processes of fractionation (loss of duplicated genes) (Force *et al.*, 1999; Lynch & Conery, 2000; Thomas *et al.*, 2006) and structural rearrangement (Tang, Bowers, *et al.*, 2008; K. H. Wolfe, 2001). Substantial difference in the levels of fractionation among subgenomes is sometimes indicative of possible ancient allo-polyploidy. Study of fractionation patterns in the three grape subgenomes produced in the γ paleo-hexaploidy showed that two subgenomes are more fractionated with respect to each other than to the third subgenome, suggesting that γ possibly involved hybridization between two somewhat divergent species, one of which had been previously autotetraploidized (Lyons, Pedersen, Kane, & Freeling, 2008). However, hybridization of differentiated progenitors is not a necessary condition for differentiated fractionation patterns between subgenomes, which could also be the results of post-polyploidy evolution. Phylogenetic trees constructed from triplets of γ paralogs and outgroup genes lack one dominant topology, suggesting that γ may also have been an auto-hexaploidy formed from a single progenitor, or an allo-hexaploidy formed from fusions of three moderately diverged genomes (Tang, Wang, *et al.*, 2008). More knowledge of the ancestral karyotypes will be needed to distinguish between those evolutionary scenarios.

Much reminiscent of the case of γ, on one hand T triplets in tomato produce a mixed population of phylogenetic trees with all the possible topologies, indicating lack of sequence divergence in the T progenitor genomes. On the other hand there is fractionation difference between the three subgenomes: T1 and T2 are less fractionated with respect to each other than to the third subgenome T3 (data not shown). These results suggested that T was possibly an auto-hexaploidy or an allo-hexaploidy of two closely related species and one more distant species. Allo-polyploidy is often thought to be more frequent in nature due to advantages in the establishment of the polyploid strains resulting from factors such as

heterosis, homeostasis, and fewer meiotic irregularities. However, the frequency of natural auto-polyploidy and its effects on species diversity may be higher than traditionally thought (Ramsey & Schemske, 1998). As with γ, because of the antiquity of the T event, a definitive conclusion cannot be drawn due to degradation of molecular signatures and loss of the progenitor genomes. However, current data are in support of T having a higher possibility to have been an auto-polyploidy than the other two paleo-hexaploidies, the γ event (discussed above) and the *Brassica* paleo-hexaploidy which appears to have been an allo-hexaploidy (Tang *et al.*, 2012). This would also be consistent with the fact that Solanaceae species do form autopolyploids in agricultural and natural settings. If T were indeed a paleo-autohexaploidy, it would be the only one known so far. Genome sequences from closely related sister taxa will aid in the test of this hypothesis.

Comparison between the tomato and potato genomes showed that about 91% of post-T gene loss is orthologous, indicating that these genes had been lost before tomato-potato divergence. Paleo-polyploidy events are usually followed by a phase of rapid genome evolution, including structural, sequence, and regulatory changes (Adams & Wendel, 2005b; Lynch & Conery, 2000; K. Song, Lu, Tang, & Osborn, 1995). Therefore it is possible that many of the shared changes in tomato and potato occurred in their common ancestor shortly after T. On the other hand, evolution of genetic content in the triplicated paleo-genome of the Solanaceae ancestor continued long after the paleo-polyploidy event. The xyloglucan endotransglucosylase/hydrolase (XTH) family gene *XTH10* that was triplicated in the T event showed differential loss between the tomato and potato genomes which diverged ~65 MY after T (Tomato Genome Consortium, 2012). Although tomato and potato genomes have maintained very similar karyotypes in ~7.3 MY of separate evolution, and 70~80% of their genes have remained orthologous (**Figure 2.7** left panels), there has been continuous rearrangement of the ancestral genome content in the two lineages. The present-day tomato and potato chromosomes differ by 9 major and several smaller inversions, and numerous local micro-synteny differences. About 4.8% (tomato) and 4.6% (potato) of the orthologous loci triplicated in T have been differentially lost between tomato and potato after their divergence. Ancestral subgenomes produced in the pan-core eudicot γ triplication had undergone

extensive rearrangement before tomato-potato divergence, but have continued to be restructured independently in their recent independent lineage histories (**Figure 2.7** right panels). Therefore paleo-polyploidy poses both immediate and long-term effects on the evolution and diversity of genome structure.

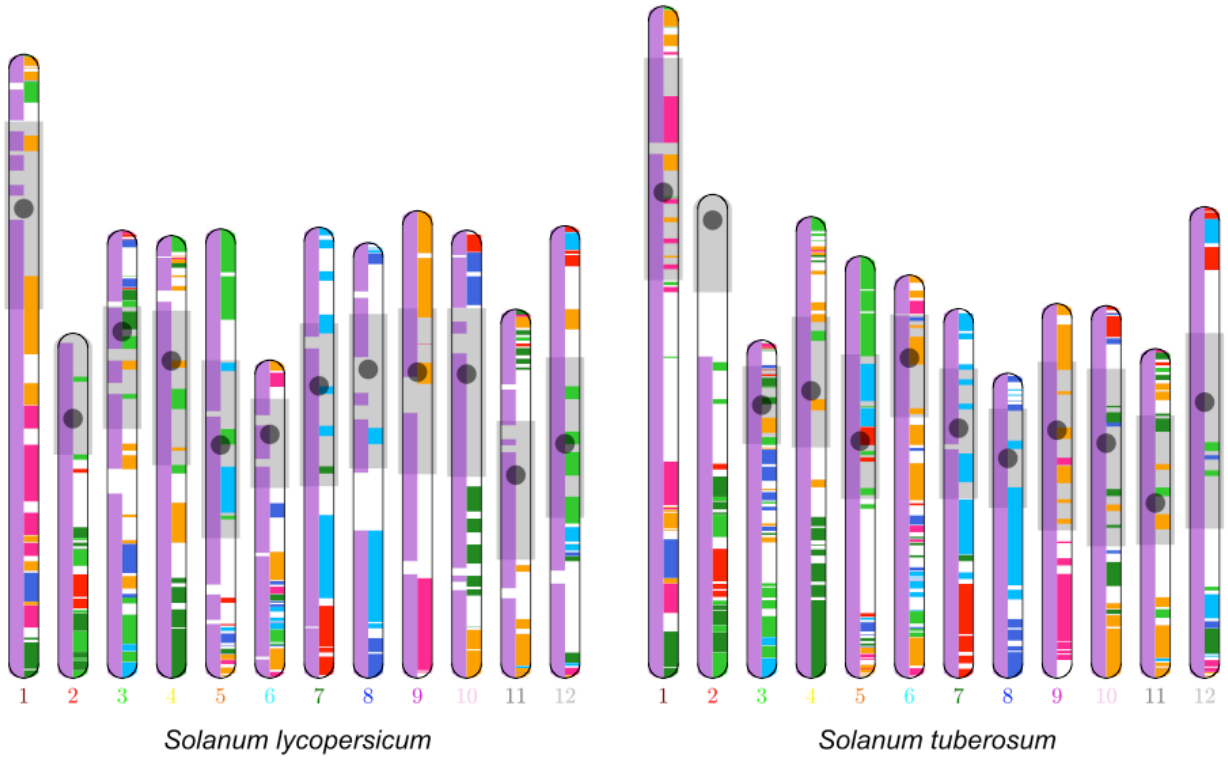


Figure 2.7 Schematic representation of orthologous and paralogous regions in tomato (*S. lycopersicum*) and potato (*S. tuberosum*) genomes. On the left side of the chromosome bars the purple regions are orthologous between tomato and potato. On the right side, 7 colors are used to paint genomic regions corresponding to 7 chromosomes in the inferred pan-core eudicot ancestral genomes (pre-γ) using grape genome data (Jaillon *et al.*, 2007). Each of the γ-triplicated (3x) ancestral regions later underwent the T triplication (3x), resulting in their dispersed and multiplied (up to 9x) pattern in today's tomato and potato genomes. The gray shades and dark gray circles mark estimated heterochromatin regions and centromeres, respectively, from cytological experiments. Corresponding linkage groups (chromosomes) between tomato and potato are labeled with same color.

In addition to the widespread effects of paleo-polyploidy, there are also important lineage-specific effects of the individual events. For example, the two ancient genome triplications in tomato have produced new gene family members that mediate important functions in its fruit ripening control, such as some transcription factors and enzymes necessary for red light photoreceptors influencing fruit quality (*PHYB1/PHYB2*) (expended in T), ethylene- and light-regulated genes mediating lycopene biosynthesis (*PSY1/PSY2*) (expended in T), and ethylene biosynthesis (*RIN*, *CNR*, *ACS*) (expended in T) and perception (*ETR3/NR, ETR4*) (expanded in γ) (Tomato Genome Consortium, 2012). More case studies like this are a clear future research interest in revealing how the expanded genetic repertoire from paleo-polyploidy events contribute to biological diversity and the evolution of unique characteristics of individual lineages.

All paleo-hexaploidy events identified so far are in eudicot lineages, including one in the core eudicot stem lineage (γ), one near the origin of the asterid Solanaceae family (T), one in the rosid *Brassica* lineages (X. Wang, Wang, *et al.*, 2011), possibly one in the *Gossypium* lineages (Paterson *et al.*, 2012) and one in the ancestral Compositae lineages (Truco *et al.*, 2013). Although some wild monocot plants such as the grass 'Timothy' (*Phleum pratense*) (NordenskiÖLd, 1953), and crops such as the bread wheat (*Triticum aestivum*) are neo-hexaploids, paleo-hexaploidy has not been found in any monocot genome studied so far. This raises curious questions about possible reasons and consequences associated with these events in the evolutionary history of some or all eudicot lineages, or alternatively, possible factors for suppressing such events in the evolution of other lineages.

2.9 Summary and perspective

Sequencing of the tomato genome was very valuable in many ways, as detailed elsewhere in this volume. With regard to angiosperm evolution, the tomato genome sequence revealed the third paleo-hexaploidy identified in plants, and the first one in asterids, adding an important sample to the small collection of paleo-hexaploids. It confirmed that the γ event shared by all sequenced rosids was also shared by asterids, unmasking a new clade for studying the effects and consequences of γ. The T paleo-hexaploidy is

possibly associated with the Solanaceae-Rubiaceae divergence, and divergence of early Solanaceae lineages, by triplicating the whole ancestral genome content, creating great potentials for subsequent diversification of homologous genomic associations and development of lineage-specific traits such as fruit ripening in tomato. Comparison of the tomato and potato genomes, both currently included in the genus *Solanum*, revealed continuous restructuring of paleo-triplicated ancestral loci long after the paleo-polyploidy events. The *Solanum* lineage is the first identified angiosperm lineage experiencing two paleo-hexaploidies but no paleo-tetraploidy. The consecutive paleo-hexaploidies γ and T are also valuable for comparative studies of the mechanisms and effects of paleo-hexaploidy and paleo-tetraploidy. Many questions about paleo-polyploidy have been answered, which nevertheless opened the door to more interesting questions.

CHAPTER 3 A WELL-RETAINED ANCIENT GENOME DUPLICATION IN THE SLOWLY EVOLVING BASAL EUDICOT *NELUMBO* (LOTUS) LINEAGE

3.1 Introduction

Nelumbo is the only genus in the Nelumbonaceae family, and has only two species: *N. nucifera* (sacred lotus) and *N. lutea* (yellow lotus), both aquatic plants. Lotus plants are well known for their divine flowers, seed longevity (more than 1000 years), self-cleaning leaf surface, and thermogenesis. They have deep roots in Asian culture and agriculture, and are widely cultivated for many uses such as gardening, food, and herbal medicine. Nelumbonaceae belongs to the basal eudicot order Proteales. Basal eudicots include the Ranunculales, Sabiales, Proteales, Trochodendrales, and Buxales orders. Their phylogenetic relationships have not been fully resolved, partly due to much diversity in their morphological and reproductive characters, and more fundamentally due to the antiquity and closeness of their divergence events and substantial variation in their lineage evolutionary rates. Nevertheless it is clear that Proteales are an outgroup of core eudicots, which include all other eudicots sequenced to date. The *Nelumbo* lineage originated around 135~125 MYA, and is considered a 'living fossil' because of both molecular and morphological stasis (Moore *et al.*, 2010; Michael J Sanderson & Doyle, 2001).

Sequencing of the sacred lotus ('China Antique' variety) genome (Ming *et al.*, 2013) revealed that *Nelumbo* diverged from the core eudicot crown group before the γ paleo-hexaploidy occurred in the latter. Therefore lotus is not only the first basal eudicot genome sequenced, but also the only eudicot genome sequenced so far that did not share γ, making it a natural reference for genome comparisons in core eudicots. Analysis of evolutionary distance between homeologous genes revealed that average nucleotide substitution rate in lotus is ~30% slower than that in grape (*Vitis vinifera*), another slowly evolving lineage in basal rosids. Lotus has experienced a paleo-tetraploidy λ in its own lineage around 76~54 MYA. In accord with its slow lineage nucleotide substitution rate, more ancestral loci were

retained in lotus after λ than in many other paleo-polyploid genomes. Compared to grape, the widely used reference in plant genome comparisons, the lotus genome aligns to more syntenic genes in both eudicots (such as *Arabidopsis*) and monocots (such as rice and sorghum). These characters made lotus a good new reference for comparisons of core eudicot genomes and monocot genomes.

3.2 A lineage-specific paleo-tetraploidy (λ) after divergence with core eudicots

Sacred lotus is a paleo-tetraploid. This whole genome duplication event, hereby named 'λ', is reliably detected by both *ab intra* alignment within the genome itself (data not shown), or when comparing against another genome such as grape (a rosid, **Figure 3.1**) and rice (a monocot, data not shown). When aligning the lotus and grape genomes, up to three homeologous regions are clear in grape, as expected from the γ triplication in grape. Reciprocally, each γ region in grape genome aligns about equally well to up to two regions, λ paralogs, in the lotus genome.

Importantly, this implies that the *Nelumbo* lineage did not experience the pan-core eudicot paleo-hexaploidy γ, at least not in its entirety. It has remained unclear whether γ is a single event or a series of two nuclear fusion events (Lyons *et al.*, 2008; Ming *et al.*, 2013; Tang, Wang, *et al.*, 2008). In the latter case, it would be possible, albeit improbable, that only the first one was shared by the ancestors of lotus (and perhaps some other basal eudicots), accounting for the low proportions of lotus loci with paleo-polyploidy depth greater than 2 as expected from λ (**Table 3.1**). An alternative to this hypothesis is that the extra depth could originate from additional duplication(s) much earlier than λ and different from γ. Current data favor the second hypothesis as both syntenic patterns and sequence divergence indicated that γ-paralogs in grape are equivalently distant to their lotus ortholog. However there is intrinsic uncertainty associated with studies of ancient events occurring several to hundreds of millions of years ago, especially if they are chronologically close to each other. Availability of more basal eudicot genomes will be helpful to verify if γ indeed occurred completely after Proteales had diverged.

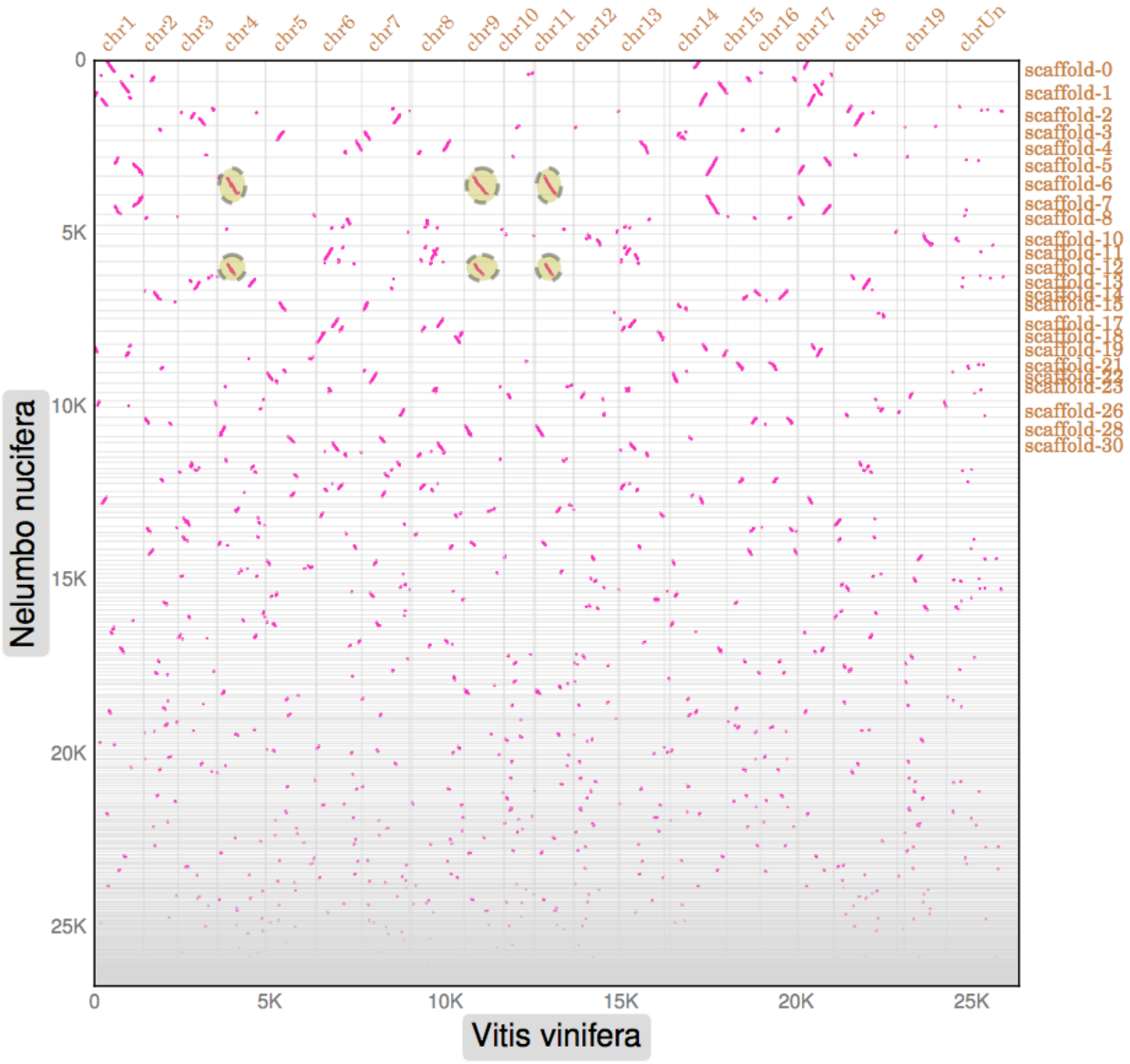


Figure 3.1 Dot plot alignment of the lotus and grape genomes. Each dot represents a pair of syntenic genes. Yellow circles with dashed borders highlight an exemplary set of syntenic regions with multiple-to-multiple (in this case 2 lotus - 3 grape) correspondences between the orthologs, reflecting lineage-specific duplication λ (2x) in lotus and pan-core eudicot triplication γ (3x) in grape. Names of small scaffolds in lotus are omitted for clarity.

Table 3.1 Distribution of lotus loci at different paleo-polyploidy depths.

Paleo-polyploidy depth	**1**	**2**	**3**	**4**	**>4**	**All**
# of ancestral loci	5279	4289	279	165	80	10092
# of genes	5279 (19.8%)	8578 (32.1%)	837 (3.1%)	660 (2.5%)	510 (1.9%)	15864 (59.4%)
Domain coverage (# of unique domains)	2263 (1112)	1861 (689)	296 (20)	174 (15)	103 (1)	3046

* Singleton homeologs (depth=1) in the sacred lotus genome were compiled from inter-genomic alignment with the grapevine and *Arabidopsis* genomes to be conservative in including sacred lotus specific genes.

Overall, 92.3% of the lotus genic regions have detectable paleo-polyploid origin. Among the homeologs (excluding tandem duplications) 5279 (33.3%) are singletons, 8578 (54.1%) are in duplex; and 2007 (12.6%) have more than 2 homeologs (**Table 3.1**). Unlike the case of a quartet of regions on rice chromosomes 11-12, and sorghum chromosomes 5-8 (Paterson *et al.*, 2009), there does not appear to be large blocks of the lotus genome experiencing concerted evolution. A total of 288 lotus genes are contained in regions with no synteny to any of the genomes of grape, *Arabidopsis*, sorghum, and itself, nor are they homologous to any other lotus genes. These single copy genes, if not accounted for by mis-annotation or technical limits of homology search, may be either ancestral genes uniquely retained in the lotus genome, or lineage-specific genes.

The λ-duplicates in lotus have a median synonymous substitution rate (Ks) distribution of 0.5428, corresponding to an age of ~27 million years ago (MYA) on the basis of an average rate of ~1*10e-8 in eudicots (Koch, Haubold, & Mitchell-Olds, 2000; Kenneth H. Wolfe, Sharp, & Li, 1989) or 54 MYA on the basis of the grape lineage rate (**Figure 3.2**). The median Ks between the grape triplets produced in γ (which we infer to have occurred after the *Vitis*- *Nelumbo* lineages diverged) is about 1.2208, which is slightly higher than the Ks for grape-lotus (median is 1.1452) despite being a more recent event (**Figure 3.2**). This suggests that the mutation rate in *Nelumbo* is lower than in *Vitis* (*Nelumbo*-*Vitis* differentiation reflecting an average of the two rates). Relative to the estimated 135~125 MYA (Moore *et al.*, 2010)

divergence of *Nelumbo* from other eudicots, this implies that the *Nelumbo* duplication occurred about 65 MYA with a range between 76 and 54 MYA.

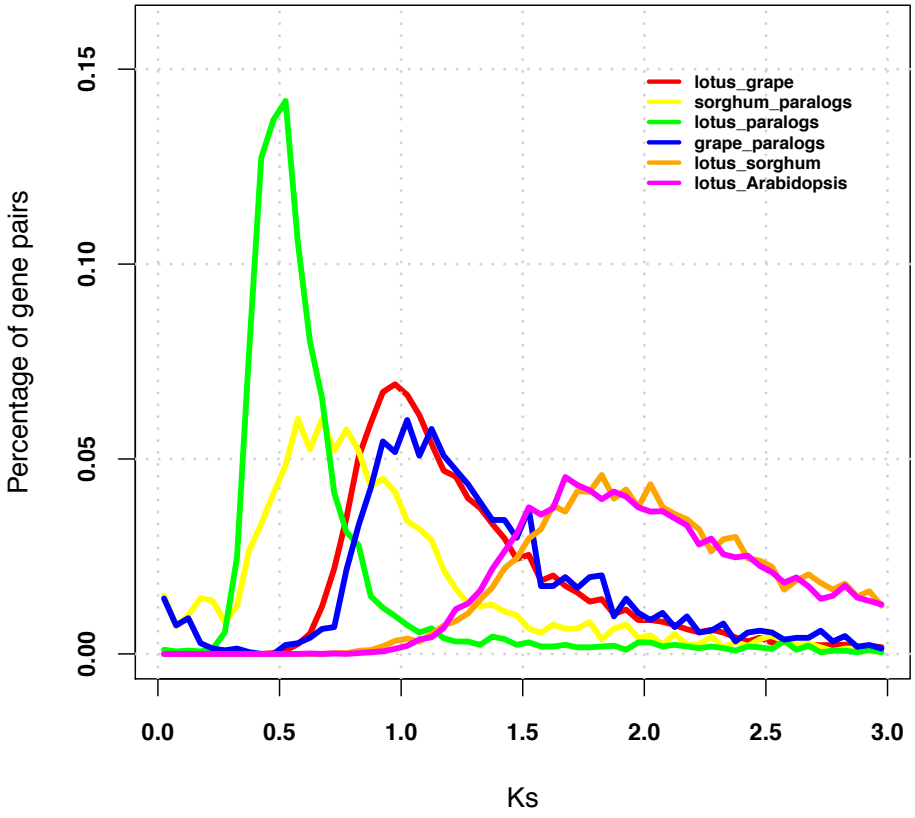


Figure 3.2 Distribution of synonymous substitution rate (Ks) between homeologous gene pairs in intra- and inter- genomic comparisons. See figure legend for details.

