Mutational Dynamics of Aroid Chloroplast Genomes

Ibrar Ahmed1,2,*, Patrick J. Biggs3, Peter J. Matthews4, Lesley J. Collins5, Michael D. Hendy6, and Peter J. Lockhart1,5

1Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand
2Department of Biochemistry, Quaid-i-Azam University, Islamabad, Pakistan
3Institute of Veterinary, Animal, and Biomedical Sciences, Massey University, Palmerston North, New Zealand
4Department of Social Research, National Museum of Ethnology, Osaka, Japan
5Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand
6Department of Mathematics and Statistics, University of Otago, Dunedin, New Zealand

*Corresponding author: E-mail: I.Ahmed@massey.ac.nz, iaqureshi_qau@yahoo.com.

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Data deposition: Chloroplast genome sequences of two morphotypes of taro (Colocasia esculenta var. GP and Colocasia esculenta var. RR) have been deposited to GenBank under accession numbers JN105689 and JN105690, respectively. The sequence alignments generated for this work will be provided on request.

Abstract
A characteristic feature of eukaryote and prokaryote genomes is the co-occurrence of nucleotide substitution and insertion/deletion (indel) mutations. Although similar observations have also been made for chloroplast DNA, genome-wide associations have not been reported. We determined the chloroplast genome sequences for two morphotypes of taro (Colocasia esculenta; family Araceae) and compared these with four publicly available aroid chloroplast genomes. Here, we report the extent of genome-wide association between direct and inverted repeats, indels, and substitutions in these aroid chloroplast genomes. We suggest that alternative but not mutually exclusive hypotheses explain the mutational dynamics of chloroplast genome evolution.

Key words: Araceae, indels, phylogeny, repeats, substitution mutations, taro.

Introduction
Comparative studies of chloroplast genome sequences have investigated divergences spanning an enormous range of evolutionary times. These have included studies of intraspecific variation in domesticated plants (Yamane et al. 2003), studies of early land plant evolution (Kugita et al. 2003) and also the earliest events of oxygenic photosynthesis (Martin et al. 2002). This range of comparisons has been possible because of the conservative nature of chloroplast (cp) genome evolution (Palmer 1985), which involves relatively slow rates of sequence evolution in some parts of the cp genome (Sammut and Huttley 2011) and elevated rates in other parts (Magee et al. 2010; Sammut and Huttley 2011).

Molecular evolution of the cp genome sequences is typically modeled as a time reversible substitution process, in which changes at any one site are independent of changes at any other site (Lio and Goldman 1998; Drouin et al. 2008). However, observations have suggested more complex processes of evolution in which both lineage-specific and nonrandom spatial patterns of substitution occur (Lio and Goldman 1998; Lee et al. 2007; Gruenheit et al. 2008; Magee et al. 2010; Wu et al. 2011; Zhong et al. 2011). Such observations have practical significance for understanding the limitations of cp genomes in phylogenetic analyses of highly diverged lineages (Gruenheit et al. 2008), and for understanding the mutational dynamics of “hotspot” regions studied in comparisons of closely related taxa (Shaw et al. 2007; Worberg et al. 2007).

In prokaryotes and eukaryotes, analyses of DNA sequence alignments show that indels commonly occur in regions that are hotspots for nucleotide substitutions. Alternative hypotheses have been proposed to explain this co-occurrence. It has been suggested that certain genome regions are predisposed to mutational events such as substitutions and insertion/deletions—"the regional difference hypothesis" (Silva and Kondrashov 2002; Hardison et al. 2003). A second hypothesis...
explaining the association between indels and substitutions is that certain (large) indels act to induce substitutions through a DNA repair process that recruits error-prone DNA polymerases—“the indel-induced mutation hypothesis” (Tian et al. 2008; Zhu et al. 2009). A third and related hypothesis is that it is the presence of repeat sequences rather than indels per se, that actually promotes replication fork arrest, causing the recruitment of the error-prone DNA polymerases, and in doing so generates nucleotide substitutions (McDonald et al. 2011).

These hypotheses have not been explicitly investigated in cp genomes yet these genomes are known to contain very high densities of direct and inverted oligonucleotide repeats. Associations between repeats, indels, and substitutions have also been reported in cp genomes (McLenachan et al. 2000; Lockhart et al. 2001 and references cited therein). Cp genome repeats include simple sequence repeats (SSRs, also known as microsatellites) and other moderate to long (8–48 bp) repeats. Contraction and expansion of the SSR units, caused by slipped strand mispairing during DNA replication (Levinson and Gutman 1987), frequently produces short indels at these SSR loci (Kim and Lee 2005; Whitlock et al. 2010). Most angiosperms also contain two large inverted repeat (IR) regions, commonly known as IRa and IRb (5–76 kb; Palmer 1987), frequently produces short indels at these SSR loci (Masood et al. 2004). The moderate-to-long repeats have also been suggested to cause indels (Kawata et al. 1997) and inversions (Kim and Lee 2005; Whitlock et al. 2010). Most angiosperms also contain two large inverted repeat (IR) regions, commonly known as IRa and IRb (5–76 kb; Palmer 1991).