3.3 Slow lineage evolutionary rate

Three lines of evidence suggested that the lineage nucleotide substitution rate in lotus is about 30% slower than that of grape. First, since syntenic and phylogenetic evidence both dated the lotus-grape divergence before the pan-core eudicot γ triplication (affecting grape but not lotus) (Ming *et al.*, 2013), if the lineage rate in lotus is equal to or higher than grape, lotus-grape orthologs should have average synonymous substitution rates (Ks) greater than γ-paralogs in grape. Instead, **Figure 3.2** showed average

Ks between lotus-grape orthologs less than that between grape γ-paralogs. Second, the lotus lineage mutation rate also appears slower (about 29.26% slower) than that of grape based on a maximum-likelihood tree of 83 plastid genes (Moore *et al.*, 2010) and expert dating of the respective speciation events (Hedges *et al.*, 2006) using the r8s program (Michael J. Sanderson, 2003) with penalized likelihood. Third, the lotus genome has retained more ancestral loci following its lineage-specific WGD (**Figure 3.3** in next section).

Nucleotide substitution rates in plants seem to have a definite trend of negative association with generation time and longevity, albeit with other complicating factors (such as breeding system, population size, speciation rate, environment), and with still unclear mechanisms (Gaut, Yang, Takuno, & Eguiarte, 2011; S. A. Smith & Donoghue, 2008). This could be a major factor underlying the slow lineage rate in *Nelumbo*.

Since its release in 2007 the grapevine genome has been widely used as a reference in angiosperm comparative genomics because of its phylogenetic position in basal rosids, slow lineage nucleotide substitution rate, and lack of reduplication. Lotus is a basal eudicot sister to all core eudicot lineages including grape, not affected by γ, and has lineage nucleotide substitution rate ~30% slower than grape. Lotus potentially represents a better reference than grape for comparative genomic studies involving eudicots and monocots.

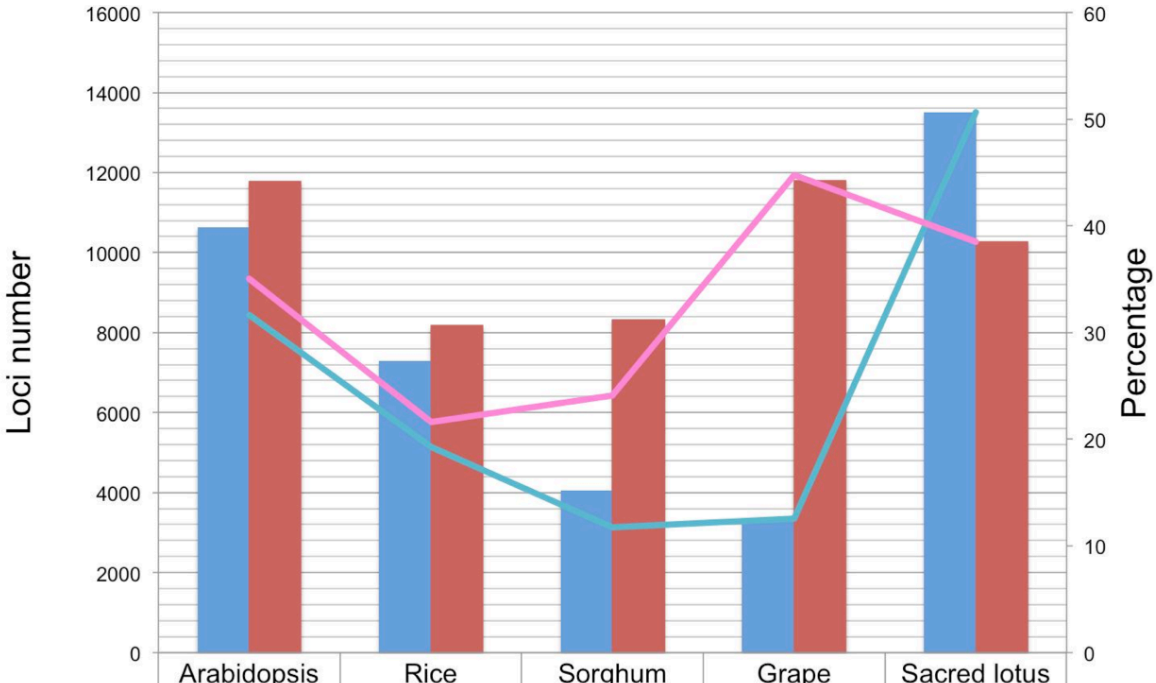
3.4 Outgroup for core eudicot and monocot genome comparisons

When analyzing synteny patterns in plant genomes with high paleo-polyploidy levels, reciprocal and differentiated gene loss in the evolution of the paleo-subgenomes sometimes render the signals of synteny elusive. In such cases an outgroup genome with few paleo-polyploidy events is used to take advantage of the smaller evolutionary distances between orthologs, and better retained ancestral gene orders. An example of such outgroup genomes is the grape genome, which has been widely used in plant genome comparisons since its release in 2007 (Jaillon *et al.*, 2007). Compared to grape, the newly sequenced lotus genome has higher proportions of homeologous genes aligned with both eudicot and monocot genomes,

exemplified in **Figure 3.3**. The lotus genome also has a high level of ancestral loci retention (**Figures 3.3, 3.4**). Therefore lotus qualifies to be a new outgroup for core eudicot and monocot genome comparisons.

Because of reciprocal loss of duplicated ancestral loci during diploidization, inter-genomic alignment (such as between the lotus and grape genomes) often recovers more synteny signals than intra-genomic alignment (such as within the lotus or grape genome). This seems true both within eudicots, such as grape-*Arabidopsis* vs. *Arabidopsis*-*Arabidopsis*; or grape-tomato VS tomato-tomato; and inside monocots, such as sorghum-maize vs. maize-maize. While showing the same trend, the difference of inter- and intra- genomic comparisons of lotus is much less dramatic (compare lotus-lotus vs. lotus-grape, and grape-grape vs. grape-lotus in **Figure 3.3**) due to higher retention of homeologs after λ.

Therefore, extensive synteny within itself, as well as with other eudicot and monocot genomes make the sacred lotus genome not only an evo-genomic reference potentially more informative than the grape genome, but also a valuable candidate for the reconstruction of the pan-eudicot genome, and improvement of distant genome comparisons between eudicots and monocots (Paterson, Bowers, & Chapman, 2004; Tang *et al.*, 2010). Such comparisons, and the critical phylogenetic position, relatively simple evolutionary history, and slow evolution rate of the sacred lotus genome, are altogether valuable for clarifying the so-far elusive relationships among the chronically close events of the pan-core eudicot γ paleo-hexaploidy (Jaillon *et al.*, 2007; Tang, Bowers, *et al.*, 2008; Tang, Wang, *et al.*, 2008), early eudicot radiation, and a paleo-polyploidy in early monocot lineages (Tang *et al.*, 2010).



	Arabidopsis	Rice	Sorghum	Grape	Sacred lotus
Grape	10638	7290	4050	3318	13508
Sacred lotus	11773	8172	8309	11801	10269
Grape(%)	31.66	19.25	11.74	12.59	50.62
Sacred lotus(%)	35.04	21.58	24.09	44.79	38.48

Figure 3.3 Number and percentage of genes in the query genomes having homeologous genes in the reference genome. Grape or sacred lotus genomes were used as reference. *Arabidopsis*, rice, and sorghum genomes were used as queries. All pairwise differences are statistically significant.

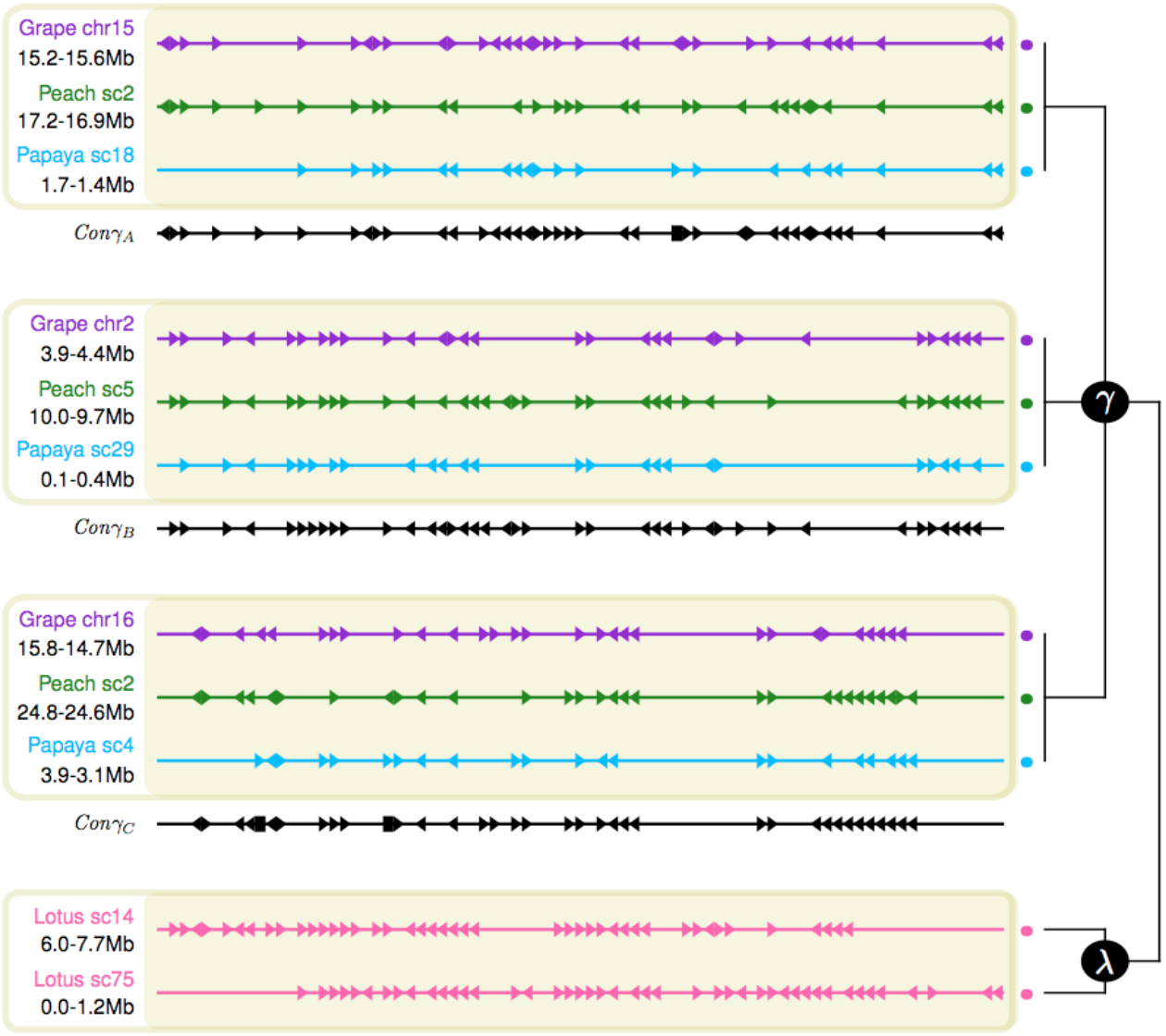


Figure 3.4 Multiple alignment of a set of syntenic regions in papaya, peach, grape and lotus. Triangles represent individual genes and their transcriptional orientations. Genes with no syntenic matches are not plotted. The event γ is the paleo-hexaploidy that occurred in ancestral eudicots, and is shared by the grape, peach, and papaya lineages. The event λ is the paleo-tetraploidy in the *Nelumbo* (sacred lotus) lineage after it diverged from the rest of eudicot lineages. The γ regions are grouped into three γ-subgenomes based on parsimony principles. Aligned genes within each γ subgenome are merged into a consensus order (Con γ_A, γ_B, γ_C respectively). Ancestral genes with uncertain orientations are represented by squares. The pair of sister λ regions in lotus is displayed at the bottom.

3.5 Discussion

Scrutiny of the sacred lotus genome revealed a paleo-tetraploidy event λ, with each of the duplicated λ regions matched to the same set of γ triplet regions in grape, indicating 2-to-3 correspondence. Sacred lotus genes are typically diverged to similar degrees from their (up to) three orthologous grape genes, with the best matching orthologs distributed evenly among triplets of γ regions. Molecular dating based on synonymous substitution rates between homeologous genes (Ks) positioned λ at about 76~54 MYA. These results indicate that the ancestral *Nelumbo* lineage diverged from core eudicot ancestors before the γ paleo-hexaploidy occurred in the latter around 125 MYA, and subsequently experienced a lineage specific paleo-tetraploidy (Ming *et al.*, 2013).

When two lineages diverged before paleo-polyploidy occurred in one of them, we would expect similar divergence of paralogous genes in one genome when compared to their shared ortholog in the other genome, as observed in previous studies (Tang, Wang, *et al.*, 2008; X. Wang, Wang, *et al.*, 2011). Comparisons of two λ paralogs and their grape orthologs generally fit this prediction (**Figure 3.5a**). Interestingly, when comparing sets of two λ paralogs with their common sorghum orthologs, there seem to be consistent differentiation in the distance of the two branches (**Figure 3.5b**). Notice that the singleton λ homeologs have overall averaged distance between the duplets, consistent with reciprocal gene loss. This discrepancy in the lotus-cereal comparison could be explained by fast evolutionary rates in cereals and/or slow evolutionary rate in *Nelumbo* and λ being older than it appears. Alternatively, this is also consistent with genome structural compartmentalization, with genes within the same genome undergoing different evolutionary trajectories (X. Wang, Wang, *et al.*, 2011). Wider taxa sampling at neighboring branches will help better distinguish the possibilities.

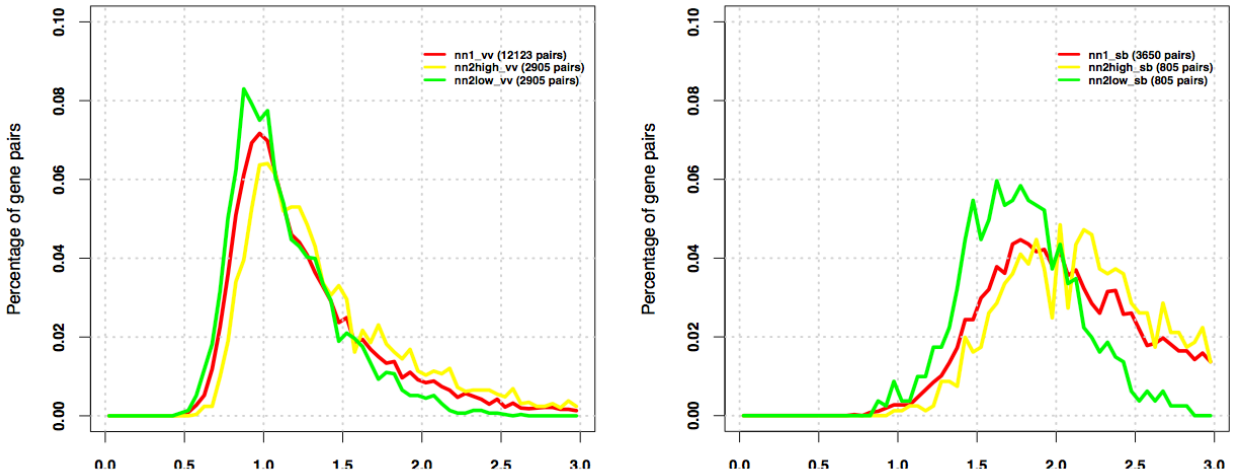


Figure 3.5 Ks distributions between orthologous homeologs gene pairs comparing the lotus genome to the grape genome (a) and sorghum genome (b). Red lines represent single copy homeologs in the sacred lotus genome. The pairs of homeologs in the lotus genome are arbitrarily assigned to the high Ks value group (yellow lines) and low Ks group (green lines). If in all the duplets both sacred lotus genes are equally distant from the grape/sorghum counterpart, it is expected the two sampling distributions should be alike.

Subfunctionalization (M. Lynch & A. Force, 2000) is a major direction in the retention and evolution of duplicated genes, where they undergo complementary divergence or loss of genic or regulatory sequences leading to eventual functional complementation. The particularly high retention rate of λ homeologs in the lotus genome suggested that subfunctionalization may have affected many of them. In order to survey such effects, we have analyzed sequences of lotus homeologs from several aspects. Comparison of pairs of homeologous genes showed that the majority have no difference in the composition of PFAM domain families, while 453 (11.6% of) gene pairs differ by up to 5 domains. Those unshared domains have mean length 17 aa in a range of 0~890 aa. The shared domains have mean bit score difference 22.08 in a range of 0 to 871.7. Between homeologous lotus gene pairs, differences of mRNA length (excluding 5' and 3' UTRs), CDS length, and intron length all follow geometric-like distributions (**Figure 3.6**), consistent with independent accumulation of small indels. The changes of length in exonic and intronic regions seem uncorrelated (**Figure 3.6**). While not an exhaustive analysis,

these results suggested that subfunctionalization of the lotus homeologs may have occurred at multiple transcriptional and post-transcriptional levels, which is an interesting subject for future studies.

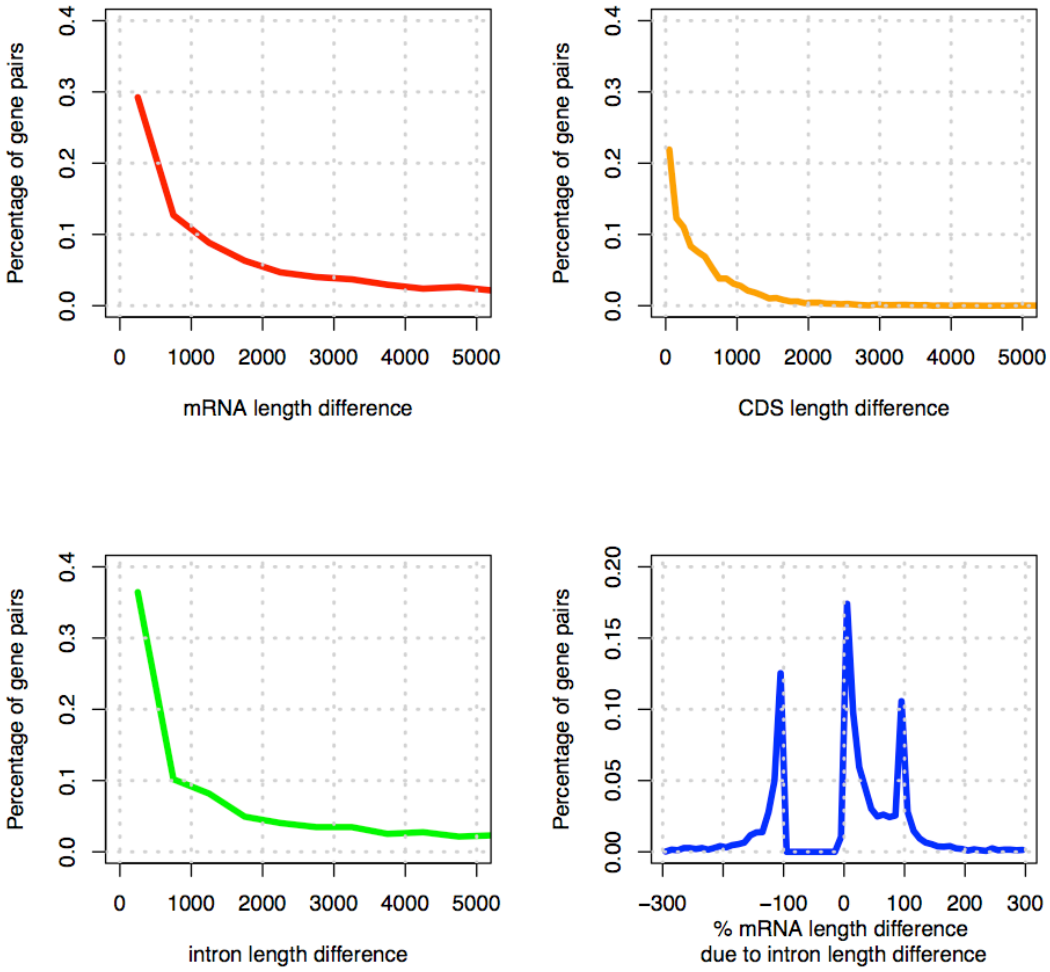


Figure 3.6 Differences in mRNA length, CDS length, intron length, and percentage mRNA length difference attributable to intron length difference. Values were measured for each pair of homeologous Sacred lotus genes. Extreme ranges on the X axes were trimmed for clarity.

Rates of molecular evolution often vary greatly among plant lineages (Gaut *et al.*, 2011). For example, nuclear gene nucleotide substitution rate in the *Vitis* lineage is estimated to be about 20% less than in *Populus* (Tang, Wang, *et al.*, 2008), while the *Nelumbo* rate is about 30% slower than *Vitis* (Ming *et al.*, 2013). Nucleotide substitution rates in plant organellar genomes also vary greatly, sometimes up to

100 fold or even more (Mower, Touzet, Gummow, Delph, & Palmer, 2007; K. H. Wolfe, Li, & Sharp, 1987). Although less explored, the frequency of genome rearrangements also varies among taxa, by at least ten-fold (Paterson *et al.*, 1996; Zuccolo *et al.*, 2011). Some major reasons underlying these variations are differences in generation time, life history, and environment of the organisms (Gaut *et al.*, 2011; S. A. Smith & Donoghue, 2008; Tuskan *et al.*, 2006; Young *et al.*, 2011).

In addition, it has been suspected that paleo-polyploidy events may accelerate lineage evolutionary rates, such as rates of nucleotide substitutions, genome structural rearrangement at macro- and micro- scales, and gene family size alteration (Adams & Wendel, 2005b; Lynch & Conery, 2000; Otto & Whitton, 2000). However, the effects of polyploidy on the genome and organism is multi-layered, including, in many instances, increased cell size, increased rate of early development, increased illegitimate recombination, increased genetic and cellular instability, increased expression regulatory complexity, transposable element expansion, nearly doubled effective population size after autopolyploidy, and morphological changes (Adams & Wendel, 2005b; Otto, 2007; Otto & Whitton, 2000). Empirical observation shows that many lineages that have been identified with slow evolutionary rate, such as grape, papaya, lotus, and poplar, have experienced relatively few WGDs. On the opposite side, many lineages that have been identified with fast evolutionary rate, such as *Arabidopsis*, bladderwort, and grasses, have experienced several WGDs. For example, the bladderwort genome, that has experienced at least 4 WGDs, has higher average nucleotide substitution rate than tomato and monkey flower, each having experienced 2 less WGDs (Jingping Li, Tang, Bowers, Ming, & Paterson, 2014). There are also exceptions. For example, the soybean (*Glycine max*) lineage has experienced at least 3 WGDs but has slow molecular evolution rate (Schmutz *et al.*, 2010); the banana (*Musa acuminata*) lineage has experienced at least 4 WGDs but has slow molecular evolution rate (D'Hont *et al.*, 2012). Paleo-polyploidy events have also been associated with species expansion episodes (Otto & Whitton, 2000; D. E. Soltis *et al.*, 2009; Van de Peer, Maere, & Meyer, 2009), and adaptation in changing environmental conditions (Fawcett *et al.*, 2009; Levin, 1983). Nonetheless, there have been very few studies that directly measure evolutionary rates before and after polyploidy events. One study showed that

in the yeast *Saccharomyces cerevisiae* following chemical treatment mutation frequency may be elevated in tetraploid but not diploid strains (Mayer, Goin, Arras, & Taylor-Mayer, 1992). At present it seems likely that while paleo-polyploidy has the potential of increasing lineage evolutionary rate, the actual rate is determined by complicated dynamic interactions of multiple biological and environmental factors.

Having preserved extensive synteny conservation from its single lineage specific paleo-tetraploidy event λ, the sacred lotus genome has also retained high levels of homeology with other plant genomes such as grape, *Arabidopsis*, rice, and sorghum. Lotus is a basal eudicot lineage and didn't share the pan-core eudicot paleo-hexaploidy. In addition, it has one of the slowest lineage evolutionary rates in flowering plants (Ming *et al.*, 2013). Lotus and the widely used reference grape genome both have high-quality assemblies, grape with a chromosome-level assembly (scaffold N50=2.0 Mb) and lotus with a megascaffold-level assembly (scaffold N50=3.4 Mb). Unique features of the lotus genome make it a valuable additional and in some cases better reference than grape in outgrouping core eudicot and monocot genome comparisons and bridging distant plant genome comparisons across eudicots and monocots.

CHAPTER 4 THE PAN-*GOSSYPIUM* PALEO-POLYPLOIDY AND TWO NUMT REGIONS IN TWO OF THE SUBGENOMES IN MODERN *G. RAIMONDII*

4.1 Abstract

Paleo-polyploidy (ancient whole genome duplication) events are a key evolutionary force of angiosperm genome organization. Paralogous regions from one or more paleo-polyploidies are detectable in all sequenced angiosperm genomes. In the lineages leading to *Gossypium* (cotton) species, a paleo-hexaploidy (γ) was shared with other core eudicots. In addition, the newly sequenced *G. raimondii* genome revealed an unusual paleopolyploidy ('C') dated to ~60 MYA, resulting in five- to six-fold duplication of the ancestral *Gossypium* genome compared to an outgroup (*Theobroma cacao*) lacking this event. Per site synonymous substitution between C-duplicates forms a bell-shaped curve with a single peak, indicating that C consists of one event or several chronologically very close events. Our results here showed that the five biggest subgenomes can be divided into two groups (SGI and SGII) based on different subgenome structure. Synteny block size, fractionation level, and homeologous sequence divergence are significantly different between the two groups. Estimated divergence based on synonymous substitution rate corresponds to ~2.7 million years separation between SGI and SGII subgenomes. On the other hand severe gene loss and complete loss of coverage in most regions of the genome suggested that the hypothetical sixth subgenome was likely produced independent of the other subgenomes. Ancestral reconstruction revealed that ancestral cotton loci tend to retain homeologs or return to single copy repeatedly following the γ and C events. We also identified two multi-gene NUMT regions on cotton chromosomes 1 and 13, belonging to SGI and SGII respectively. NUMT1 is longer and better preserved than NUMT13, consistent with SGI-SGII comparison. Sequence conservation between syntenic genes in the NUMT regions is far better than in other genomic regions. Our results revealed evolutionary and functional compartmentation in the *G. raimondii* genome that is worth further studies.

4.2 Introduction

Gossypium raimondii is a wild diploid cotton species (2n=26) that resembles the D progenitor genome of the domesticated tetraploid cottons. *Gossypium* (cotton) of the Malvaceae family includes 45 diploid, and five allopolyploid species *G. hirsutum*, *G. barbadense* (two commercial cotton), *G. tomentosum*, *G. mustelinum*, and *G. darwinii*. The tetraploid cottons each contain two divergent progenitor genomes, A (African) and D (Mexican). The two progenitor genomes diverged about 5-10 MYA (million years ago) and then reunited about 1-2 MYA in the New World (J. F. Wendel, 1989). Therefore sequencing its genome (Paterson *et al.*, 2012) is of interest to many basic and practical questions.

Analysis of the *G. raimondii* genomes revealed two paleo-polyploidy, or ancient whole genome duplication (WGD) events in its lineage history, one in the core eudicot ancestral lineage, and one in the *Gossypium* lineage shortly after it diverged from a common ancestor shared with the *Theobroma* lineage (Paterson *et al.*, 2012). Paleo-polyploidy events are duplications of whole genome content, commonly via unreduced gametes or hybridization, which occurred millions to hundreds of millions of years ago. Although polyploidized species experience dramatic mutations in the genome and often go extinct (Arrigo & Barker, 2012), paleo-polyploids that have survived long evolution have been identified in the eukaryotic kingdoms of Animalia (Dehal & Boore, 2005; Jaillon *et al.*, 2004), Fungi (Kellis *et al.*, 2004; K. H. Wolfe & Shields, 1997), and Chromalveolata (Aury *et al.*, 2006), and are widespread in Plantae, especially in flowering plants. Paleo-polyploidies incur immediate effects such as increased genetic content, increased cell volume, and altered chromosomal pairing; and long-term effects such as gene family expansion, rewired biochemical networks, and karyotype change, usually through the diploidization process. Therefore it is not surprising that paleo-polyploidies are associated with origination, radiation, and evolution of a wide range of angiosperm taxa (for some recent reviews see (D. E. Soltis *et al.*, 2009; Van de Peer *et al.*, 2009)).

The *Gossypium* lineage has experienced repeated paleo-polyploidies (Paterson *et al.*, 2012). Besides having shared the pan-core eudicot paleo-hexaploidy γ (Bowers *et al.*, 2003; Jaillon *et al.*, 2007; Tang, Bowers, *et al.*, 2008), it also had a lineage-specific paleo-polyploidy, herein called ‘C’. Particularly

interesting is that C is a paleo-(do)decaploidy (5~6x duplication) of the ancestral *Gossypium* genome (Paterson *et al.*, 2012). Homeologous genes duplicated in the C event all have similar sequence divergence, indicating that C was a single polyploidy or several events close in time. The word 'subgenome' is sometimes used to refer to the A and D progenitor genome components in a tetraploid cotton. We specifically note that in this paper 'subgenome' refers to pre-duplicated ancestral *Gossypium* genome components that have been substantially restructured (during diploidization) in forming the extant diploid genome, the same way as used in other such studies (J. C. Schnable *et al.*, 2011).

We report here that the five major C-subgenomes can be divided into two groups (SGI and SGII) based on different subgenome structure. Three subgenomes in SGI cover 75.13%, and two subgenomes in SGII cover 15.34% of the cotton genome. Synteny block size, extent of fractionation (post-WGD gene loss), and homeologous sequence divergence are significantly different between the two groups. Difference in average synonymous substitution rates corresponds to about 2.7 million years separation between SGI and SGII subgenomes. The hypothetical sixth subgenome has completely lost coverage in most regions of the genome, suggesting that it may have been produced independent of the other subgenomes. Using the aligned subgenomes and two outgroup genomes (cacao and grape) we reconstructed the ancestral cotton gene order, which helped clarify synteny patterns from the nested γ event in *G. raimondii*. It also helped reveal repeated tendency to retain or lose homeologs among ancestral cotton loci following the γ and C events, respectively.

We also revealed two NUMT (nuclear mtDNA) regions on cotton chromosomes 1 and 13, belonging to SGI and SGII respectively. NUMT-I is longer and better preserved than NUMT-II, consistent with SGI-SGII comparison. NUMT-I is syntenic to two NUMT regions in cacao, while NUMT-II is syntenic to no NUMT region in cacao but to the *Arabidopsis thaliana* NUMT region on chromosome 2. Sequence conservation between syntenic genes in the NUMT regions is exceptionally better than in other genomic regions. Our results will facilitate further analyses on the formation of the C paleo-(do)decaploidy, and structural and functional compartmentation in the evolution of the *G. raimondii* genome.

4.3 Methods

4.3.1 Data sources.

Nuclear genome assembly and annotation of *Gossypium raimondii*, *Theobroma cacao*, *Vitis vinifera*, *Arabidopsis thaliana* were downloaded from Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html#, v10). The mitochondrial genome of *Arabidopsis thaliana* was downloaded from TAIR 10 (ftp://ftp.arabidopsis.org/home/tair/Sequences/). The mitochondrial genome of *Gossypium hirsutum* was downloaded from NCBI with accession number JX065074.

4.3.2 Similarity search and synteny detection.

Matching gene pairs were identified by LASTZ (Harris, 2007) with default settings. Similarity between nucleotide sequences was searched with LAST under default settings. Synteny blocks were identified and quota screened by QUOTA-ALIGN (Tang *et al.*, 2011) with block chaining distance 20, cotton-cacao quota ratio 5:1. Only blocks with at least 5 anchor gene pairs were retained for further analysis.

4.3.3 Subgenome division.

Synteny blocks in the *G. raimondii* genome were chained by dynamic programming into 5 major subgenomes based on structural constraints (no overlapping regions within a subgenome), and in favor of longer blocks with more anchor genes, which are preferentially selected. The pairwise alignments were resolved by a topological sorting algorithm to produce multiple alignment of the cotton subgenomic regions (**Supplementary File 1**). The cacao genome from the sister clade was used as outgroup reference of gene order to guide the chaining process.

4.3.4 Reconstruction of ancestral gene order before the C event.

The above aligned subgenomic regions were merged into pre-duplication segments by interpolation following methods in previous studies (Bowers *et al.*, 2003). We reconstructed the ancestral cotton genome from two data sets. The high confidence ancestral genome contains 13,772 loci, each of which is

supported by 2 or more cotton homeologs, or 1 cotton homeolog with its ortholog retained in cacao or grape (assuming independent syntenic duplications are unlikely). The low confidence ancestral genome was reconstructed under relaxed criteria that permit including singleton cotton genes, which resulted in 25,651 loci. Because of the lack of outgroup genomes (cacao is the only other sequenced genome in Malvales), we were not able to validate many loci in the low confidence ancestral genome. Therefore, we used the high confidence ancestral cotton genome in downstream analyses. The reconstructed gene order is provided in **Supplementary File 1**.

4.3.5 Fractionation level calculation.

The reconstructed ancestral gene order was divided into 200 gene sliding windows. Each sliding step is 100 genes. Then, fractionation level in each of the 5 subgenomes was calculated from the alignments in each window as the proportion of ancestral loci retained.

4.3.6 Synonymous substitution rate (Ks) calculation.

Protein sequences of homologous gene pairs were aligned using CLUSTALW2 (Larkin *et al.*, 2007), which was then used to guide their CDS alignment by PAL2NAL (Suyama, Torrents, & Bork, 2006; Yang, 2007). To calculate Ks, we used the Nei–Gojobori method implemented in the yn00 program in the PAML package (Yang, 2007). A Python script was used to create a pipeline for all the calculations, as described in (Tang, Wang, *et al.*, 2008).

4.3.7 Phylogenetic analysis.

Genes within the same homeolog group were aligned using MUSCLE v3.8.31 (Edgar, 2004). Codon alignments were edited with Gblocks (Talavera & Castresana, 2007) to remove poorly aligned regions. Phylogenetic trees were constructed using RAxML v7.3.5 (Stamatakis, 2006) with the fast bootstrapping algorithm, and cross validated with NEIGHBOR in PHYLIP (Felsenstein, 1989) with 100 bootstrapping replicates. The procedure is pipelined by an in-house python script.

4.4 Results

4.4.1 An ancestral *Gossypium* genome experienced 5~6x lineage-specific duplication

Paleo-polyploidies produce multiple homeologous regions in extant diploid genomes. The number of homeologous regions is called homeolog depth, duplication depth, or simply depth. For example, a paleo-tetraploidy (ancient genome doubling, 2x) produces up to homeolog depth of 2 in an extant genome (with some regions losing homeologs during evolution). We identified homeolog depth of 5~6 in the extant *G. raimondii* genome. It is visible in *ab intra* cotton alignment, and in inter-genomic alignment with the cacao (*Theobroma cacao*; diverged about 70-50 MYA) or grape (*Vitis vinifera*) genomes (diverged about 110 MYA). Both grape and cacao have not experienced lineage-specific paleo-polyploidy. Individual grape or cacao regions have five (infrequently six) best-matching cotton regions (and secondary matches resulting from the pan-core eudicot paleo-hexaploidy), supporting a paleo-(do)decaploidy in the *Gossypium* lineage.

Using the cacao genome as a reference, inter-genomic aligned synteny blocks in cotton were chained by dynamic programming into 5 major subgenomes based on structural constraints (no overlapping regions within a subgenome), and in favor of longer blocks with more anchor genes, which are preferentially selected. The pairwise alignments are then resolved by a topological sorting algorithm to produce multiple alignment of the subgenomes threaded by the cacao genome (**Supplementary File 1**). The majority of local alignments involve five different cotton chromosomes. A local alignment example is shown in **Figure 4.1**.

The sixth hypothetical subgenome, which appeared to have suffered severe gene loss and lost coverage completely in many regions of the genome, is of questionable existence. Alternatively the depth 6 regions may have been produced in independent segmental duplications. There are too few anchor genes (179) in those regions to make confident inferences. The five ‘major’ subgenomes collectively cover 90.48% of the cotton genome (total 37,505 genes) in 628 synteny blocks containing 21,390 anchor genes. The blocks span ranges from 5 to 679 genes (not counting tandem and proximal duplications less than 10 genes apart).

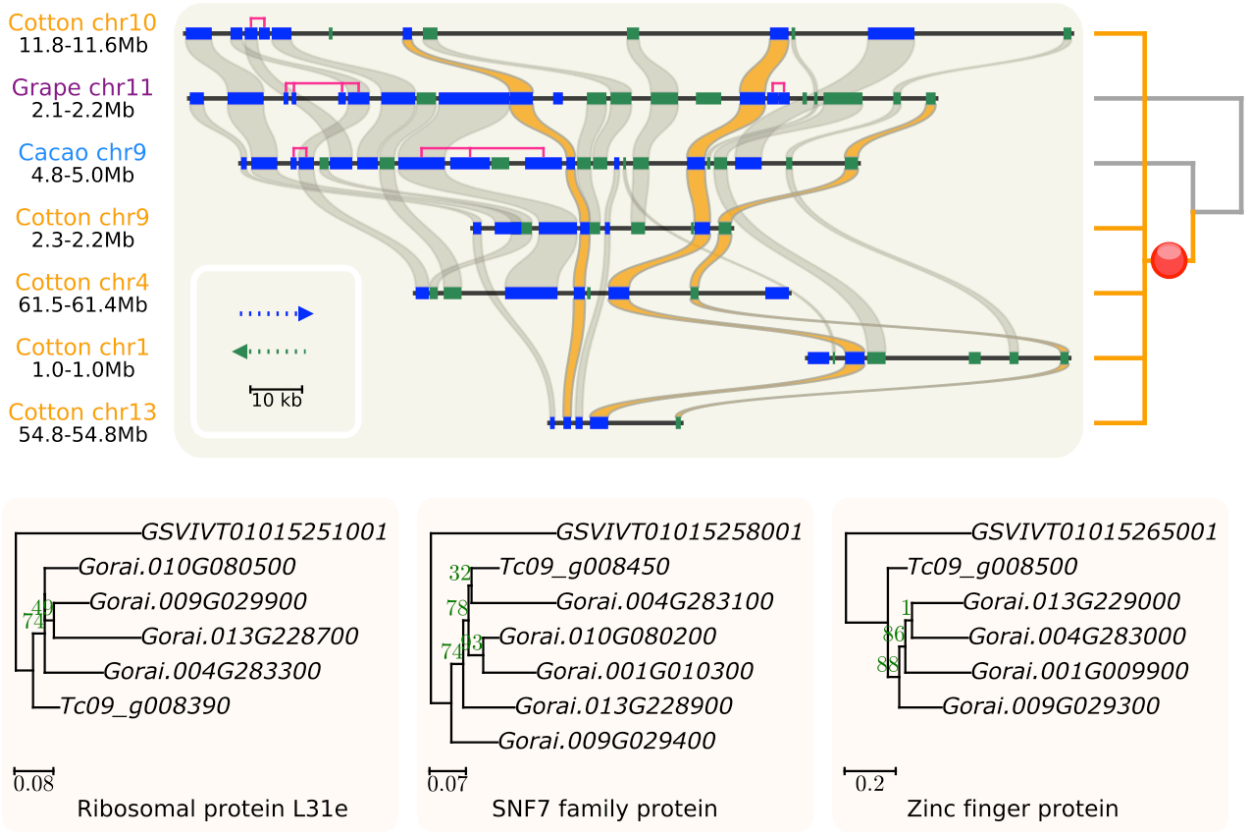


Figure 4.1 Example alignment showing syntenic relationships among five cotton subgenomic regions and their single orthologous regions in cacao and grape, respectively. Three well-retained gene clusters along these syntenic groups (highlighted by orange curves) are plotted to show overall consistency between the gene phylogenies and the evolutionary history of the genomes. The pan-*Gossypium* paleo-polyploidy is represented by the red ball. The sample trees were constructed by PhyML with aLRT SH-like procedure using default parameters.

4.4.2 Differential evolution of subgenome structure following the paleo-(do)decaploidy

Among the 5 major cotton subgenomes, a one-way ANOVA test of the effect of subgenome on synteny block size gave a p-value of 1.2e-4. The 5 subgenomes can be roughly divided into two groups, subgenomes 1-2-3 (SGI) and 4-5 (SGII). SGI has average synteny block span (measured as number of genes) 1.8x that of SGII (**Figure 4.2**). One-way ANOVA test of subgenome effect on synteny block

median synonymous substitution rate (Ks) gave a p-value of 0.0020. The block median Ks values of SGI and SGII differ by 0.032, which is roughly equivalent to 2.7 million years separation using an estimated *Gossypium* synonymous substitution rate of about 6e-9 (Kenneth H. Wolfe *et al.*, 1989).

SGI and SGII have different average block size, 53.57 and 29.7 respectively (three SGI subgenomes: 54.94, 58.10, 46.53; two SGII subgenomes: 32.52, 25.51), giving Mann-Whitney test p-value 0.0055. We recognize that the subgenome chaining algorithm preferentially selects longer blocks (when other criteria are equally met), which is one contributing factor to this observation. However this is not the sole rule of chaining. In fact the first rule of the chaining process is non-overlapping regions within a subgenome. Therefore although the chaining process may partially account for the differentiated patterns, there is no simple causality between the chaining process and the block size difference in the two groups. Furthermore, in addition to differential block sizes, the two groups are also different in their median block Ks values, having median values 0.416 and 0.448, respectively (three SGI subgenomes: 0.416, 0.410, 0.423; two SGII subgenomes: 0.440, 0.465), with Mann-Whitney p-value of 2.88*10e-6 (**Figure 4.2**).

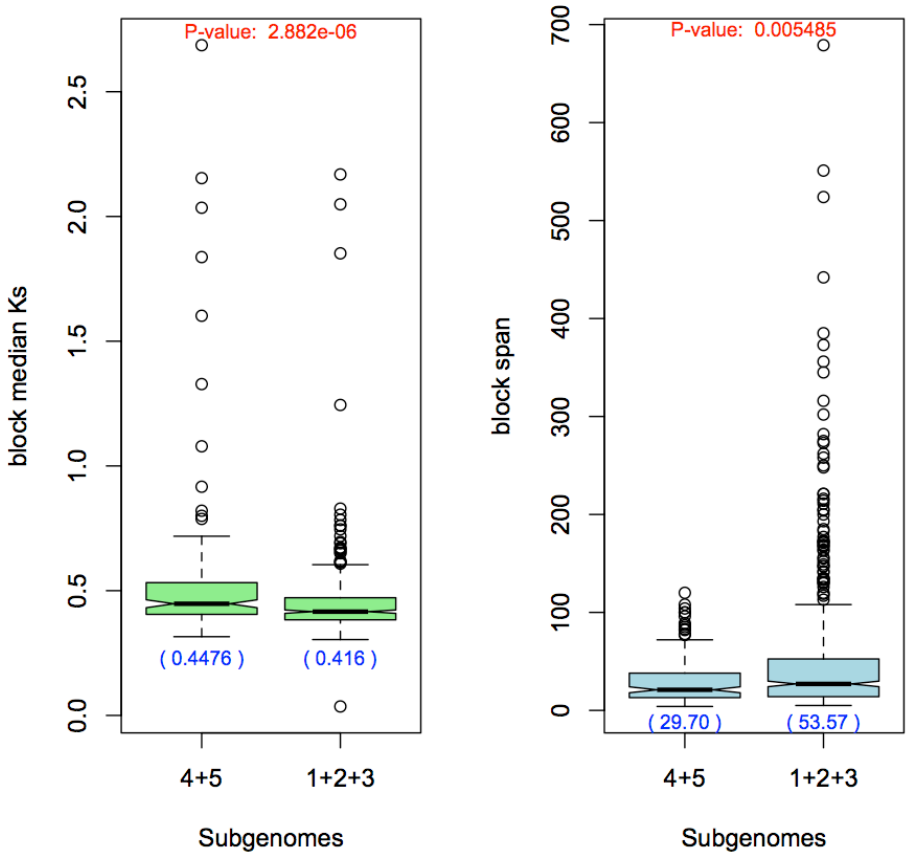


Figure 4.2 Box and whisker plots showing distributions of synteny block median Ks and block span. On the left are synteny block median Ks values between cotton genes and their orthologs in cacao. On the right are block spans (measured as gene counts) in cotton (right). Each dot represents measurement from one synteny block. The p-values (red) are from two sample Mann-Whitney tests. Median (for Ks) and mean (for span) values are shown in blue. Notches are centered at sample medians.

The two subgenome groups SGI and SGII have also retained different proportions of ancestral cotton loci, averaging about 0.42 for SGI and 0.13 for SGII (two sample Student's t-test p-value <2.2e-16). The average retention rates of the inferred ancestral cotton genome are 0.5317, 0.4260, 0.3124, 0.1714, 0.0889 for the five subgenomes, respectively (**Figure 4.3**). Pairwise Mann-Whitney tests are all significant. Such biased post-duplication gene loss (fractionation) observed in the 5 cotton subgenomes is consistent with previous reports of lineage-specific paleo-polyploidies in *Brassica rapa* (X. Wang, Wang, *et al.*, 2011) and *Zea mays* (J. C. Schnable *et al.*, 2011). The cotton C event (~60 MYA, see below) is also much more ancient than the *Brassica* (17~13 MYA) and maize (12~5 MYA) events. The higher paleo-

polyploidy level and longer evolutionary history make the C-subgenomes a valuable addition to research in biased evolution of paleo-subgenomes.