Here, we report the cp genome sequences of two morphotypes of taro (Colocasia esculenta; var. RR and var. GP; Matthews 1985) and examine the genome wide association of repeats (excluding IRa and IRb), indels and substitutions in the cp genomes of these taro morphotypes and four other regions, commonly known as IRa and IRb (5–76 kb; Palmer 1991).

The overall gene arrangement was similar between taro (C. esculenta) and the duckweed (Lemna minor, Mardanov et al. 2008) cp genomes. However, notable differences were as follows:

(a) trnH gene is reported in the LSC region in duckweed, whereas the 5’-end of this gene extended into the IRa region in taro.

(b) infA gene is completely missing in duckweed, but a pseudo-copy of this gene with internal stop codons was observed in taro.

(c) A single functional rpl2 gene spanning the IRb–LSC boundary is reported in duckweed, whereas two functional copies of this gene were found in taro, one in each of the IR regions.

(d) A pseudo-copy of ycf68 gene is reported in duckweed; however, a functional copy of this gene was observed in each IR region in taro.

(e) Duckweed has ycf1 and rps15 genes within its IR regions, whereas these genes were placed within the SSC region in taro.

The infA gene is considered to be among the most mobile cp genes. Multiple independent gene transfers from cp to nuclear genomes are thought to have occurred during angiosperm evolution (Millen et al. 2001). The ycf68 gene is present in a range of plant families as a functional or a pseudo-gene, and may have functional significance even in its noncoding form (Raubeson et al. 2007). Other genes showing variation in comparison with L. minor include trnH, rpl2, ycf1, and rps15. These are located at or near the boundaries of IRs with single copy regions. These boundaries are well known to exhibit expansion and contraction in angiosperms (Whitlock et al. 2010) as well as in gymnosperms (Lin et al. 2012). A comparison of

The Colocasia esculenta cp Genome

Colocasia esculenta (L.) Schott, commonly known as taro, is an ancient root crop in subfamily Aroidaeae of the monocot family Araceae. This species is distributed in the tropical to subtropical and some temperate regions of the world (Bown 1988).

Gene arrangement and other features of the C. esculenta cp genome are shown in figure 1. Size of the cp genome was 162,546 bp (GC content: 36.1%) in var. RR, and 162,424 bp (GC content: 36.2%) in var. GP. The GC content varied from 42.4% in IRs to 34.4% in the large single copy (LSC) and only 28.4% in the small single copy (SSC) regions of the taro cp genomes. Higher GC content in the IR regions corresponded to the presence of the ribosomal DNA locus. Pair-wise sequence alignment between the taro cp genomes revealed 99.5% identical sequence, 241 substitutions, and 92 indels. The LSC region contained 141 (58.6%) substitutions and 65 (71%) indels, the SSC region contained 83 (34.4%) substitutions and 25 (27%) indels, whereas the IRa and IRb regions collectively contained only 17 (7%) substitutions and 2 (2%) indels, indicating that the IR was the most evolutionarily stable region. Prominent differences between the two taro cp genomes were found at the IRb–SSC boundary (numerous indels making up a 91 bp difference in size), and at the SSC–IRa boundary (a shift of 64 bp in the repeat boundary without causing indels). Thus, the IR boundaries at both ends of the SSC region were polymorphic at intraspecific level in taro. Polymorphism between the two taro cp genomes included 59 substitutions in 29 protein coding genes. Among these, the most polymorphic gene was ycf1 even when normalized for its size, showing 16 substitutions between the two genomes. Some protein coding genes (including atpH, psbM, and psbZ) and tRNA genes (including trnH, trnG, and trnW) in particular showed a relatively high density of substitutions and indels within 20bp upstream of their respective coding regions. Whether this observation has functional significance needs to be further explored. A set of 30 functional tRNA genes covering all 20 amino acids required for protein synthesis was present in the taro cp genome.

The overall gene arrangement was similar between taro (C. esculenta) and the duckweed (Lemna minor, Mardanov et al. 2008) cp genomes. However, notable differences were as follows:

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the size and percentage proportions of LSC, SSC, and IR regions in taro and other aroid cp genomes is given in Table 1. Characterization of these boundaries is likely to provide useful insights into the dynamics of single copy—IR boundary shifts in *Colocasia* and other aroid cp genomes.