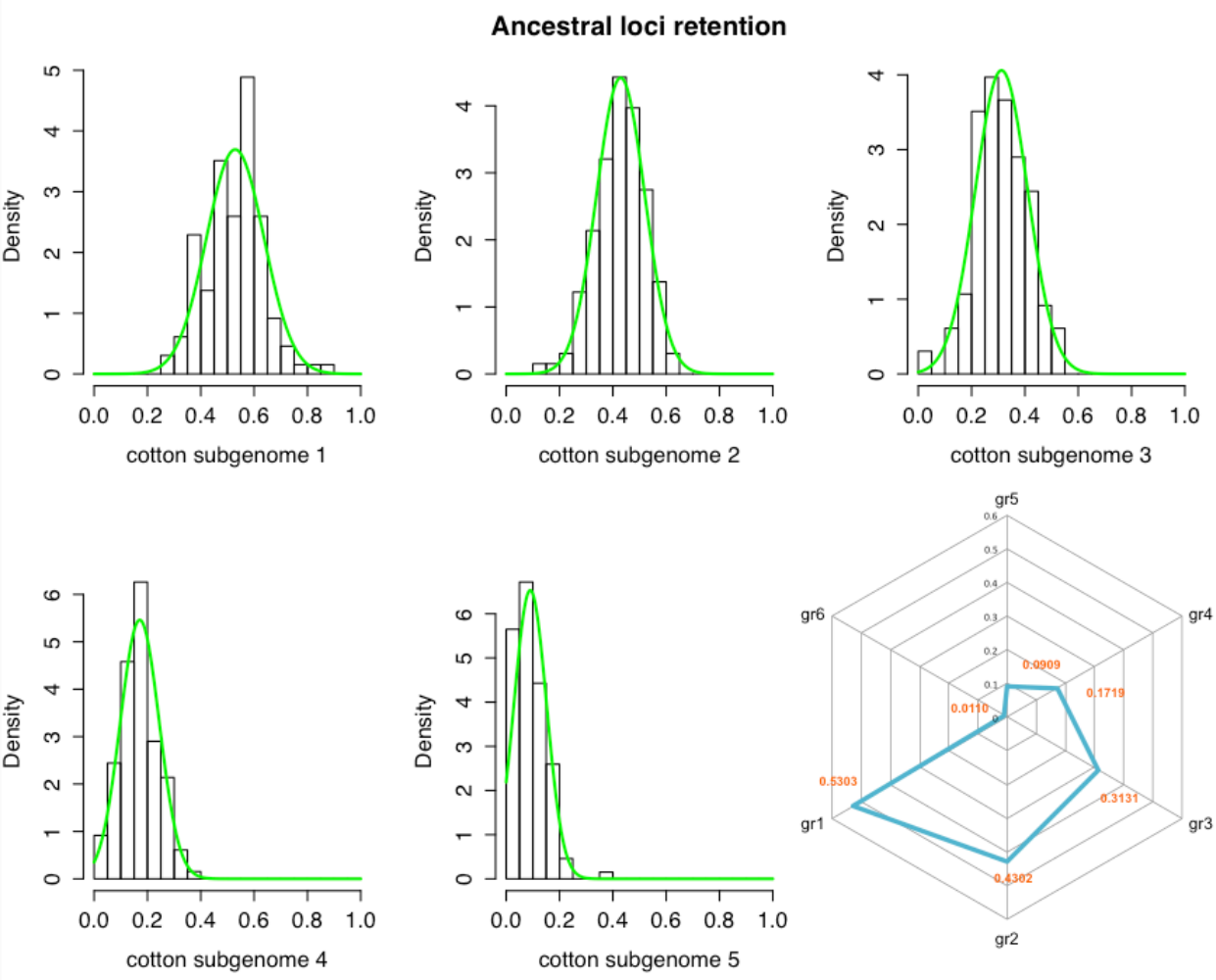


Figure 4.3 Histograms of ancestral loci retention rates in the 5 C-subgenomes. Ancestral gene order was reconstructed using cacao as reference, and divided into 200-gene sliding windows. On the lower right is a spider plot showing mode values of the distributions.

4.4.3 Differential subgenomic sequence divergence following the *Gossypium* paleo-polyploidy

In addition to differentiated structural evolution, SGI and SGII are also different in their sequence divergence patterns. As shown above, median block Ks values are 0.416 in SGI and 0.448 in SGII, respectively, which are significantly different between the two groups (Mann-Whitney test p-value

2.88*10e-6). Genes in the two subgenome groups also exhibit different distributions in exon number and third codon GC content (GC3), but not gene or protein length (**Figure 4.4**). SGI genes on average have fewer exons than SGII genes, with median values 3 and 4, and Mann-Whitney test p-value 0.018. SGI genes on average also have slightly lower GC3 content than SGII genes, with mean values 41.5% and 42.0% respectively, and Mann-Whitney test p-value 1.6*10e-4. Such patterns of differentiated exon number and GC3 content may indicate possible regulatory divergence, such as in expression levels (T. Tatarinova, Elhaik, & Pellegrini, 2013), between SGI and SGII genes. On the other hand, gene length and protein length show no significant difference between the two groups of subgenomes.

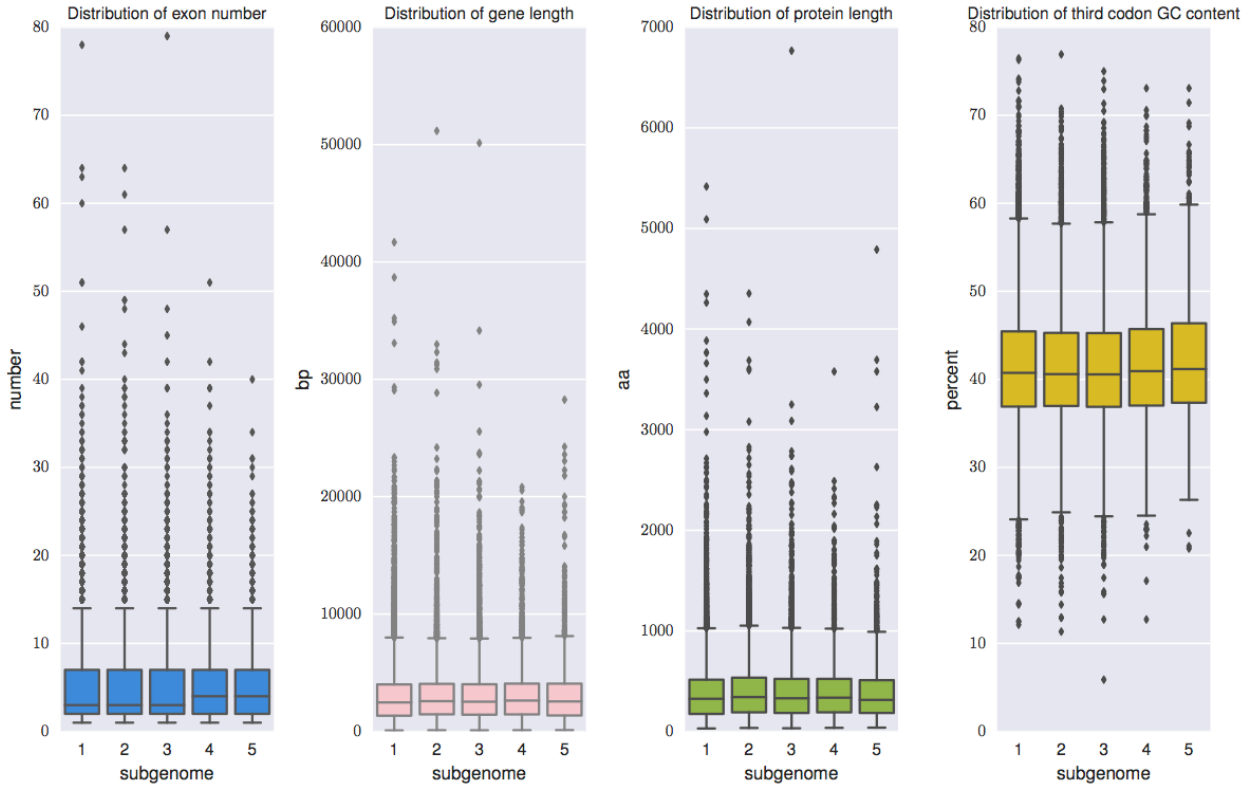


Figure 4.4 Box and whisker plots showing distributions of exon number, gene length, protein length, and third codon GC content among genes in the 5 cotton subgenomes. Boxes display the data between the first and third quartiles. The horizontal line inside the box represents the median. The two whiskers represent 1.5 IQR extensions from the first and third quartiles, respectively. Beyond the whiskers, data are considered outliers and are plotted as individual dots.

An attractive hypothesis to explain these differences is that the two groups of subgenomes were joined in the ancestral *Gossypium* genome at slightly different times, and/or having evolved under different constraints. These results provide directions to further dissect the pan-*Gossypium* (do)decaploidy event C.

4.4.4 A highly conserved NUMT-containing synteny block between cotton (in SGI) and cacao, and a less conserved one (in cotton SGII)

Plant organellar genomes have been actively rearranged, exchanging DNA with each other and transferring DNA into the nucleus (Richardson & Palmer, 2007). Insertion of organellar DNA into the nuclear genome is an important factor in reshaping plant genomes (Bock & Timmis, 2008; Hazkani-Covo, Zeller, & Martin, 2010; Richly & Leister, 2004). While most previous studies focused on single gene transfers across different taxa, when analyzing the *G. raimondii* nuclear (NU) genome we noticed two synteny blocks, on chromosomes 1 and 13 respectively, that contains two arrays of genes with homologs in plant mitochondrial (MT) genomes. The Chr1 block consists of 82 genes spanning 636,155 bp of sequence, and is called NUMT1 below. The Chr13 block consists of 8 genes spanning 105,934 bp of sequence, and is called NUMT13 below.

LASTZ comparisons between the *G. raimondii* nuclear genome and 13 selected plant mitochondrial genomes (*G. hirsutum*, *Arabidopsis thaliana*, *Brassica rapa*, watermelon, papaya, soybean, grape, rice, sorghum, maize, date palm, moss and green algae) identified 757 matching gene pairs involving a total of 188 *G. raimondii* nuclear genes. Among these genes 148 are dispersed throughout the nuclear genome, while 40 genes are located in the two NUMT blocks. Of 82 NUMT1 genes, 20 have homologs in the *G. hirsutum* mitochondrion, while 34 have homologs in at least one of the selected mitochondrial genomes. Of 8 NUMT13 genes, 3 have homologs in the *G. hirsutum* mitochondrion, while 6 have homologs in one of the selected mitochondrial genomes. The remaining genes are aligned to non-coding proportions of the *G. hirsutum* mitochondrial genome. Three genes in the *G. hirsutum* mitochondrion have homologous genes on both NUMT1 and NUMT13.

NUMT1 locates on subgenome 1 of SGI, while NUMT13 locates on subgenome 4 of SGII. NUMT1 has 92.6% sequence matched with 91.4% of *G. hirsutum* mitochondrial genome with 99.6% average identity. NUMT13 has 56.8% sequence matched with 9.4% of *G. hirsutum* mitochondrial genome with 97.5% average identity. These numbers are similar to the ~270 kb *A. thaliana* NUMT region, ~99% of which has 99.0% average identity with ~75% of its mitochondrial genome, supporting a recent insertion event (Lin *et al.*, 1999). Average Ks between *G. hirsutum* mitochondrial and NUMT regions (**Table 4.1**) are similar to those between *A. thaliana* mitochondrial and NUMT regions (0.047). Therefore insertion of *G. raimondii* NUMT1 and NUMT13 likely occurred no more than a few million years ago, similar to the *A. thaliana* NUMT. Insertion of NUMT13 was likely earlier than NUMT1.

The synonymous substitution rate between best matching NUMT regions in cotton and cacao is substantially less than between average orthologous genomic regions (**Table 4.1**), reflecting slower evolution rates in the mitochondrial genomes. The same is true between cotton and *Arabidopsis*. Cotton NUMTs show better gene order conservation with cacao NUMTs than with the *G. hirsutum* mitochondrial genome, with the proportion of anchor genes being 50.0% between NUMT1 and cacao Chr 5 NUMT versus 25.3% between NUMT1 and *G. hirsutum* MT. This likely reflects more structural constraints in the nuclear genomes.

Table 4.1 Mean synonymous substitution rate (Ks) values between syntenic gene pairs. The genes involved are located on *G. raimondii* NUMTs, *G. raimondii*, *Theobroma cacao*, *Arabidopsis thaliana* nuclear genomes, and *A. thaliana, G. hirsutum* mitochondrial (MT) genomes. For comparisons between nuclear genome regions (such as *G. raimondii* genomic – *T. cacao* genomic) orthologous gene pairs were used.

	G. hirsutum MT	*A. thaliana* MT	*T. cacao* genomic	*A. thaliana* genomic
G. raimondii NUMT1	0.037	0.15	0.055	0.18
G. raimondii NUMT13	0.014	0.13	.	0.15
G. raimondii genomic	-	-	0.39	1.70
G. hirsutum MT	-	0.086	0.045	0.18

.: no matches.
-: not applicable.

4.5 Discussion

4.5.1 Inference on the cotton evolutionary rate and timing of the paleo-(do)decaploidy

The pan-*Gossypium* C event was estimated to be ~47 MY old based on descendant paralogs in cotton having a *Ks* distribution of mode ~0.56 (**Figure 4.5**) and average plant evolutionary rate of 6e-9 substitutions per synonymous site per year (Kenneth H. Wolfe *et al.*, 1989). This is likely an underestimated age as *Gossypium* lineage rates in the past few million years were higher than 6e-9 (Senchina *et al.*, 2003). Fossil records indicated that the Byttnerioideae lineage (containing cacao) diverged from the Malvoideae (containing cotton) ~60 MYA (Carvalho, Herrera, Jaramillo, Wing, & Callejas, 2011). Taking into consideration the uncertainties in molecular evolutionary rate estimation, such as rate variations and availability of paleontological records, the cotton C event likely occurred sometime in the late Cretaceous or early Paleogene, shortly following Byttnerioideae - Malvoideae divergence. This event is almost certainly shared by all Gossypieae lineages, which originated about 40~20 MYA (Jonathan F Wendel & Cronn, 2003). More sequence data in the future will help circumscribe the exact phylogenetic position of this event.

The Ks distributions of cotton-*Arabidopsis* and cacao-*Arabidopsis* orthologs (**Figure 4.5**) revealed that average nuclear gene synonymous substitution rate in cotton is slightly higher than in cacao. This is in accordance with previous study showing that *Gossypium* lineage has one of the fastest plastid molecular evolution rates in the Malvaceae (Baum *et al.*, 2004). Studies indicated that paleo-polyploidy events may accelerate evolutionary rates, such as rates of nucleotide substitutions, genome structural rearrangement at macro- and micro- scales, and gene family size alteration (Adams & Wendel, 2005b; Lynch & Conery, 2000; Otto & Whitton, 2000). Empirical observation shows that many lineages with slow evolutionary rates, such as grape, papaya, lotus, and poplar, have experienced relatively few WGDs. Further, many lineages that have been identified with fast evolutionary rate, such as *Arabidopsis*, bladderwort, grasses, and cotton, have experienced several WGDs. Paleo-polyploidy events have also been associated with species expansion episodes (Otto & Whitton, 2000; D. E. Soltis *et al.*, 2009; Van de Peer *et al.*, 2009), and adaptation to changing environmental conditions (Fawcett *et al.*, 2009; Levin,

1983), which in turn affect lineage evolutionary rates. One study directly showed that tetraploid *Saccharomyces cerevisiae* tends to have more chemical treatment-induced mutations than diploid strains (Mayer *et al.*, 1992). Therefore, while the actual lineage evolutionary rate is determined by multiple interacting biological and environmental factors, the pan-*Gossypium* paleo-(do)decaploidy is likely to have played an important role in the high levels of diversification observed in cotton lineages.

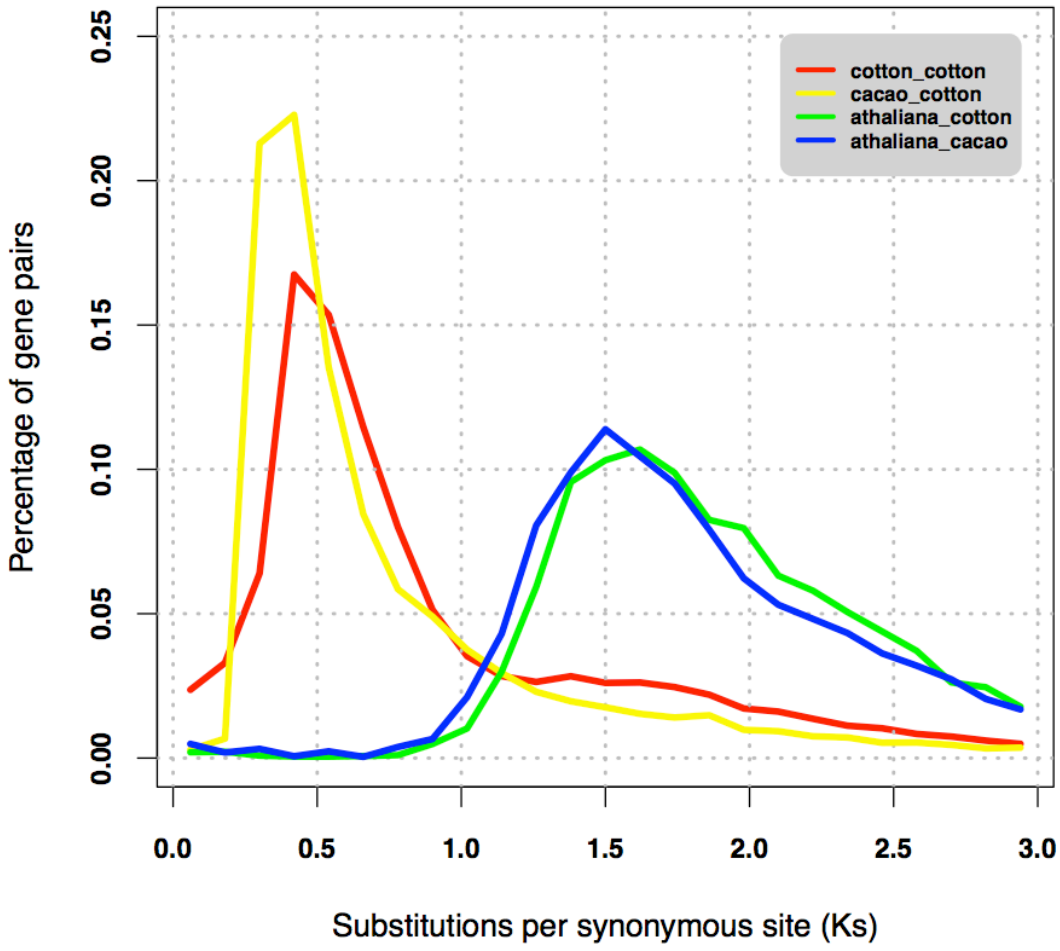


Figure 4.5 Distributions of synonymous substitution rates (Ks) among groups of cotton homeologous gene pairs and orthologous gene pairs between cotton-*Arabidopsis*, cotton-cacao, cacao-*Arabidopsis*. The average rate is higher in cotton than cacao. The cotton C event has Ks mode at 0.56, corresponding to a time close to but postdating cotton-cacao divergence (see text).

4.5.2 Reconstruction of the pre-C ancestral cotton genome

By interleaving the gene orders in the 5 cotton subgenomes we reconstructed an approximation of the ancestral cotton genome predating the C paleo-(do)decaploidy (methods, **Supplementary File 1**). This reconstruction facilitates structural analysis of γ in cotton, the signals of which were substantially obscured by the superimposed C event, a common problem with nested paleo-polyploidies. As expected, the pan-core eudicot paleo-hexaploidy γ is more evident in the ancestral cotton genome with much post-C gene loss mitigated by the reconstruction.

Interestingly we found that genes preferentially retained from γ were also more frequently retained after C, and genes returned to single copy after γ were more likely to return to single copy after C. Comparing ancestral cotton loci retained in 2 copies (2,124) and the full 3 copies (543) from γ, their average retained loci following C is 1.67 and 1.77 respectively, giving Mann-Whitney test p-value (two-sided) significant at 0.02355. The proportions of retained C homeolog number being 3 or more are 18.4% and 14.8%, with p-value 0.04341 (two-sided test of equal proportions). Comparing ancestral cotton loci retained in 2 or 3 copies (2,667) and 1 copy (11,105) from γ, their average retained loci following C is 1.69 and 1.51 respectively, giving Mann-Whitney test p-value (two-sided) significant at < 2.2e-16. The proportions of retained C homeolog numbers being 1 are 50.0% and 61.2%, p-value < 2.2e-16 (two-sided test of equal proportions).

4.5.3 Patterns of paleo-*Gossypium* genome evolution

The Malvaceae family, which contains cotton (*Gossypium*) and cacao (*Theobroma*), is a big eudicot family with complicated phylogenetic relationships. Several biogeographic events happened in the time frame of the paleo-(do)decaploidy C. In the late Cretaceous the first split within Malvaceae occurred between Byttnerioideae (containing *Theobroma*), Grewioideae, and the core Malvaceae lineages, which mainly include the Malvatheca lineages (including Malvaceae *sensu stricto* and Bombacaceae) and the remaining lineages (Brownlowioideae, Dombeyoideae, Helicteroideae, Sterculioideae, and Tilioideae) (Nyffeler *et al.*, 2005; The Angiosperm Phylogeny Group, 2009). There are two models for the

subsequent divergence of Malvoideae (containing the ancestor of *Gossypium*) and Bombacoideae. The Gondwana vicariance model (Pfeil, Brubaker, Craven, & Crisp, 2002) proposed a widely distributed Malvoid ancestor which diverged into two lineages following the Gondwana breakup. The dispersal model (Baum *et al.*, 2004) proposed that an ancestral lineage in South America migrated across the Pacific to Australasia and/or Southeast Asia, and then radiated into Malveae, Gossypieae, and Hibisceae. More complete fossil records are needed to distinguish between the two models, while the time of the divergence is likely to be before the mid-Cenozoic (Carvalho *et al.*, 2011). The eumalvoideae clade (including the Hibisceae, Malveae, and Gossypieae tribes) then experienced a boost in molecular evolutionary rate, making them evolve up to nine-fold faster than other Malvaceae lineages (Baum *et al.*, 2004). These changes might be associated with or have interacted with the paleo-polyploidy C event.

In cotton-cacao comparison, genes in the two groups of C subgenomes, SGI and SGII, differ in average Ks by 0.032, corresponding to about 2.7 million years. SGI and SGII also differ in structural and sequence divergence patterns. Compared to SGI, SGII subgenomes in average contain shorter synteny blocks with higher fractionation levels, and tend to host genes with more exons and slightly higher GC3 content. Although not proven, it has been suggested that paleo-allopolyploidy may be more likely than paleo-autopolyploidy to cause subgenome dominance and biased fractionation (Garsmeur *et al.*, 2014). Our observations suggested that C was possibly a multi-step event with at least one allo-polyploidy component. In addition, ancestral reconstruction revealed that many ancestral loci that had expanded after γ further expanded after C. These and other effects of C in the late Cretaceous or early Paleogene may have prepared the paleo-cotton lineages for later radiation and dispersal, such as following a proposed lag-time model(Schranz *et al.*, 2012).

If the C paleo-(do)decaploidy occurred in more than one step, as is likely the case according to our analysis, the evolutionary transit genomes of intermediate ploidy nonetheless seem to have gone extinct, because to our knowledge there is no *Gossypium* species of other paleo-polyploidy level. Similarly, the pan-core eudicot hexaploidy γ has been suggested to have occurred in two steps (Lyons *et*

al., 2008), but no extant 'intermediate' paleo-tetraploid genomes have been found. Whether this indicates evolutionary advantages in the highest paleo-polyploidy level lineages awaits further studies.

While there are studies on individually transferred NUMT genes across wide plant taxa (such as (Adams, Qiu, Stoutemyer, & Palmer, 2002; Leister, 2005; Liu, Zhuang, Zhang, & Adams, 2009)), to our knowledge only a few cases described multi-gene NUMT regions, including a ~270 kb region (or ~620 kb (Stupar *et al.*, 2001)) on *Arabidopsis thaliana* chromosome 2 consisting of 75% of its mitochondrial genome (Arabidopsis Genome Initiative, 2000; Lin *et al.*, 1999), and five NUMT regions in rice with size ranging from 25~223 kb (Noutsos, Richly, & Leister, 2005). All NUMT regions currently identified seem to have inserted relatively recently in evolution. Flanking regions of the NUMTs in cotton, cacao, and *Arabidopsis* are not aligned to each other, favoring the hypothesis that the NUMTs in these taxa result from independent insertions. Therefore it seems that ancient common insertions, if occurred, have already been lost. On the other hand, comparison among several surveyed plant nuclear and mitochondrial genomes revealed low copy NUMT regions across species from all major clades (data not shown), indicating that this is a widespread phenomenon in plants. Structural and sequence comparisons suggested multiple insertions of the NUMTs at different times.

Many orthologous NUMT genes are exceedingly conserved compared to genome-wide averages, likely reflecting the low nucleotide mutation rates among plant mitochondrial genes. Gene order of the NUMTs is more stable than that of their mitochondrial counterparts, reflecting frequent rearrangements in angiosperm mitochondrial genomes resulting in highly variable gene order and genome size (Kubo & Newton, 2008). Gene expression data in cotton (Paterson *et al.*, 2012), *Arabidopsis* (Wada *et al.*, 2012) and maize (**Figure 4.6**) all supported coexpression of at least some of the NUMT region genes across different tissues. In addition, our previous results showed that of 105 genes upregulated in 10 DPA fibre of wild tetraploid *G. barbadense*, 30 (37%; $P < 0.001$) are in the NUMT1 region, all of which are within the QTL hotspot D_t01 that affects fiber fineness, length, and uniformity (Paterson *et al.*, 2012). Based on those results we propose that the low copy conserved NUMT regions were transferred from mitochondrial to nuclear genomes multiple times during plant evolution, and possibly contain co-regulated genes.

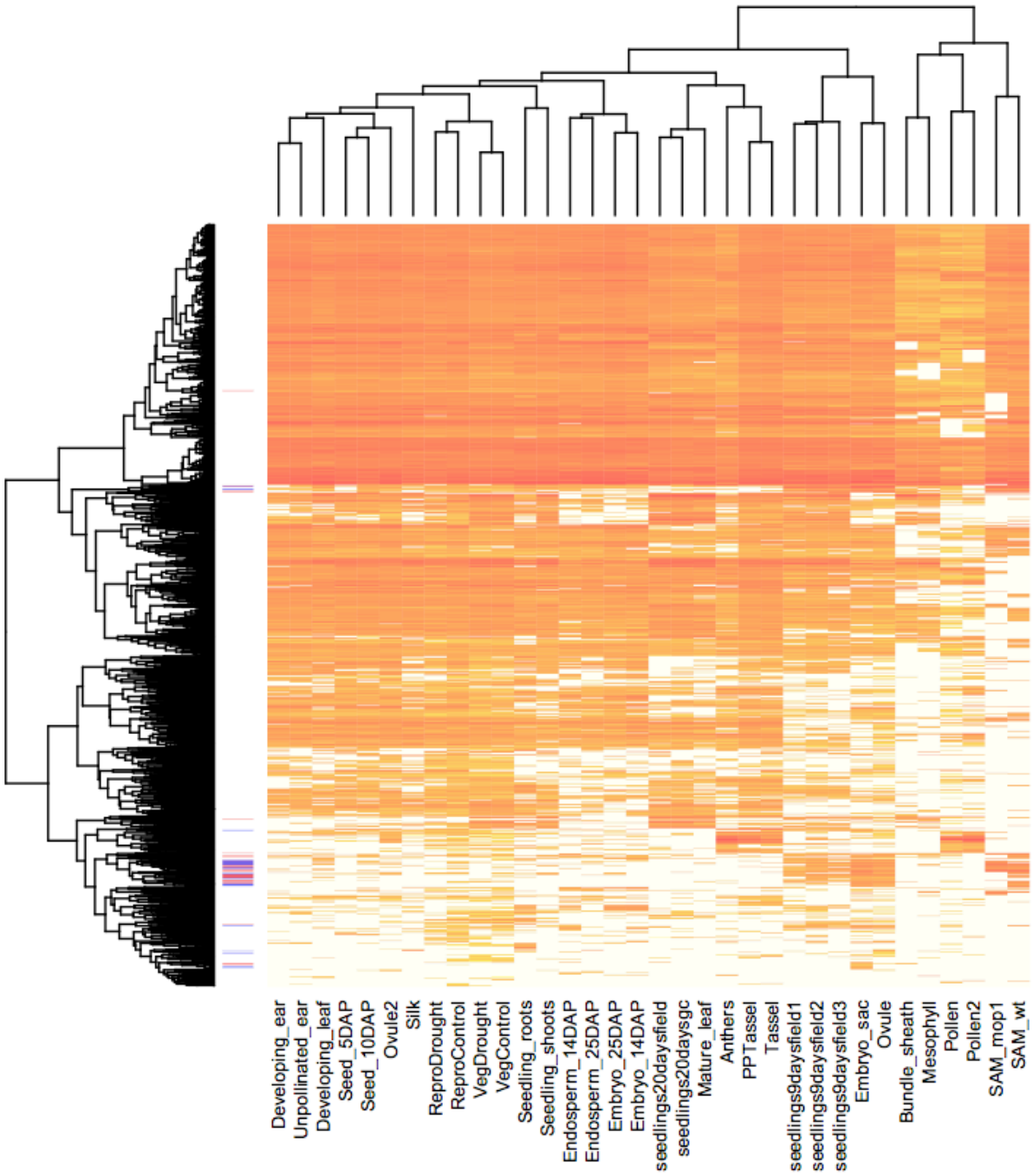


Figure 4.6 Cross tissue heatmap of gene expression levels in maize NUMT and control regions. On the y-axis, genes in two maize NUMT regions are colored in red and blue respectively. Also included in the comparisons are 100 genes flanking each NUMT region, and first 100 genes on each maize chromosome. On the x-axis are names of the tissue types. Maize expression data were downloaded from qTeller (http://qteller.com/).

The effects of polyploidy on the genome and organism are multi-layered, including, in many instances, increased cell size, increased rate of early development, increased illegitimate recombination, increased genetic and cellular instability, increased expression and regulatory complexity, transposable element expansion, nearly doubled effective population size after autopolyploidy, and morphological changes (Adams & Wendel, 2005b; Freeling, 2009; Otto, 2007; Paterson *et al.*, 2010; Schranz *et al.*, 2012; D. E. Soltis *et al.*, 2009; Van de Peer *et al.*, 2009). Those effects may be vital for lineage survival under certain stressful conditions or environmental changes (Arrigo & Barker, 2012; Vanneste, Baele, Maere, & Van de Peer, 2014). Detailed analysis of paleo-polyploidies is valuable and essential for understanding plant genomes. In particular, paleo-polyploidies are a major factor in the evolution of plant genome structure. There is increasing evidence for subgenomic biases in post-polyploidy (especially allo-polyploidy) evolution (Sankoff & Zheng, 2012; J. C. Schnable *et al.*, 2011; Thomas *et al.*, 2006; X. Wang, Wang, *et al.*, 2011; Yoo, Szadkowski, & Wendel, 2013), which are supported by our results. In addition, operon-like structures and co-regulated gene clusters have been identified in plants (Field *et al.*, 2011; Field & Osbourn, 2008) and animals (Boutanaev, Kalmykova, Shevelyov, & Nurminsky, 2002). Our results also suggested that multiple incidences of multi-gene NUMT regions may indicate possible advantages of such clustered transfer, while the NUMT regions seem affected by genomic context after their integration into the nuclear genomes. More studies are needed to understand evolutionary and functional compartmentation in paleo-polyploid plant genomes.

4.6 Conclusion

Based on structural patterns, we divided the cotton *G. raimondii* genome into 5~6 subgenomes corresponding to the pan-*Gossypium* C (do)decaploidy event. A 6th hypothetical subgenome, of tiny size compared to the other 5, is considered uncertain at this point. The 5 major subgenomes can be classified into two groups SGI and SGII, which are differentiated in several structural and sequence features. Compared to SGII subgenomes, SGI subgenomes have longer synteny blocks, smaller Ks values, higher ancestral locus retention, fewer exons per gene, and slightly lower GC3 content. Average gene and

protein length are similar between the two groups. Using a reconstructed ancestral cotton genome we found that duplicated genes retained from the pan-core eudicot γ were also more likely to be retained after the *Gossypium* C event. We also identified two apparent NUMT blocks, on chromosome 1 of SGI and chromosome 13 of SGII. NUMT1 is longer than NUMT13, and is more conserved with NUMT regions in cacao and *Arabidopsis*. Our results provided direction for finer dissection of the *Gossypium* paleo-polyploidy and suggested evolutionary and regulatory compartmentation in the *G. raimondii* genome.

CHAPTER 5 TWO PALEO-TETRAPLOIDIES IN ANCIENT GRASS AND MONOCOT LINEAGES

5.1 Introduction

In the ancestral lineage leading to modern cereals (Poaceae family) it has long been known that a whole genome duplication (WGD) event, named "ρ", occurred an estimated 70 MYA (million years ago) (Paterson, Bowers, & Chapman, 2004). The Poaceae, or grass family, has evolved as separate lineages for about two-thirds of the time since ρ. Nonetheless synteny conservation continues for hundreds to thousands of genes between rice and sorghum, the first two sequenced grasses. The two genomes also shared 97-98% of post-ρ gene loss in orthologous regions (Paterson *et al.*, 2009). The relatively extensive synteny conservation is typical of genome comparisons among cereals (Bennetzen *et al.*, 2012; 2010; P. S. Schnable *et al.*, 2009). However, hidden behind the recent ρ-homeologous regions are additional paralogies from more ancient paleo-polyploidy events that have been obscured by subsequent re-duplications and re-diploidizations. Comparing rice with the grape genome (Tang *et al.*, 2010), and recently with the banana and oil palm genomes (Jiao, Li, Tang, & Paterson, 2014) revealed two additional WGDs in early monocot lineages, designated as "σ" and "τ", which are nested within the "ρ" duplication, making rice and other grasses paleo-hexadecaploids (three rounds of ancient genome doubling). Lineage specific paleo-polyploidies have been detected in several sequenced monocot genomes. The maize genome has experienced an additional WGD about 5~12 MYA, after ρ and σ (P. S. Schnable *et al.*, 2009). The banana lineage has undergone three successive rounds of WGD after separation with grasses (D'Hont *et al.*, 2012). The oil palm genome has experienced one additional WGD in the palm lineage (Singh *et al.*, 2013).

In this chapter the two early WGD events in ancient monocot lineages, "τ" and "σ", which occurred before the divergence of Commelinids and Poaceae respectively, were circumscribed using currently available monocot genomes, and studied in detail.

5.2 Overview of synteny conservation in studied genomes

We aligned six sequenced angiosperm genomes (**Table 5.1**) using gene markers. In total 97.66% (38,136) of rice genes, 98.70% (34,046) of sorghum genes, 96.50% (35,272) of banana genes, 86.10% (27,746) of oil palm genes, 95.76% (25,228) of grape genes, and 92.87% (24,782) of sacred lotus genes were in the aligned regions.

Alignments of four monocot genomes (rice, sorghum, banana, oil palm) and two eudicot genomes (grape, sacred lotus) revealed clear patterns of the "σ" and "τ" events (the two early monocot WGDs). **Table 5.2** summarizes multiplicity ratios between pairs of the studied genomes. The lotus and oil palm genomes showed a 2-to-4 correspondence, indicative of the previously identified "λ" duplication in the lotus lineage (Ming *et al.*, 2013), and two paleo-tetraploidy events in the lineages leading to oil palm (the more recent one being palm-specific (Singh *et al.*, 2013)). On the other hand, 2-to-4 correspondence between oil palm and rice genomes suggest one WGD in oil palm and two WGDs (the recent one being "ρ") in rice after their separation. Collectively, these findings indicated one additional WGD ("σ") in the lineages leading to rice, and one ("τ") in the common ancestor of Poaceae and Arecaceae. These events conferred a total of 8x paleo-multiplicity in the rice genome, confirmed by lotus-rice and grape-rice comparisons. Using similar cross-species synteny comparisons we can also confirm the three WGDs identified in the banana lineage (D'Hont *et al.*, 2012), and the pan-core eudicot γ event that occurred after their divergence with the sacred lotus (a basal eudicot) lineage (Jaillon *et al.*, 2007; Ming *et al.*, 2013; Tang, Bowers, *et al.*, 2008; Tang, Wang, *et al.*, 2008).

With successive WGDs followed by loss of most duplicated genes, ancestral gene orders become progressively more fragmented and discernible synteny blocks become smaller (**Table 5.2**). This is a consequence of usually extensive gene loss and genome rearrangement in the post-WGD diploidization process. The number of anchor genes also shows a tendency to decrease with increased species divergence (**Table 5.2**), indicating that reciprocal gene loss is a continuous process on a large evolutionary scale. Nonetheless, differential retention of ancestral loci in early Poales branches has slowed down in the cereal genomes after the most recent WGD, ρ. About 4.68% of banana genes and

6.85% of oil palm genes have no homeologs in the studied monocot genomes, but are syntenic to some grape and lotus (both eudicots) genes. This percentage is substantially smaller in rice and sorghum (3.00% and 3.51% respectively). Because rice and sorghum are from the same taxonomic family we excluded their mutual matches in this calculation. Therefore this indicates that different proportions of angiosperm ancestral genes may have contributed to divergence of different monocot groups. The majority (~ 80%) of ancestral loci that had been differentially retained in the lineage leading to cereals (since their divergence from other Commelinids 120~83 MYA (The Angiosperm Phylogeny Group, 2009)) are still syntenic in present-day rice and sorghum genomes. The remaining ~ 20% have been differentially lost or transposed since the divergence of the two species 80~50 MYA (The Angiosperm Phylogeny Group, 2009). Therefore positional evolution of ancestral loci seems to be slower more recently.

Table 5.1 Sources and basic information of angiosperm genomes used in this study.

Species	**Common name**	**Family**	**Order**	**1x**	**Genome size (Mb)**	**Protein coding genes**	**Scaffold N50 (Kb)**	**Release**
Oryza sativa	rice	Poaceae	Poales	12	420	39,045	29,958	MSU7
Sorghum bicolor	sorghum	Poaceae	Poales	10	818	33,032	62,400	JGI_2.1
Elaeis guineensis	oil palm	Arecaceae	Arecales	16	1,800	34,802	1,270	Singh *et al.*, 2013
Musa acuminata	banana	Musaceae	Zingiberales	11	523	36,542	1,311.1	D'Hont *et al.*, 2012
Vitis vinifera	grape	Vitaceae	Vitales	19	475	26,346	2,065	Genoscope 12x
Nelumbo nucifera	sacred lotus	Nelumbonaceae	Proteales	8	929	26,685	3,435	Ming *et al.*, 2013

Table 5.2 Summary of synteny blocks in the six studied genomes. Multiplicity ratios between pairs of genomes resulting from independent WGDs are in lower triangle. Numbers of anchors (number of synteny blocks) are in upper triangle.

	rice	sorghum	banana	oil palm	grape	sacred lotus
rice	-	18377 (57)	18871 (1779)	15879 (826)	10582 (956)	11518 (1015)
sorghum	1 : 1	-	18681 (1755)	15447 (802)	10242 (913)	11001 (958)
banana	8 : 4	8 : 4	-	20481 (1546)	12718 (1363)	12725 (1327)
oil palm	2 : 4	2 : 4	2 : 8	-	16504 (1007)	17798 (1056)
grape	3 : 8	3 : 8	3 : 16	3 : 4	-	18003 (685)
sacred lotus	2 : 8	2 : 8	2 : 16	2 : 4	2 : 3	-

5.3 Circumscribing the pan-grass σ duplication event

Comparison between grape and rice genomes showed that there are more paralogous regions in rice than those produced in the pan-grass ρ, indicating more ancient paleo-polyploidy events before ρ (Tang *et al.*, 2010). Such nested WGDs are often best revealed by 'bottom-up' approaches (Bowers *et al.*, 2003), attempting to reverse the changes resulting from more recent events (in this case ρ) superimposed on them. Using such bottom-up reconstruction a total of 146 p (palm WGD) blocks covering 71.74% of the oil palm genome were merged into pre-p ancestral gene orders. Similarly, 140 ρ (most recent grass WGD) blocks covering 70.63% of the rice genome were merged into pre-ρ gene orders. Direct comparison between the ancestral orders resulted in 209 synteny blocks covering 96.34% of the pre-p order and 72.77% of the pre-ρ order, each pre-p region aligning with up to two paralogous pre-ρ regions, as exemplified in **Figure 5.1B**. This clearly revealed that the σ event occurred before the pan-Poaceae ρ event (~70 MYA; (Paterson, Bowers, & Chapman, 2004)), but after the Poaceae-Arecaceae split (120~83 MYA; (The Angiosperm Phylogeny Group, 2009)).

As the second largest monocot family and fifth largest angiosperm family, the Poaceae is rich in morphological and ecological diversity. Much of the underlying genetic diversity may have resulted from σ and ρ WGDs. The events may also have contributed to some grass-specific characters. Genomes from basal Poales species such as pineapple could be useful to further narrow the dating of ρ and σ.

5.4 Circumscribing the pre-commelinid τ duplication event in early monocots

It has been discovered that a paleo-tetraploidy ("p") predated modern oil palm (Arecaceae family) (Singh *et al.*, 2013), and that the sacred lotus (Nelumbonaceae family) lineage had a paleo-tetraploidy ("λ") after it diverged from other eudicots (Ming *et al.*, 2013). We merged 146 "p" synteny blocks covering 71.74% of oil palm genome into pre-p gene order. Similarly 178 "λ" blocks covering 74.05% of lotus genome were merged into pre-λ gene order. Alignment of the reconstructed genomes resulted in 203 ancestral synteny blocks covering 69.44% of pre-λ order and 73.53% of pre-p order. Comparison between the approximated ancestral pre-p and pre-λ orders reveals synteny structures consisting of one pre-λ region and one to two orthologous pre-p regions. This indicated that an ancestor of oil palm experienced another more ancient paleo-tetraploidy event before "p", but after it diverged with the ancestral eudicots. Syntenic regions with more than two homeologous copies retained are distributed over all 16 oil palm chromosomes, indicating that this event was genome-wide. Since we have shown that oil palm did not share "σ", this additional paleo-tetraploidy is inferred to be the "τ" WGD event (**Figure 5.1**).

Traces of the "τ" paleo-tetraploidy were first discovered in the rice genome (Tang *et al.*, 2010). By comparing the rice genome a relatively clean outgroup genome of grape, which only had "γ" in its own lineage, up to eight orthologous regions can be identified in rice, indicating three WGDs that have occurred in the monocot lineages leading to rice. However, fast evolutionary rates and three WGDs have severely increased paleo-paralog loss in the rice genome, with about 10,877 paralogous copies of pre-"τ" loci retained (excluding tandem duplications) among 39,049 rice genes. In contrast, oil palm, whose genome was recently published, has evolved at a much slower rate (about one half that of grasses on average), and has had one less WGD in its lineage history. As a result about 16,092 paralogous copies of pre-"τ" loci are retained (excluding tandem duplications) among its 32,225 genes. In both genomes recurring WGDs severely eroded signals of paleo-paralogy produced in "τ", with pre-"τ" loci retained at 1.40 of 8 possible copies on average in rice, and 1.49 of 4 possible copies on average in oil palm.

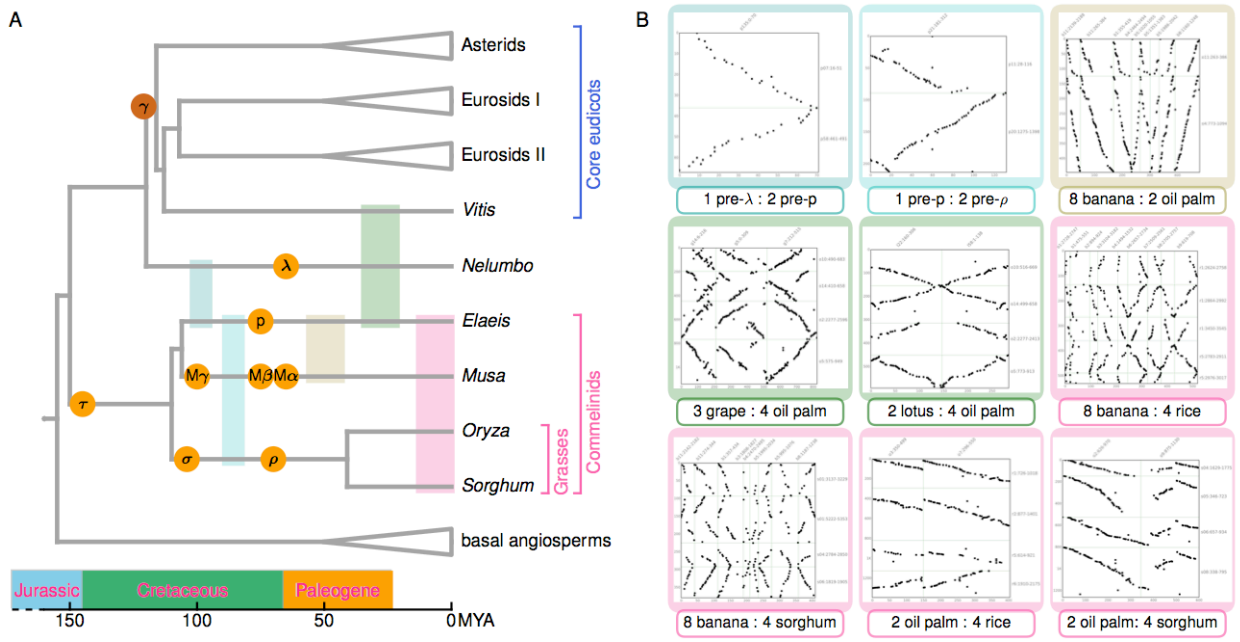


Figure 5.1 Genome comparison on a phylogeny of paleo-polyploidized angiosperms. (A) Paleo-polyploidy events are represented by orange (duplication) or brown (triplication) circles on the angiosperm phylogeny, filled with their name codes. Color shades on tree branches indicate positions of genome comparison and whether they precede or postdate the adjacent polyploidies. (B) Dot plots (dots represent matching genomic loci on two axes) of example regions from each of the designated genome comparisons are shown, outlined by the same color as on the tree. Quota ratios of the alignments are shown below the dot plots (detailed in the text). Paleo-polyploidy events underlying the alignment patterns are detailed in the text. Geologic timing is based on molecular clock-based estimation rather than values from physical evidence (which are usually unknown for ancient events).