**Table 1**

| Species               | GenBank ID | Genome Size | LSC     | SSC     | IR       |
|-----------------------|------------|-------------|---------|---------|----------|
| *Colocasia esculenta* var. GP | JN105689   | 162,424     | 89,670  | 22,208  | 25,273   |
| *C. esculenta* var. RR  | JN105690   | 162,546     | 89,817  | 22,075  | 25,327   |
| *Lemna minor*          | NC010109   | 165,955     | 89,906  | 13,603  | 31,223   |
| *Spirodela polyrhiza*  | JN160603   | 168,788     | 91,222  | 14,056  | 31,755   |
| *Wolffiella lingulata* | JN160604   | 169,337     | 92,015  | 13,956  | 31,683   |
| *Wolffia australiana*  | JN160605   | 168,704     | 91,454  | 13,394  | 31,930   |

**Note.**—Percentage proportions of the LSC, SSC, and IRs are given in parenthesis.

**Correlations among Repeats, Indels, and Substitutions in Aroid cp Genomes**

We have visualized the extent to which indel and substitution mutations are nonrandomly distributed between taro and other aroid cp genomes, using a Circos
(Krzywinski et al. 2009) plot as given in figure 2. This plot shows that substitutions are very closely correlated in their distribution with moderate (15 bp) to long (48 bp) repeat sequences mainly found in noncoding regions. Correlation ($r$) and related values for these data are given in table 2.

Correlations were highly significant in comparisons of three types of mutations, including 1) repeats and substitutions, 2) substitutions and indels, and 3) repeats and indels. In a pairwise comparison of the two closely related taro genomes, the strength of correlations was greatest for...
“repeats and indels” followed by “substitutions and indels” and then “repeats and substitutions.” In contrast, when pairwise comparison was made between a taro genome and a more distantly related aroid genome, the strength of correlations reversed. The strongest correlation was for “repeats and substitutions” followed by “substitutions and indels” and then “repeats and indels.”

The strongest correlation value observed was for “repeats and indels” in comparison of the two taro genomes. Similar observations have previously been reported in prokaryotes and eukaryotes (Kawata et al. 1997; McDonald et al. 2011) and have led to a hypothesis that repeat sequences play a pivotal role in generation of indel and substitution mutations (McDonald et al. 2011).

Since Tian et al. (2008) proposed that moderate-to-large-sized indels induce substitutions in their surrounding sequences, we also investigated this relationship in a multiple sequence alignment (parental alignment) of all six aroid cp genomes. From the this parental alignment, we extracted data partitions containing distinct indel location points (ILPs) to make mutually exclusive partitions with respect to locations of the ILPs. Partition A contained ILPs associated with SSR indels in both coding and noncoding regions. Partition B contained ILPs associated with large (oligonucleotide long, non-SSR) indels in both coding and noncoding regions. Partition C contained ILPs in noncoding regions, associated with both SSR indels and large indels. Partition D contained ILPs in coding regions, associated with both SSR and large indels. The density of substitutions in all partitions was highly dependent upon inverse of distance from the ILPs ($r^2$ ranged from 0.85 to 0.97 for all bin sizes; supplementary fig. S1, Supplementary Material online). Higher substitution density in bins closer to the ILPs was a general trend in all five comparisons above, including the partition in which coding regions were removed (partition C); however, in this case, distance from the ILPs was relatively shorter than in the other four comparisons. The indel-induced mutation hypothesis was further explored in a comparison including the parental alignment and partitions A and B, as shown in figure 3. From this comparison, it is evident that the partition B (containing ILPs associated with large indels) displayed a higher density of substitutions closer to ILPs, and the density of substitutions decreased with an increase in distance from the ILPs. In contrast, the partition A (containing ILPs associated with SSRs) exhibited a low density of substitutions close to ILPs, and the density of substitutions showed a net increase with increase in distance from the ILPs. These observations are consistent with the indel-induced mutation hypothesis suggested for diploid eukaryote (Tian et al. 2008) as well as bacterial genomes (Zhu et al. 2009).