5.5 The value of ancestral gene order in genome comparisons

Inferred or approximate ancestral gene order often provides the best reference to thread genomic alignments in taxa with WGDs (Bowers *et al.*, 2003; Kellis *et al.*, 2004; Zheng, Chen, Albert, Lyons, & Sankoff, 2013). While development of new algorithms provides increased sensitivity to detect ancient nested paleopolyploidy events, approximated ancestral gene order provides the best reference to thread inter-genomic alignments in such taxa for several reasons. Firstly, it compensates for gene loss, increasing the alignability and proportion of aligned genes among homeologous regions. Secondly, it makes longer synteny blocks as lineage specific breakpoints are removed. Thirdly, it “reverses” and therefore masks

more recent WGDs. Finally, it helps better reveal the interleaving pattern of gene loss (as illustrated in Figure 1 (Kellis *et al.*, 2004)). Therefore although paleopolyploidies can be unequivocally identified by integrated detection of positional and sequence signals, ancestral gene order is necessary to recover full syntenic mapping among homeologous regions. When a "clean" (having no WGD) outgroup genome is not available, as is the case in many plant clades, ancestral gene order can be approximated through reconstruction. Reconstructed ancestral genomes may not be the same as the true ancestral genome, but likely will have high structural similarity (Tang, Bowers, *et al.*, 2008; Zheng, Albert, Lyons, & Sankoff, 2012), and are an irreplaceable reference in whole genome alignments.

Recurring polyploidization and diploidization, ubiquitous in plant evolution, greatly obscure the network of homology in plant genome comparisons. More than 20 ancestral and independent paleo-polyploidies have been identified in ~50 sequenced angiosperms, collectively affecting 100% of lineages. Consequently many angiosperm genomes contain populations of homeologous regions of different depth resulting from combination of different levels of paleo-polyploidization and post-polyploidy gene loss. Inter-genomic comparisons are complicated as well. *Arabidopsis* and rice, for example, have 829 synteny blocks averaging 43 genes, resulting from 6 WGDs, 3 in each lineage. A comparison between diploid *Brassica rapa* and banana genomes would involve as many as 52 copies involving 8 WGD events (3x2x2x3=36 in Brassica and 2x2x2x2=16 in banana). It is beyond current technology to directly find and align all the homeologous regions in such genomes. Instead, ancestral reconstruction based on an established framework of historical WGDs is able to effectively recover the grand hierarchy of homeology.

5.6 Variation of lineage nucleotide evolutionary rates and estimated ages of σ and τ

Nucleotide evolutionary rates can vary greatly among sites, gene families, nuclear and organellar genomes (Gaut *et al.*, 2011; Mower *et al.*, 2007; K. H. Wolfe *et al.*, 1987; Zhang, Vision, & Gaut, 2002), and lineages with different life history traits and population characters (Gaut *et al.*, 2011; S. A. Smith & Donoghue, 2008). Shared paleo-polyploidy events provide inherent reference points to calibrate

evolutionary rates among affected lineages. Homeologs bearing synteny information are also intrinsically more accurate phylogenetic markers for multi-copy gene families. By applying such reasoning, the *Vitis* lineage nucleotide substitution rate is estimated to be less than half that of *Arabidopsis* (Tang, Wang, *et al.*, 2008), and the *Nelumbo* lineage rate is 30% slower than *Vitis* (Ming *et al.*, 2013). The synonymous site substitution rate between paralogs formed in the shared τ WGD (**Figure 5.2**) is ~1.7 times larger in rice than oil palm. This is much less than the ~5 fold difference between grasses (faster) and palms estimated from chloroplast *rbcL* genes (Gaut *et al.*, 2011), and ~4 fold difference estimated from a combination of rDNA, chloroplast, and mitochondrial genes (S. A. Smith & Donoghue, 2008). These differences in rate estimates emphasize the heterogeneous nature of molecular evolution in plant genomes. Reliable WGD and homeology identification is clearly essential for detailed evolutionary analysis on a genome-wide scale.

The pan-grass ρ event was estimated to be ~70 MY old (Paterson, Bowers, & Chapman, 2004) based on descendant paralogs in rice having a *Ks* distribution of mode ~0.86 and estimated rice lineage rate of 6.5e-9 substitutions per synonymous site per year. Using the same rate estimate the age of σ (paralogous *Ks* mode ~1.65) is approximately 127 MYA. The palm WGD p event was estimated to be ~75 MYA (D'Hont *et al.*, 2012; Singh *et al.*, 2013), with descendant paralogs in oil palm having a *Ks* distribution of mode ~0.36, giving an estimated oil palm lineage rate of ~2.4e-9. Paralogs of τ have *Ks* distributions of mode ~1.13 in oil palm and ~1.87 in rice. Taking the average rate of rice and oil palm lineages (4.45e-9) to be the approximate substitution rate on their MRCA lineage, the age of τ can be estimated to be ~73 MYA before Arecaceae-Poaceae split using *Ks* distribution of oil palm paralogs, or ~64 MYA using rice *Ks* distribution. Taking into consideration the uncertainties in molecular evolutionary rate estimation, and availability of paleontological records, τ likely occurred in primitive monocot branches. Due to a lack of fully sequenced early-diverging monocot genomes and limitation of resolution in current sequence-based dating methods, further refinement of the exact timing of σ and τ awaits future studies.

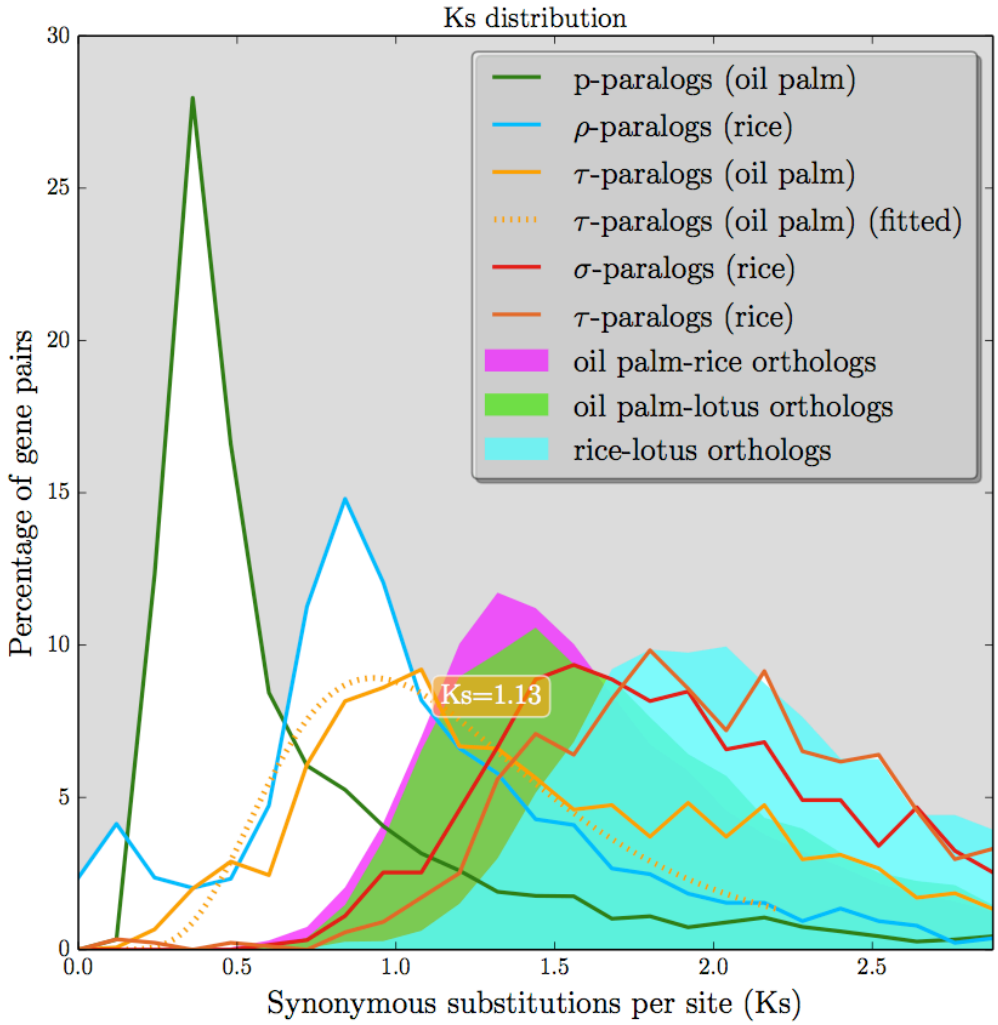


Figure 5.2 Distributions of synonymous substitution rates (Ks) among groups of orthologous or paralogous (homeologous) gene pairs. Curves for paralogous gene pairs are not filled, while curves for orthologous gene pairs are filled with colors. The orange dotted line is a fitted log-normal distribution for the Ks values between oil palm paralogous gene pairs duplicated in the "τ" event, which has a modal value of 1.13. The small peak near Ks=0.1 in rice "ρ" duplicates correspond to the gene conversion regions on rice chromosomes 11-12 terminal regions ("ρ" paralogous regions) (X. Wang, Tang, & Paterson, 2011). The Ks rate in the oil palm lineage is similar to or slightly higher than in the lotus lineage, but much smaller than that of the rice lineage (see text for details). Therefore shared events in the common ancestor of oil palm/lotus and rice are represented by shifted curves in the descendant genomes, although in theory they should overlap if lineage rates were the same. For example, "τ" paralogous gene pairs in oil palm (orange) and in rice (brown) have accumulated different amount of synonymous substitutions in the same amount of time. Similarly, although rice and oil palm diverged with lotus at the same time, oil palm-lotus orthologous gene pairs have smaller Ks values.

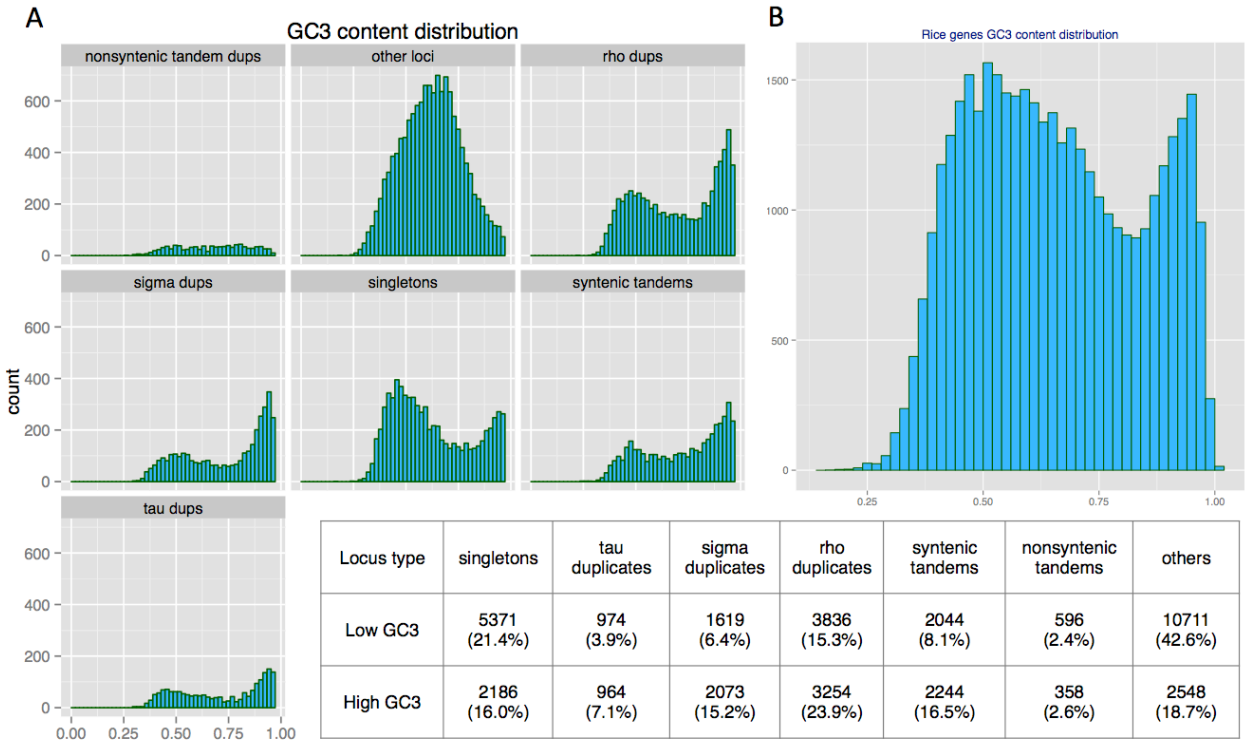
5.7 Origins of high GC3 genes in grasses

The frequency of genes with different G+C percentage at third codon positions (GC3) is not randomly distributed in the genome. The GC3 distributions are also not random among different taxa. Grasses and warm-blooded animals are known to have bimodal GC3 distributions, while cold-blooded animals and most other plants have unimodal GC3 distributions. Previous studies showed that genes with high GC3 content (GC3 % > 0.75~0.8 in grasses) tend to have lower intron density, more methylation targets, more variable expression, over-representation from certain gene families (such as electron transport, stress response, signal transduction and transcription factors), and higher than average regulatory complexity (Carels, Hatey, Jabbari, & Bernardi, 1998; Shi *et al.*, 2006; T. Tatarinova *et al.*, 2013; T. V. Tatarinova, Alexandrov, Bouck, & Feldmann, 2010). We noticed that some of these characters are also exhibited in post-WGD loci with preferentially retained homeologs. It is therefore one possible reason for the observed enrichment of high GC3 genes among rice loci that have remained duplicated after ancient WGDs (Chi-square test p-value is 1.8e-180).

Duplicated homeologous loci harbor about 80% of all high GC3 loci in syntenic regions in rice. On the other hand, we also observed a significant enrichment of high GC3 genes in ancestral loci in rice (loci that have homeologs in at least one of the sorghum, oil palm, banana, lotus and grape genomes) (harboring about 80% of all high GC3 genes) versus the loci that appeared to be specific to the rice lineage (**Figure 5.3**), with a Chi-square test p-value close to 0. This indicates that many of the high GC3 loci are ancient. Since the τ event was shared by grasses, palms, and bananas, while the increased high GC3 gene fraction is specific to grasses, it is most likely that the grass lineage WGDs, σ and ρ, have contributed more to the expansion of high GC3 genes in grasses. However, it has been known and also observed in our study that Zingiberales and Arecales of the Commelinids have genomes with GC3 distributions intermediate between clear unimodality and clear bimodality. Consistent with evident enrichment of high GC3 genes also in rice homeologous loci duplicated in the shared τ event, this indicates that expansion of high GC3 loci likely started as early as the τ event. In addition, the average GC3 difference between rice τ duplicates is 28.2% more than that between σ duplicates (0.157 versus

0.123), which is in turn 46.9% more than that between ρ duplicates (0.084). This indicates that diversifying of paralogous GC3 content has been on-going in the rice genome for a very long time, a trend not observed in the non-grass oil palm and eudicot lotus genomes. Major grass lineages have been evolving independently for about 50 MYA. The parallel maintenance of high GC3 gene frequency in the genomes of this widely distributed diverse group of species appears to argue against a hypothesis that such patterns have been formed solely by chance.

Collectively, our findings suggested that many high GC3 genes are possibly ancient and functionally important, and may have been involved in the diversification of ancestral grass and perhaps Commelinid lineages. Further studies of GC3 gene evolution in grass genomes may increase our knowledge particularly about the effects of the σ and ρ events on shaping the modern grass genomes and diversity, as well as facilitating applications in grass genetic engineering.



Locus type	singletons	tau duplicates	sigma duplicates	rho duplicates	syntenic tandems	nonsyntenic tandems	others
Low GC3	5371 (21.4%)	974 (3.9%)	1619 (6.4%)	3836 (15.3%)	2044 (8.1%)	596 (2.4%)	10711 (42.6%)
High GC3	2186 (16.0%)	964 (7.1%)	2073 (15.2%)	3254 (23.9%)	2244 (16.5%)	358 (2.6%)	2548 (18.7%)

Figure 5.3 Histograms of third codon position GC content (GC3) in rice genes. (A) Frequencies of GC3 in different types of rice genes maintained as duplicates or singletons from the ρ, σ, τ WGDs, tandem

duplicates at syntenic or non-syntenic regions, or other likely recently gained genes in the rice lineage. (B) Overall GC3 distribution in rice genome, showing the grass-specific bimodal shape. Counts and percentage decomposition of high and low GC3 genes are shown in the table. The cutoff between low GC3 and high GC3 rice genes is 0.75 (Carels *et al.*, 1998; Tang *et al.*, 2010). Singletons, τ/σ/ρ duplicates, and syntenic tandems are collectively called the ancestral loci, while non-syntenic tandems and other loci are collectively called the rice lineage specific loci (see text for more details). High GC3 genes were preferentially evolved from ancestral loci, especially duplicated ancestral loci.

CHAPTER 6 GeDupMap: SIMULTANEOUS DETECTION OF MULTIPLE ANCIENT GENOME DUPLICATIONS AND A SYNTE-MOLECULAR FRAMEWORK

6.1 Abstract

Observations of paleo-polyploidy (ancient genome duplication) events in all sequenced flowering plant lineages revealed their multifarious effects on genome and species evolution, from molecular to populational. Rapidly accumulating genomic data make it an opportune time to exploit and dissect these effects, some common and some lineage-specific. Because of abundant paleo-polyploidy derived paralogs (homeologs) in flowering plant genomes, they must be correctly accounted in many studies such as genome structure comparisons, ortholog identification, and gene family analyses. So far, paleo-polyploidy detection and genome comparisons in flowering plants are largely manual and involve only two lineages at a time. In this paper we describe the GeDupMap approach for simultaneous detection of all paleo-polyploidies in multiple lineages. Inputs to GeDupMap are genome annotations and a phylogenetic tree (topology only) of the studied taxa, both readily available for sequenced genomes. The program analyzes synteny maps between the genomes and counts the numbers of homeologous regions after each speciation event, which are integrated to estimate the paleo-polyploidy levels on branches of the species tree. The program also compiles groups of putative orthologous regions (PORGs) after divergence of each pair of genomes, forming a network of syntenic mappings to frame structural and molecular analyses in those paleo-polyploid genomes. Most steps in the pipeline are accompanied by visualization to facilitate inspection of the results. Such automatic paleo-polyploidy detection and a synte-molecular framework of homeologous regions are useful in comparative studies involving several plant genomes. A set of 8 eudicot and monocot genomes were used to illustrate the functions of GeDupMap.

6.2 Introduction

The past century since the first discovery of polyploidy (whole genome duplication) (DeVries, 1915; Lutz, 1907; Muller, 1914; Winge, 1917) marked greatly increased recognition of its frequency, breadth, and importance, especially in the past 30 years with new large-scale genome mapping and sequencing technology. Remarkably, not only are polyploids frequently formed in nature, some of the events have survived several to hundreds of millions of years' evolution, producing paleo-polyploid organisms which were once polyploids but have been returned to diploid transmission genetics, as seen today. Among the eukaryotic lineages that have been identified with paleo-polyploidies, including vertebrates (Dehal & Boore, 2005; McLysaght, Hokamp, & Wolfe, 2002; Ohno, 1970; J. J. Smith *et al.*, 2013), fungi (Kellis *et al.*, 2004; K. H. Wolfe & Shields, 1997) and ciliates (Aury *et al.*, 2006; McGrath, Gout, Doak, Yanagi, & Lynch, 2014), the angiosperms (flowering plants) are most affected. More than 50 paleo-polyploidy events have been described across all studied angiosperm lineages (D. E. Soltis, Visger, & Soltis, 2014). About 15% of angiosperm speciation events are estimated to be directly accompanied by polyploidy (Wood *et al.*, 2009). Moreover, most sequenced angiosperm lineages have experienced repeated paleo-polyploidization. For example, *Arabidopsis thaliana*, the first sequenced model plant genome, had one paleo-hexaploidy (genome triplication) and two paleo-tetraploidies (genome doubling) totaling 12x duplication (Bowers *et al.*, 2003; Tang, Bowers, *et al.*, 2008). Realization of widespread paleo-polyploidization has fundamental impacts on angiosperm genome comparison (Blanc & Wolfe, 2004; Langham *et al.*, 2004; Paterson, Bowers, Chapman, *et al.*, 2004; Douglas E Soltis, Soltis, & Tate, 2004; Van de Peer, 2004).

Paleo-polyploid genomes return to diploid heredity (and typically lower chromosome number) during evolution, in the 'diploidization' process (M. Lynch & A. G. Force, 2000; K. H. Wolfe, 2001) characterized by extensive sequence loss and structure rearrangement. Consequently, despite having experienced varied paleo-polyploidy histories, contemporary angiosperm genomes often have similar gene and chromosome numbers, typically varying less than five-fold. Zooming in, genome-wide contraction of post-duplication genomes has created complex networks of homology and synteny

(conservation of gene order) among multiple restructured homeologous regions. Such networks complicate both inter- and intra- genomic comparisons, especially the later ones due to frequent reciprocal gene loss between paleo-duplicated regions (Freeling, 2009; Scannell, Byrne, Gordon, Wong, & Wolfe, 2006).

GeDupMap (Genome Duplication Map) serves two purposes. One is to estimate all paleo-polyploidy events on a phylogeny of species. The other is to organize putative orthologous regions in those genomes in a systematic manner to form the synte-molecular framework for many comparative genomic analyses. These tasks are currently not available in any other programs, and will facilitate detailed analysis of multiple paleo-polyploid plant genomes.

6.3 Methods

The six steps of GeDupMap are illustrated in **Figure 6.1**. GeDupMap requires no more than the annotations of the input genomes that are available for all sequenced species, and their species phylogenetic tree (topology only) that can be easily obtained from the NCBI Taxonomy database, or from literature. It is highly recommended to use genomes with chromosome or mega-scaffold level assemblies because low contiguity compromises confidence in detecting synteny patterns, especially when comparing multiple paleo-polyploid genomes.

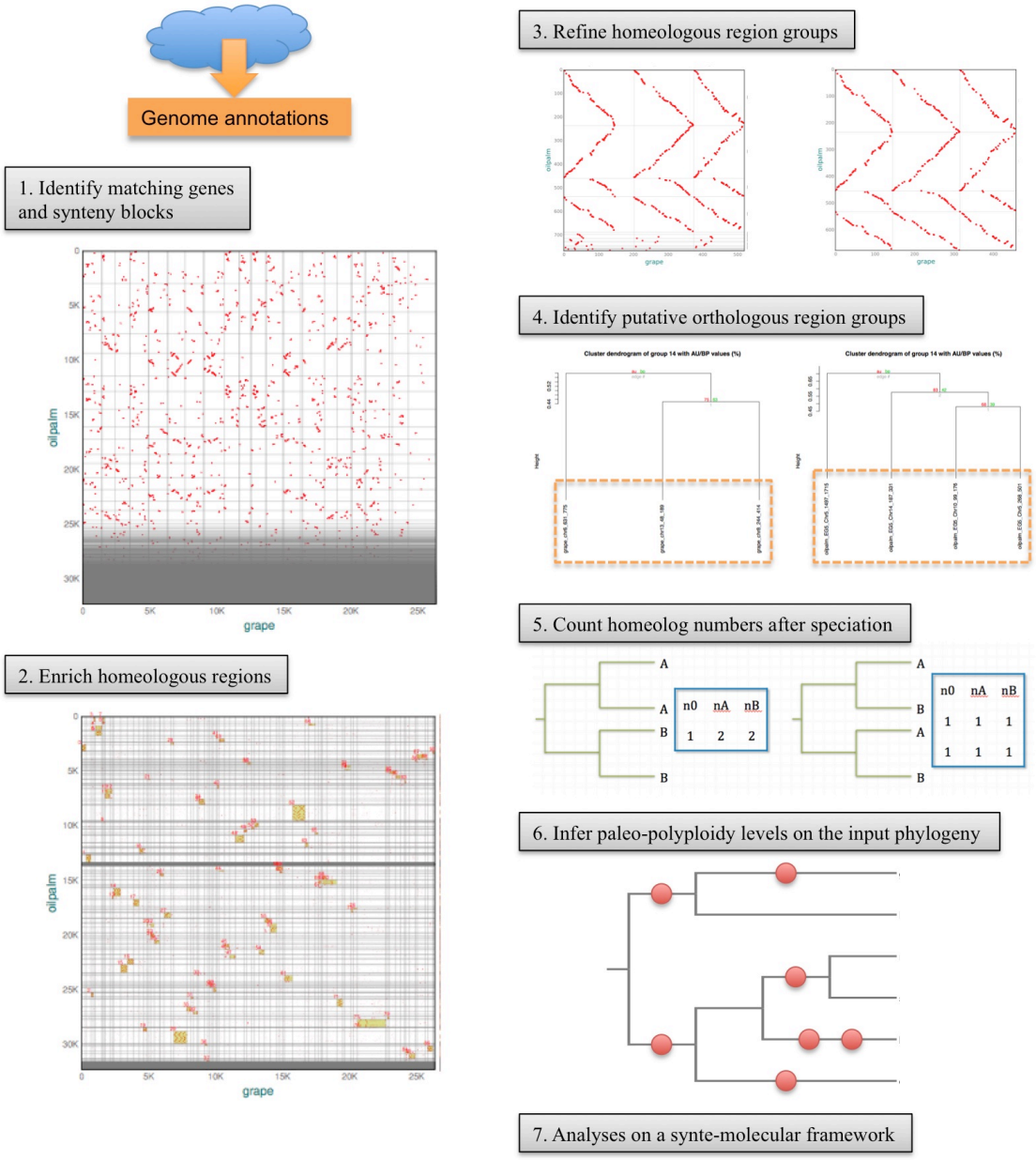


Figure 6.1 Overview of GeDupMap procedure. Step one involves downloading annotated gene location and sequence files (from Phytozome, genome portals, or project websites), and identification of synteny blocks. Step two involves segmentation and clustering of synteny blocks using the PAR algorithm (Tang *et al.*, 2010). Step three involves refinement of PAR clusters by removing spurious regions and unaligned boundary regions, and splitting conflict regions. Step four involves identification of putative orthologous region groups (PORGs) containing regions duplicated after divergence of two species. Step five involves collecting pairwise PORG counts into a distance matrix. Step six involves inference of paleo-polyploidy levels using the Fitch-Margoliash algorithm. Finally the above steps form the synte-molecular framework for downstream analyses. See more details in the text.

6.3.1 Matching gene sets and synteny blocks identification

Annotated gene sequences and location in the studied genomes can be downloaded from Phytozome website or their respective genome project websites. LASTZ (Harris, 2007), LAST (Kielbasa, Wan, Sato, Horton, & Frith, 2011), or BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) can be used to search sequence similarity between genes and identify matching gene pairs. The representative CDS sequence from each gene locus is used. While for divergent homologous gene pairs BLASTP (using protein sequences) is expected to be more sensitive than LASTZ/LAST (using CDS sequences) because of synonymous mutations, in practice we observed that using BLASTP only resulted in a negligible increase in syntenic anchors, while run time is several fold slower, and data pre-processing is more cumbersome and prone to occasional frame-shift errors in annotations. In balancing benefits and cost for angiosperm genome comparisons we decided that LASTZ/LAST search of CDS sequences is the recommended approach. However, BLAST outputs are also fully compatible with the program.

Many studies suggested filtration of weak matches from raw BLAST/LASTZ output by applying additional cutoffs, typically for hits identity, length, coverage, or c-score (Putnam *et al.*, 2007; Tang *et al.*, 2010; Tang, Wang, *et al.*, 2008; Vandepoele *et al.*, 2002). These filters are available in GeDupMap. By default the GeDupMap filters raw LASTZ output by identity >= 50%, coverage >= 30%, c-score >= 0.5, which we found suitable for our studied genomes, and can be a good starting point for analyzing other flowering plant genomes.

Identification of synteny blocks follows previous methods published by our lab (Tang *et al.*, 2010; Tang, Wang, *et al.*, 2008). Tandem gene families, defined as clusters of genes within 10 intervening genes from one another, were filtered out by keeping only one representative member with longest peptide. LASTZ matches within 30~40 Manhattan distance units were clustered into synteny blocks, with those blocks containing 5 or more anchor gene pairs retained for further analysis.

6.3.2 Chromosome segmentation and homologous region enrichment

Chromosome segmentation and PAR (putative ancestral regions) clustering follow (Tang *et al.*, 2010). For each pair of input genomes the chromosomes were cut into segments at boundaries of synteny blocks. After segmentation the genomes are divided into regions less affected by genome rearrangements and are therefore suitable for defining simple synteny patterns in next steps. Each segment in the query genome was compared to subject genome segments, and profiled with the probability of the observed homeolog number modeled with a Poisson distribution. The profile matrix was used as input of the CLUSTER software (V3.0) (de Hoon, Imoto, Nolan, & Miyano, 2004; Eisen, Spellman, Brown, & Botstein, 1998), which conducts two-way hierarchical clustering of the segments in the two genomes using Pearson correlation coefficient (r) as distance and average linkage method. By default clusters were harvested at r=0.3, which should be adjusted to ensure patterns of single cluster (no stacking clusters) on both axes of the dot plot. Harvested clusters are further filtered by requiring that the probability of getting the total observed number of homeologs by chance is less than 1e-30. As a result the "dense" (syntenic) portions of the inter-genomic dot plot are concentrated into the PARs.

The collected PARs were then refined for three criteria: 1. Remove unaligned margins; 2. Split conflicting regions; 3. Merge adjacent regions (**Figure 6.2**). Conflicting regions are those in a PAR cluster but not syntenic to each other. Such PAR clusters can therefore be further split into several groups that have no conflicting regions. Removal of unaligned region margins and merge of adjacent regions ensure precise region boundary definition for further analysis.

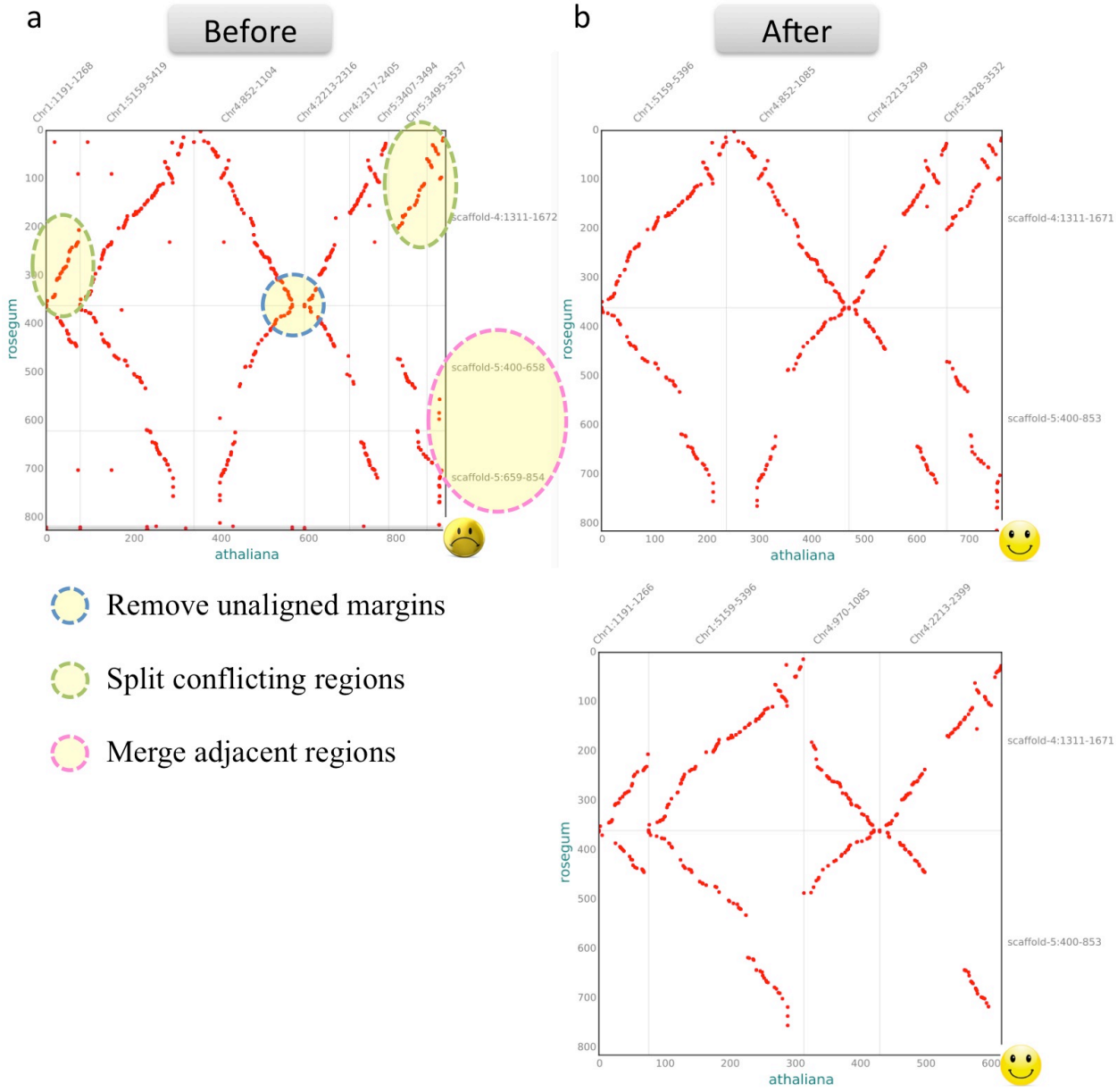


Figure 6.2 Refinement of PAR groups. (a) Purposes of the refinement are to remove unaligned margins, split conflicting regions, and merge adjacent regions. (b) After refinement the two groups of regions both correctly reflect the 4: 2 ortholog ratio between *Arabidopsis* and rose gum.

6.3.3 Putative orthologous region groups (PORGs) identification

Paleo-polyploidy produces variation in ortholog ratios. When a paleo-polyploidy was shared by two lineages, there is 1: 1 ortholog ratio. In cases of lineage-specific (post-speciation) paleo-polyploidies, there are 1: multiple or multiple: multiple ortholog ratios. Those ratios can be used to estimate differences of paleo-polyploidy levels between the two genomes.

We define a 'synteny distance' between two homeologous segments as -ln(nhits / alnlen), where nhits is the number of hits (anchored gene pairs) between the two aligned segments, and alnlen is the length of alignment. The synteny distance is derived as an analog to the Jukes-Cantor distance for nucleotide alignment. This is because loss of one of the anchored genes is sufficient to cause loss of the matching in the gene order alignment, a situation analogous to multiple substitutions in nucleotide alignment. For each PAR group the regions in the query and subject genomes were aligned pairwise. Their synteny distances were used as input to the PVCLUST program (Suzuki & Shimodaira, 2006) which identifies statistically significant clusters in hierarchical clustering analysis. For each PORG, paralogous regions in each genome are clustered by PVCLUST, from which the largest cluster that cannot be further divided (containing two leaves or two insignificant nodes) is identified as the first duplication node since speciation (FDN).

While we identify PORGs based on synteny patterns, GeDupMap also provides three additional types of phylogenetic reconstruction based on homeologous gene families located in each PAR group, including those that use concatenated full CDS sequence, synonymous substitution sites, or fourfold degenerate sites. Outgroup genes are identified by BLASTN search against basal angiosperm mRNA sequences in NCBI GenBank or a database of users' choice. Such data are not all available for all region groups because of severe post-WGD gene loss. But when available, the alternative sequence-based trees may be used to cross-validate synteny-based trees.

6.3.4 Estimating the number of unshared paleo-polyploidies between two genomes

Using the PORGs, we then calculate copy number ratios as the number of paralogous segments under

each FDN (designated as nA and nB) divided by the ancestral copy number before speciation (n0=1 for PORG). Assuming most paleopolyploidies are or consist of duplications (genome doublings), the number of unshared WGDs between the two genomes can be calculated as $\log_2[(nA/n0)*(nB/n0)]$. Because of typically extensive sequence loss following paleo-polyploidies, ortholog ratios in most PORGs are expected to be smaller than their theoretical values. Because of potential small segmental duplications and some uncertainties in synteny block and region tree inferences, occasionally ortholog ratios may also be larger than the theoretical values. Therefore we empirically choose to use the 90th percentile of copy number ratios from the population of PORGs between each pair of the genomes.

6.3.5 Inferring paleo-polyploidies on branches of the input phylogeny

The numbers of unshared paleo-polyploidies between the input genomes were compiled into a full distance matrix. The distance matrix and the input species tree topology are then input to the Fitch-Margoliash algorithm (Fitch & Margoliash, 1967) implemented in FITCH of PHYLIP package (Felsenstein, 1989), which minimizes the difference between the tree and the distance matrix based on sum of squares measurement. The resultant branch lengths are the paleo-polyploidy level estimation on the branches. PORG ortholog ratios were bootstrapped 1000 times to estimate the 95% confidence intervals of the paleo-polyploidy levels.

6.3.6 GeDupMap implementation

While this paper emphasizes more the procedure than a 'black box' software, our GeDupMap implementation is available from https://github.com/Jingping/GeDupMap under BSD License. The code was developed in Python programming language (v2.7). No compilation is needed, though third party software and packages need to be installed separately (usually with a package manager). A README file provides more details. The inputs are pairwise LASTZ (or BLAST, LAST) output files between studied genomes in BLAST "—outfmt 6" format (http://www.ncbi.nlm.nih.gov/books/NBK1763/#CmdLineAppsManual.Cookbook), BED files

(http://genome.ucsc.edu/FAQ/FAQformat.html#format1) of genome annotation coordinates, and phylogeny of the taxa under study in NEWICK format (http://evolution.genetics.washington.edu/phylip/newicktree.html). A run.sh file used for running analyses in this study is also provided as an example.

6.4 Results

6.4.1 Simultaneous multiple paleo-polyploidy detection

For demonstration of GeDupMap functionality a set of 6 eudicot genomes (common bean, *Arabidopsis thaliana*, cotton, rose gum, sugar beet) and 2 monocot genomes (oil palm, duckweed) were selected for analysis (**Table 6.1**). All of these genomes have an assembly at chromosome or mega-scaffold level.

Table 6.1 Sources and basic information of angiosperm genomes used in this study.

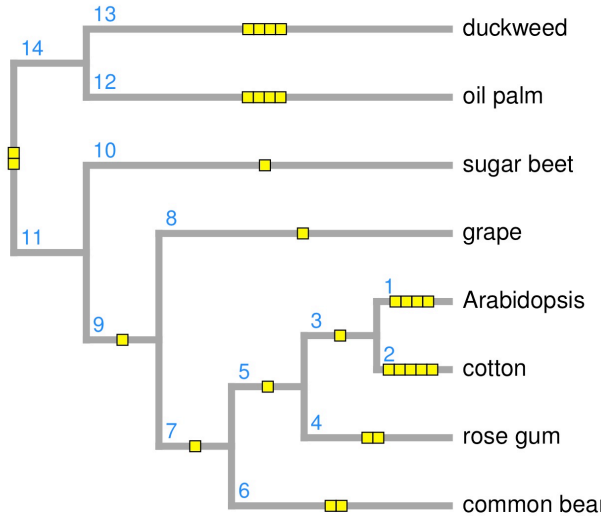
species	common name	family	1x	protein coding genes	assembly level	version	publication
Phaseolus vulgaris	common bean	Fabaceae	11	27,197	chromosome	Phytozome 218	Schmutz *et al.*, 2014
Arabidopsis thaliana	thale cress	Brassicaceae	5	27,416	chromosome	TAIR 10	AGI, 2000; Swarbreck *et al.*, 2008
Gossypium raimondii	cotton	Malvaceae	13	37,505	chromosome	Phytozome 221	Paterson *et al.*, 2012
Eucalyptus grandis	rose gum	Myrtaceae	11	36,376	chromosome	Phytozome 201	Myburg *et al.*, 2014
Vitis vinifera	grape	Vitaceae	19	26,346	chromosome	Genoscope 12X	Jaillon *et al.*, 2007
Beta vulgaris	sugar beet	Amaranthaceae	9	27,421	chromosome	RefBeet 1.1	Dohm *et al.*, 2013
Elaeis guineensis	oil palm	Arecaceae	16	34,802	chromosome	MPOB EG5	Singh *et al.*, 2013
Spirodela polyrhiza	greater duckweed	Araceae	20	19,623	megascaffold	Phytozome 290	Wang *et al.*, 2014

In total 4013 PORGs from 28 pairwise comparisons were analyzed. The paleo-polyploidy inference is summarized in **Figure 6.3**. Inference from our automatic approach confirmed all the

previously described events in individual studies. Our results also showed that by comparing to each other and to outgroup eudicot genomes, it is clear that the common ancestor of duckweed and oil palm did not experience paleo-polyploidy, or the affected lineages did not survive, since monocot-eudicot divergence. This indicated that there is no paleo-polyploidy in the monocot branch before the divergence of Commelinids (containing oil palm) and Alismatales (containing duckweed), which spanned ~ 30 million years in the early Cretaceous (Hedges *et al.*, 2006). This is an interesting evolutionary time associated with divergence of the two major flowering plant groups in the early days of angiosperms. Following monocot-eudicot divergence both groups experienced one or more paleo-polyploidies in most or all of their lineages.

A limitation of GeDupMap is that the paleo-polyploidy levels on the two oldest nodes of the species tree (such as 11 and 14 in our dataset) cannot be separately inferred. This is however easily resolved by directly looking at the dot plot alignments between the genomes, which clearly indicated that the paleo-polyploidy inferred on 11+14 belongs to the eudicots. The ploidy level of the pan-core eudicot γ event is estimated as 2, which is an under-estimate compared to its true ploidy level of 3 (Jaillon *et al.*, 2007; Tang, Bowers, *et al.*, 2008). This is because of lack of a good outgroup to γ in our dataset. In a dataset that include the genome of sacred lotus (*Nelumbo nucifera*), which is a basal eudicot having not experienced the γ event, the ploidy level of γ is correctly inferred as 3 (data not shown). Inclusion of an outgroup genome and careful selection of input taxa are important to GeDupMap results.

a



b

Branch	Name	Mean	95% CI		Estimate	Inferred event name
1	Arabidopsis	3.94	3.43	4.44	4	β,α
2	cotton	5.01	4.43	5.74	5	C
3		1.00	1.00	1.00	1	
4	rose gum	2.32	1.99	2.65	2	EG
5	Eurosids II	1.00	1.00	1.00	1	
6	common bean	2.17	1.95	2.47	2	L
7	Eurosids	1.00	1.00	1.00	1	
8	grape	1.11	1.00	1.29	1	
9	Rosids	1.06	1.00	1.24	1	
10	sugar beet	1.33	1.10	1.60	1	
12	oil palm	4.16	3.70	4.72	4	τ,P
13	duckweed	4.22	3.76	4.80	4	βSP,αSP
11+14		1.92	1.58	2.27	2	γ

Figure 6.3 Paleo-polyploidy inference on each branch of input species phylogeny. Genome duplication (doubling) has paleo-polyploidy level of 2. Genome triplication has paleo- polyploidy level of 3. (a) Tree branches are named by blue numbers, which correspond to those in the table in panel (b). Yellow squares represent estimated paleo- polyploidy levels on the individual branches. For example two squares represent paleo-polyploidy level of 2 (resulted from a paleo-tetraploidy, or genome doubling). Numerical values of mean, 95% CIs and estimate are given in (b). Branches 11 and 14 are currently not separable in the tree due to lack of an outgroup. The last column in the table contains inferred paleo-polyploidy names from the literature that described the events. See text for more details.

6.4.2 Putative orthologous region groups (PORGs) and synte-molecular comparison

With contributions from repeated paleo-polyploidies, angiosperm genomes are highly variable in genome size, content and structure (Kejnovsky *et al.*, 2009; Salse *et al.*, 2009; D. E. Soltis *et al.*, 2003). A direct multiple genome alignment, like the 28-way vertebrate alignment (Miller *et al.*, 2007), is not feasible in plants because the number of common anchors soon diminishes with taxa from a few families added to the alignment, and numbers of homeologous regions vary both within the same genome and across different genomes (see next section). Therefore, a more fruitful way to formulate multiple genome comparisons in angiosperms may be a system of pairwise comparisons, such as formed by GeDupMap. The collection of putative orthologous region groups (PORGs) between each pair of the input genomes is useful to many downstream analysis. It also forms the synteny backbone of a synte-molecular framework of systematic genome comparison.

Once paleo-polyploidy levels on all species tree branches have been inferred, a combined use of intergenomic and intragenomic synteny blocks will enable extraction of homeologous pairs on a given branch. For example, grape and soybean (*Glycine max*) genomes have ortholog ratio of 1:4 due to two paleo-tetraploidies (the Papilionoideae duplication (Young *et al.*, 2011) and the *Glycine* duplication (Schmutz *et al.*, 2010)) in the lineages leading to soybean after the divergence with the grape lineage. Soybean and common bean genomes have ortholog ratio of 2:1 due to the *Glycine* duplication experienced by soybean. Therefore subtracting soybean-common bean comparison from the grape-soybean comparison will produce soybean paralogs duplicated in the Papilionoideae duplication event.