It is well known that certain regions of the chloroplast genome show different rates of mutations (Lee et al. 2007; Gruenheit et al. 2008; CBOL Plant Working Group 2009; Zhong et al. 2011). These are observations consistent with a regional difference hypothesis (Silva and Kondrashov 2002; Hardison et al. 2003) and the suggestion that purifying selection operates at both coding and noncoding regions (Petersen et al. 2011). However, these explanations are alone insufficient to explain substitution and indel patterns of the chloroplast genome. The extent of genome wide correlations reported here for indels, repeats, and substitution provides further support for the hypothesis by McDonald et al. (2011), which emphasizes the evolutionary importance of the repeats in causing mutations. In addition, our observations on substitution densities also provide support for an indel-induced mutation hypothesis (Tian et al. 2008;
Zhu et al. 2009) and further our understanding for the sometimes poor fit between time reversible substitution models and chloroplast sequence data. Perhaps, most interestingly, the relationship between repeats, substitutions, and indels implies that, if the distribution of repeat sequences in a chloroplast genome is determined, there is a possibility to predict the mutational hotspot regions and other sequences that are most appropriate for population genetic, phylogeographic, and phylogenetic analyses.

Materials and Methods

Taro plants (C. esculenta var RR; voucher number MPN:46548, and var GP; voucher number MPN:46549 in the Dame Ella Campbell Herbarium, Massey University, New Zealand) were obtained from the University of Auckland campus. Chloroplasts were enriched following procedure given in Atherton et al. (2010). DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, USA) and quantified using a Qubit Fluorometer (Invitrogen) and Quant-iT-ds DNA HS Assay kit (Invitrogen). Illumina sequence reads were generated using the GAIIx platform at the Massey Genome Service, Massey University, New Zealand. Illumina sequencing produced 33 million reads of 75 base long (16.5 million paired-end reads) for var. RR, and 26.4 million reads of 75 base long (13.2 million paired-end reads) for var. GP. The reads were mapped to the duckweed cp genome (L. minor; Mardanov et al. 2008) using BWA mapping tool (Li and Durbin 2009). Mapping results were visualized using Tablet (Milne et al. 2010). The reads from var. RR were de novo assembled into contiguous sequences (“contigs”) of variable lengths using Velvet (v.0.7.60; Zerbino and Birney 2008), as described elsewhere (Collins et al. 2008). These contigs were BLAST-searched (Altschul et al. 1997) to determine homology to the duckweed cp genome. The contigs of cp origin were assembled in Geneious Pro (Drummond et al. 2009) to deduce the cp genome of the taro var. RR morphotype. The two IRs were distinguished by visual inspection of the boundaries between the repeat and single copy regions. Genome annotation was carried out using Dual Organellar GenoMe Annotator (DOGMA;Wyman et al. 2004) and also by direct comparison with the duckweed cp genome. Contigs were generated similarly for the var. GP morphotype. The completed var. RR cp genome was then used as our reference genome to help assemble the var. GP cp genome. To verify integrity of the de novo assembly process, the original 75 base long reads from both taro samples were mapped back to their respective, assembled cp genomes. Summary statistics for the BWA mapping of 75 base long reads to the L. minor cp genome, as well as to their respective assembled var. RR and var. GP genomes are given in table 3.

The var. RR cp genome was pairwise aligned to the var. GP cp genome, as well as to four aroid cp genomes from the Lemnoideae subfamily, using DIALIGN alignment (Morgenstern 2004). The four aroid cp genomes included L. minor (GenBank ID: NC010109; Mardanov et al. 2008), Spirodela polyrhiza (GenBank ID: JN160603), Wolffiella linguata (GenBank ID: JN160604), and Wolffia australiana (GenBank ID: JN160605; Wang and Messing 2011). Selecting C. esculenta var. RR cp genome as a reference for the coordinate positions, indels, and substitutions were counted in pairwise comparisons in nonoverlapping bins of 250 bp through the entire length of the aligned cp genomes (partitioning each of the five alignments into 651 bins). For the
Table 3
Summary Statistics for BWA Mapping of 75 Base, Paired-End Reads Obtained from the Colocasia esculenta var. RR and var. GP Morphotypes to the Lepna minor Chloroplast Genome and to Their Assembled Chloroplast Genomes

| Parameter                        | L. minor | C. esculenta var. RR | C. esculenta var. GP |
|----------------------------------|----------|----------------------|----------------------|
| Genome coverage (%)              | RR1      | RR2                  | RPE                  |
|                                  | 68.5     | 68.1                 | 85                   |
| Average coverage depth           | 129      | 128                  | 337                  |
| Maximum coverage depth           | 674      | 623                  | 1,531                |

Note.—The acronyms RR1, RR2, and RPE represent mapping with the read 1, read 2, and paired-end (reads 1 and 2 taken together) reads obtained from the var. RR morphotype. Similarly, GR1, GR2, and GPE represent mapping with the read 1, read 2, and paired-end reads obtained from the var. GP morphotype.

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