6.4.3 Deletion-resistant and duplication-resistant regions

Following paleo-polyploidy gene and domain families from different functional groups are often differentially retained, sometimes in the same direction repeatedly (Freeling & Thomas, 2006; Gout, Duret, & Kahn, 2009; Paterson *et al.*, 2006; Tang *et al.*, 2010; Tang, Wang, *et al.*, 2008). For example, protein domains such as the Glycine-rich domain (PF07172), G-patch domain (PF01585), and SpoU rRNA methylase (PF00588) are repeatedly restored to singleton state in both *Arabidopsis* and rice

(Paterson *et al.*, 2006). Four functional groups (transcriptional factor activity (GO:0003700), ligand binding (GO:0005488), DNA binding (GO:0003677), and transcriptional regulator activity (GO:0030528)) remained the most enriched functional groups across both σ-duplicates and ρ-duplicates in rice (Tang *et al.*, 2010). In addition to "duplication-resistant" and "deletion-resistant" genes (Paterson *et al.*, 2006), paleo-duplicated genomic regions also show different propensity to be retained or lost. For some regions this propensity may persist across multiple paleo-polyploidy events, resulting in higher depth (homeolog copy number) in the extant genome than other regions without such property. The differential post-duplication retention patterns among genomic regions produce a spectrum of homeolog depth in the extant genomes (**Figure 6.4**), with regions of depth 1 being strict "duplication-resistant" (DupR), and regions with high depths up to the genome's paleo-multiplicity level being "deletion-resistant" (DelR). For example, following a paleo-hexaploidy (γ) and a paleo-tetraploidy (L), an ancestral common bean genomic region can retain all 6 copies (depth 6) at one extreme, or have repeatedly lost homeologous regions and been restored to singleton (depth 1) at the other extreme.

The patterns of DelR and DupR regions seem specific to paleo-polyploidy, as simulation showed that independent massive single gene duplications of similar scale are not likely to form such regions (**Figure 6.5**). The size of DelR regions is often larger than the size of DupR regions, partly reflecting the greater power to identify DelR regions as more of them are retained. Identification of DupR regions is conservative because those DupR regions that were retained in only one sequenced genome are indistinguishable from lineage-specific regions, and therefore cannot be classified. Available sequences from more related genomes will help unmask those regions in future studies.

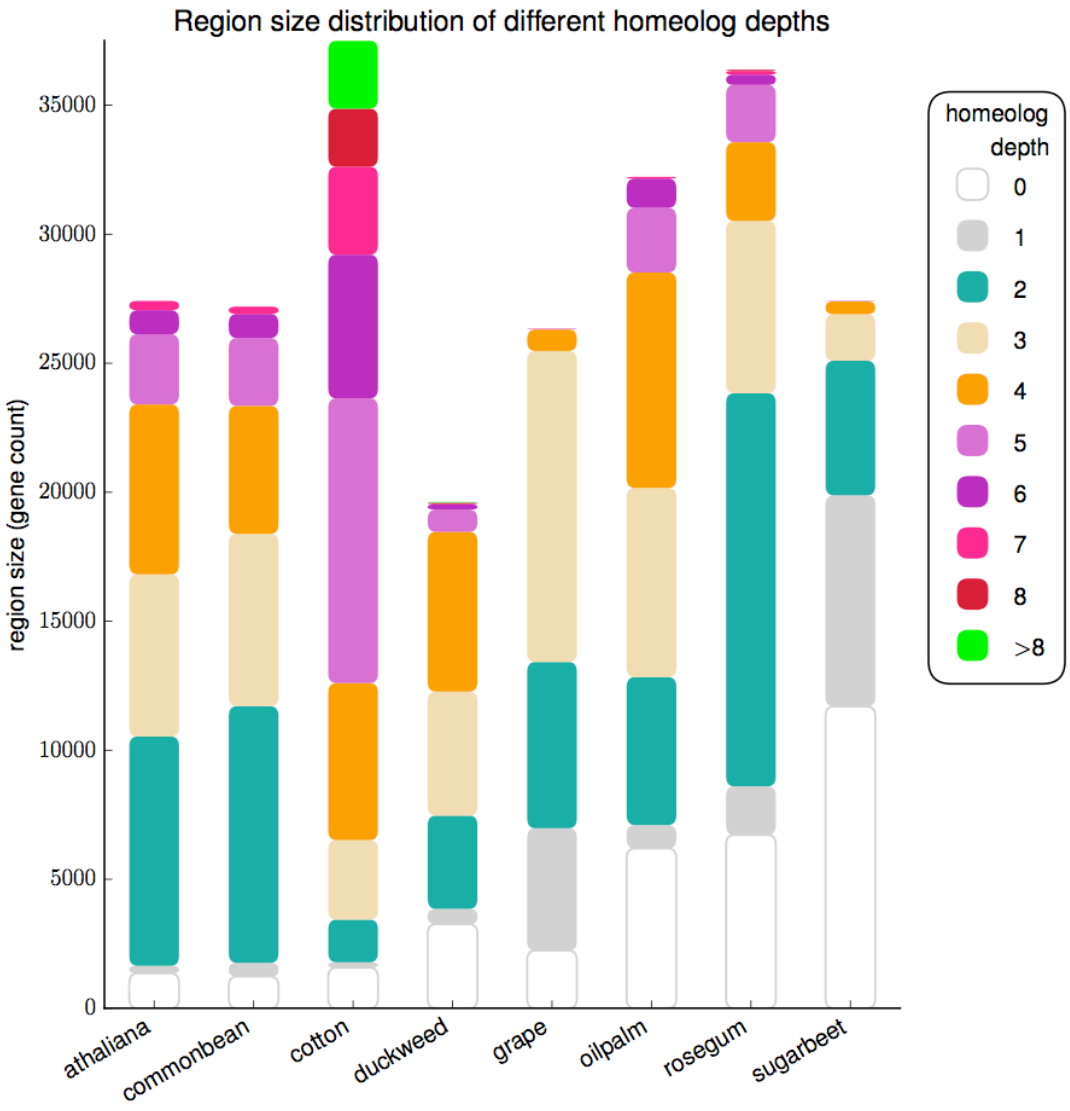


Figure 6.4 Homeolog depths (paleo-polyploidy levels) of genomic regions in the eight genomes. One paleo-tetraploidy (doubling) without further loss leaves two homeologous regions (depth 2). Two paleo-tetraploidies without further loss leave four homeologous regions (depth 4). Depth 0 represents lineage-specific singleton regions that have no homeologous regions in the studied genomes. For example, in the lineages leading to common bean there were 2 ancient WGDs: γ (3x), L (2x). Therefore differential gene loss and lineage-specific gene gain together resulted in the extant common bean genome consisting of regions having 0~6 homeologs. Additional depths greater than 6 are due to calculation artifacts or earlier paleo-polyploidies in pre-angiosperm lineages.

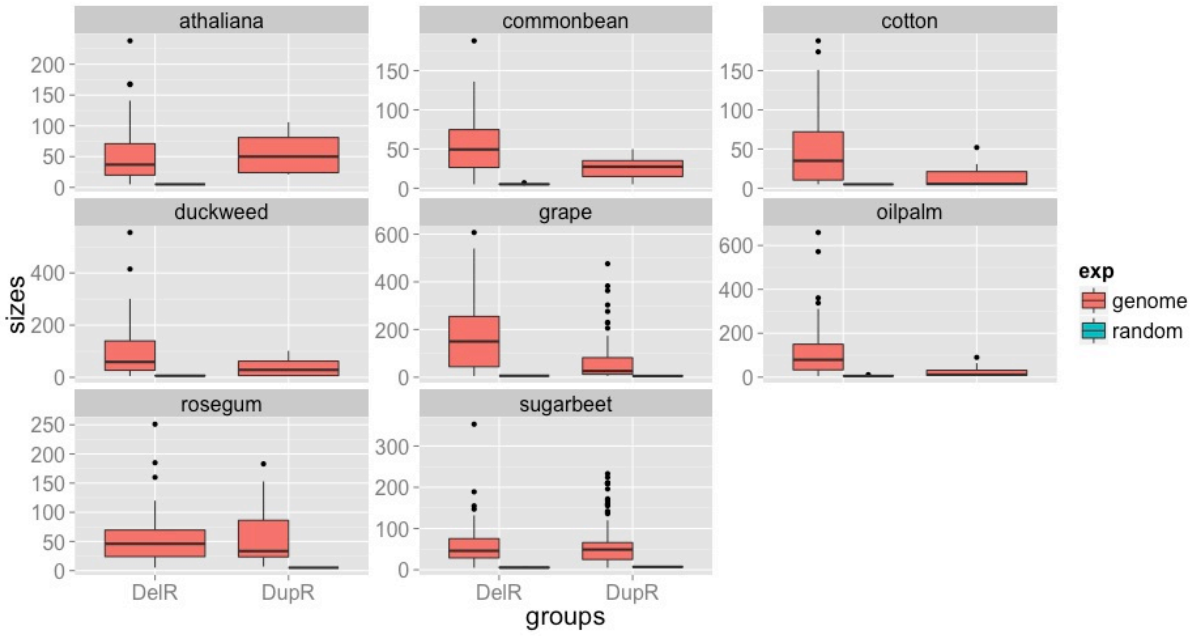


Figure 6.5 Size distributions of DupR (duplication resistant) and DelR (deletion resistant) regions in studied genomes and simulated random genomes. Regions identified in 20 simulated random genomes are combined and used as control. Sizes are measured as gene counts. (a) In randomized control genomes (green boxes on the right) typically only zero or a few short gene clusters were found, indicating lack of DupR and DelR regions. (b) Number of regions identified in each of the studied genomes and 20 control genomes. DupR regions are defined as having homeolog depth 1. DelR regions have high homeolog depths in each of the genomes: *Arabidopsis* depth >= 5, common bean depth >= 5, cotton depth >=9, rose gum depth >= 5, grape depth >= 3, sugar beet depth >= 3, oil palm depth >= 4, duckweed depth >= 4.

6.5 Discussion

Repeated paleo-polyploidization which is characteristic of angiosperm genome evolution, and diploidization which is specific to the genomic 'big bang' (polyploidy) have made genome comparison in angiosperms fundamentally different from other groups of organisms, such as mammals. Often one-to-multiple or multiple-to-multiple ortholog ratios, and numtiple secondary associations form a network of hierarchical synteny and homology mapping that make angiosperm genome comparisons one of the most complex.

Knowledge of the timing (phylogenetic positioning) and ploidy levels of paleopolyploidy events are essential in many plant studies. Conventionally this is usually done by investigating the individual lineages. However, this approach will not scale well with rapidly expanding genome data. Therefore we designed part of the GeDupMap pipeline to fill in this gap and automate simultaneous paleo-polyploidy circumscription on multiple lineages. In addition, GeDupMap provides three levels of regions clustering: PARs, Groups, PORGs. The PORGs are tentative and do not replace rigorous orthology definition by phylogenetics, but they provide a starting and visualized solution that may be directly useful in some studies.

Inter-genomic alignment is the fundamental method to reveal similarities and differences among genomes. In particular, alignment with orthologous regions in the genomes of model organisms facilitates accurate knowledge transfer to and accelerates studies of non-model organisms. Systematic multi-way syntenic mapping provides an effective framework for genome alignment, with the unique advantage of tolerating long evolutionary distance and extensive genome rearrangement. Nucleotide-level alignments can in turn be conducted to enable more evolutionary analyses, such as nucleotide-level conservation, categorization of indels in coding and regulatory regions, discovery of candidates for new and lineage specific genes and other functional elements, recovery of ancestral functional elements that are lost in extant sequences, and detection of genomic selection patterns. Phylogenetic trees of gene families are often vulnerable to nucleotide substitution rate variation among lineages or different parts of gene, biased sequence composition, saturation of sequence divergence, homoplasy, and gene loss. Largely exonerated from these complications, synteny conservation or deviation is regarded as a more reliable phylogenetic character (Rokas & Holland, 2000). The synte-molecular framework we proposed, also a flavor of combined local and global (or glocal) alignment, is a naturally advantageous way to dissect fine structural homology in divergent angiosperm genome comparisons.

Alignment of multiple gene order in homologous regions can be represented in several ways. Traditional linear row-column alignment representation, although straightforward, does not store information of micro-rearrangements of gene order. Partial order graph (POA) representation of multiple

alignments was developed to take advantage of directed acyclic graphs in handling gaps more naturally, and incorporating more information than linear profiles (C. Lee, Grasso, & Sharlow, 2002). However, POA is not useful in the presence of repeats or shuffled anchors, which create cycles in the graph and void the acyclic property of POA. To mitigate this disadvantage, the A Bruijn graph, a weighted directed graph related to the de Bruijn graph widely used in short read sequence assembly, was introduced in 2004 (Pevzner, Tang, & Tesler, 2004; Raphael, Zhi, Tang, & Pevzner, 2004). Different from the de Bruijn graph which requires exact l-tuple matches, a situation seldom seen in the world of gene order alignment, in the A Bruijn graph nodes represent individual loci, directed edges represent locus order, and "dark edges" connect aligned loci. Cycles are allowed to represent local repeat, inversion, and shuffling. After the A Bruijn graph representation of gene order alignment is constructed, heuristic procedures such as "threading" (Raphael *et al.*, 2004) and "modification" (Pham & Pevzner, 2010) work to simplify the graph. Having the distinct advantage in handling local repeated and shuffled segments, A Bruijn graph qualifies to be an ideal representation of homeologous region alignments that will provide a useful interface for downstream analyses such as visualization, molecular evolution analyses, and ancestral reconstruction. In addition, several other graphs related to the A Bruijn graph are discussed in depth in a recent study (Kehr, Trappe, Holtgrewe, & Reinert, 2014). While graph representations of PORGs are not part of GeDupMap, they can be reasonably easily applied to the program output.

6.6 Conclusion

We described a systematic approach, GeDupMap, to simultaneously infer all paleo-polyploidy events on an input phylogeny of species with available genome assembly and annotation. This has great advantage over previous manual inferences in view of rapidly increasing sequencing efforts on many taxa. The analyses of synteny patterns behind the paleo-polyploidy inferences then form the synte-molecular framework for downstream comparative analyses of the involved genomes. Such a framework will also facilitate identification of high dimensional structural orthologs between the genomes. We demonstrated the functions of GeDupMap using 8 eudicot and monocot genomes. Our results confirmed individually

identified paleo-polyploidy events in those lineages. We also identified groups of putative orthologous regions, and duplication resistant (DupR) and deletion resistant (DelR) regions in the studied genomes. The framework described here will facilitate studies of genome structure hierarchy in biochemical and evolutionary settings.

CHAPTER 7 CONCLUSION AND PERSPECTIVE

7.1 Structural evolution of paleo-polyploid angiosperm genomes

Repeated paleo-polyploidies distinguish angiosperm genome evolution from that of other organisms. Those dramatic whole genome events have been a key contributor to genetic changes underlying phenotypic novelties and diversity in angiosperm lineages. Major findings of mechanisms by which paleo-polyploidies affected angiosperm genome evolution include structural rearrangements (Kishimoto *et al.*, 1994; Kowalski *et al.*, 1994; Shoemaker *et al.*, 1996), fractionation (Thomas *et al.*, 2006), subfunctionalization and neofunctionalization (Kellis *et al.*, 2004; M. Lynch & A. Force, 2000; Ohno, 1970), subgenomic dominance (J. C. Schnable *et al.*, 2011; Tang *et al.*, 2012), rewired biochemical pathways (Arabidopsis Interactome Mapping Consortium, 2011; Bekaert *et al.*, 2011), new gene combinations (De Bodt *et al.*, 2005; Rieseberg *et al.*, 2003), favored gene retention explained by 'gene balance theory' (Birchler *et al.*, 2001; Papp *et al.*, 2003; Thomas *et al.*, 2006), increased genetic robustness explained by the 'functional buffering theory' (Chapman *et al.*, 2006; Gu *et al.*, 2003; Paterson *et al.*, 2006), increased regulatory complexity (Freeling & Thomas, 2006), altered gene expression (Adams & Wendel, 2005a; J. C. Schnable *et al.*, 2011), and illegitimate/homeologous recombination (Gaeta & Chris Pires, 2010; X. Y. Wang, Tang, Bowers, & Paterson, 2009) (for some reviews see (J. J. Doyle *et al.*, 2008; Freeling, 2009; Paterson *et al.*, 2010; Van de Peer *et al.*, 2009; K. H. Wolfe, 2001)). Chapters 2-5 of this dissertation described several newly identified paleo-polyploidies, contributing to knowledge of angiosperm paleo-polyploidies and genome evolution from several aspects.

There are also other aspects of plant paleo-polyploidies and genome evolution that will likely benefit from more research in the near future. While paleo-tetraploidy events appear to be most common, a few paleo-hexaploidy events have been identified in eudicots, including one in the core eudicot stem lineage ("γ"), one in the rosid *Brassica* stem lineage, and one in the asterid Solanaceae stem lineage

("T"). The *Gossypium* paleo-(do)decaploidy may also contain a hexaploidy component. The *Solanum* genomes (such as tomato and potato) are the only ones identified so far that had two consecutive paleo-hexaploidies but no paleo-tetraploidies. In contrast, although some wild monocots such as the grass 'timothy', and crops such as bread wheat are neo-hexaploids, paleo-hexaploidy has not been found in any monocot genome studied so far. At present there are 3~4 cases of paleo-hexaploidy in 19 eudicot paleo-polyploidies, while the 12 monocot paleo-polyploidies are all paleo-tetraploidy. If such bias is not due to lack of sampling artifacts, this would raise curious questions about possible reasons and consequences associated with these events through eudicot evolution, or alternatively, possible suppression of such events in the evolution of other lineages.

The content of heterochromatin, which is rich in transposable elements (TEs), is well known to account for a large proportion of the substantial genome size differences among angiosperm species (Bennetzen, 2005; Bowers *et al.*, 2005; Tenaillon, Hollister, & Gaut, 2010). Colinearity conservation is much less in heterochromatin than in euchromatin regions (Bowers *et al.*, 2005). TE content can vary greatly between closely related genomes having similar genic fractions (Hawkins, Kim, Nason, Wing, & Wendel, 2006; Hu *et al.*, 2011; X. Wang, Wang, *et al.*, 2011; Wu *et al.*, 2013) or even from the same species (Morgante, De Paoli, & Radovic, 2007). In plants much of the genome expansion and contraction owing to retrotransposon activity seems to take place rapidly in the evolutionary timescale (Bennetzen, 2005; Morgante, 2006). TEs and heterochromatin have once been thought of as more or less dispensable in genomes. However, TEs have also been found to stimulate genome rearrangements by mechanisms such as chromosome-breaking, aborted transposition, and ectopic recombination (Bennetzen, 2005). After polyploidization the genome restructuring effects of TEs may occur in a subgenome biased manner (D. E. Soltis & Soltis, 1999). Increased heterochromatin restructuring after polyploidization may even be selectively advantageous, and may occur in parallel in sister species (Bowers *et al.*, 2005). Therefore, better knowing the history and mechanisms of TE activity may facilitate understanding of genome structure evolution.

One fundamental question about genome structure is whether gene order is random. Most eukaryotic genomes lack the operon structures in prokaryotes, and therefore were suspected to have little constraint in gene order. However, most studies so far seem to support the hypothesis that eukaryotic gene order is non-random, especially at larger regional scales (Davila Lopez, Martinez Guerra, & Samuelsson, 2010; Hurst, Pal, & Lercher, 2004). In particular, studies in recent years reported evidence for operon-like structures and functional gene clusters in Drosophila (Boutanaev *et al.*, 2002) and plants (Field *et al.*, 2011; Field & Osbourn, 2008), large co-expression gene clusters (Boutanaev *et al.*, 2002; Paterson *et al.*, 2012; Wada *et al.*, 2012), and selection on local organization of human and mouse genome structure (Boettger, Handsaker, Zody, & McCarroll, 2012; Singer, Lloyd, Huminiecki, & Wolfe, 2005). Our own studies also suggested evolutionary and functional compartmentation in plant genomes. Like hierarchical chromatin packaging, it is plausible to think that organization of genomic elements may also be hierarchical. In angiosperms, ubiquitous retention of syntenic regions following tens to hundreds of millions of years' divergence argues against random gene organization. Indeed, repeated paleo-polyploidies and diploidization are a logical favorable factor for structural compartmentation in angiosperm genomes. Availability of more genomes in the near future may provide the opportunity to test this hypothesis.

7.2 Effective synte-molecular comparisons of related plant genomes

Genes are the core functional units of the genome. Although essential, it is no easy task to study gene function and regulation, and to transfer such knowledge between related species. Angiosperm species are substantially variable in genome size, content, and arrangement, and have retained different levels of conservation with one another and their ancestors. These observations form the basis, and a central objective, of plant comparative genomic studies (Gale & Devos, 1998; Paterson *et al.*, 1996). Knowledge of genome structure conservation has two fundamental applications. It enables accurate transfer of hard-won biological information from model organisms to many additional organisms. It also provides for evolutionary inferences of ancestral and derived states of structure, sequence, and function.

However, as discussed above, the extent of large-scale structural conservation at chromosome and region scales often does not correlate with the level of micro-synteny conservation over a few genes' distance, the latter of which is often disrupted by local non-colinear elements. Therefore, global and local alignments often complement each other in comparative studies. Combination of the two increases power in identifying orthologous genes and delineating gene family genealogy.

On the other hand, compared to intra-genomic alignment, inter-genomic alignment is more advantageous in revealing homology (and divergence) among genomes because of often smaller evolutionary distance between orthologous regions. Concealing of homology due to reciprocal loss and subfunctionalization between paralogs is often revealed by comparing to an orthologous region in a sister lineage that lacked the duplication. Therefore, multi-way syntenic mapping aided by 'intermediate' genomes provides an effective backbone for genome alignments. Local and nucleotide-level alignments can in turn be conducted to enable more evolutionary analyses, such as nucleotide-level conservation, categorization of indels in coding and regulatory regions, discovery of candidates for new and lineage specific genes and other functional elements, recovery of ancestral functional elements that are lost in extant sequences, and detection of genomic selection patterns. The GeDupMap program (Chapter 6) provides a starting solution to apply such synte-molecular comparisons among plant genomes.

7.3 Closing remarks

The past decade has seen exponential increase in the number of published genomes. Advancing sequencing technology and plummeting cost assure continuing proliferation of genome data in the future. The golden age of genome informatics is here with unprecedented challenges and opportunities. Novel tools and deepened knowledge of paleo-polyploidy in recent years have greatly facilitated comparisons among diverse plant genomes, including those that were once thought to be beyond the reach of such comparisons. There is clear need and opportunity for future comparative genomic studies to take more depth. For example, the frequency of structural alterations is not uniform throughout a genome. Hotspots of micro-rearrangements are found near centromeres, telomeres, duplications, and interspersed repeats

(Eichler & Sankoff, 2003; Murphy *et al.*, 2005). Rates of recombination vary along genomic regions of different nucleotide composition, gene density, repeat content, chromatin packing, and other potential factors (Cirulli, Kliman, & Noor, 2007; Giraut *et al.*, 2011; McVean *et al.*, 2004; Pal & Hurst, 2003). Arrangement and diversification of functional elements in the genome are also different in organisms with different reproductive types, life styles, habitats, and other factors. Systematic detailed studies of many genomes are needed to fully understand the rules and consequences of genome organization in plants and other organisms.

The biggest difference between plant and animal genome structure, biologically and methodologically, is the extra duplicity in plant genomes due to repeated paleo-polyploidies. Consequently essential genomic analyses, such as genome alignment, ortholog identification, and allelic sequence mapping, are often more complicated or require separate tools in plants. However, by systematically accounting for duplication in plant genomes, they can then be naturally connected with genomic studies in other eukaryotic kingdoms, and therefore help unify and synergize knowledge of genes and genomes across the eukaryotic phylogeny.

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