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Analysis of plastid and nuclear DNA data in plant phylogenetics—evaluation and improvement

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Correct combination of plastid (cp) and nuclear (nr) DNA data for plant phylogenetic reconstructions is not a new issue, but with an increasing number of nrDNA loci being used, it is of ever greater practical concern. For accurately reconstructing the phylogeny and evolutionary history of plant groups, correct treatment of phylogenetic incongruence is a vital step in the proper analysis of cpDNA and nrDNA data. We first evaluated the current status of analyzing cpDNA and nrDNA data by searching all articles published in the journal *Systematic Botany* between 2005 and 2011. Many studies combining cpDNA and nrDNA data did not rigorously assess the combinability of the data sets, or did not address in detail possible reasons for incongruence between the two data sets. By reviewing various methods, we outline a procedure to more accurately analyze and/or combine cpDNA and nrDNA data, which includes four steps: identifying significant incongruence, determining conflicting taxa, providing possible interpretations for incongruence, and reconstructing the phylogeny after treating incongruence. Particular attention is given to explanation of the cause of incongruence. We hope that our procedure will help raise awareness of the importance of rigorous analysis and help identify the cause of incongruence before combining cpDNA and nrDNA data.

combined analysis, plastid DNA, incongruence, nuclear DNA, phylogenetics

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Tremendous progress has been made in our understanding of phylogenetic relationships at all taxonomic levels across all plant groups with developments in molecular phylogenetics. As an example, the order- and family-level phylogenetic framework of land plants has been largely resolved [1–5]. Phylogenetics has become a powerful tool and a starting point in many areas of biology, such as taxonomy, physiology, ecology, biogeography, paleobiology, genomics, and developmental genetics [5–9]. A robust and well-supported phylogenetic tree is a prerequisite for understanding and explaining many life phenomena, otherwise erroneous conclusions will be generated in an incorrect phylogenetic context.

Plastid (cp) and nuclear (nr) genomes are the most frequently used sources of genetic data for reconstructing the phylogeny of plant groups [10–14]. However, certain evolutionary events, such as gene duplication, hybridization, and lineage sorting of ancestral polymorphisms, may result in conflicting topologies based on data sets from these two genomes at all taxonomic levels [12,15,16]. It is well known that hybridization and lineage sorting of ancestral polymorphisms have occurred much more frequently in nature than previously envisioned [17,18]. The issue of combining cpDNA and nrDNA data sets is not new, but it is of greater practical concern today, with the vast amount of molecular data now available, especially as an increasing number of low-copy nuclear genes are used in plant phylogenetics [13,14,19,20]. Careful analysis of cpDNA and nrDNA data and assessment of their combinability is a vital step toward accurate reconstruction of phylogenetic relationships in a

*Corresponding author (email: wangwei1127@ibcas.ac.cn) plant group. If significant incongruence exists, the data should not be combined without further justification, otherwise a phylogenetic tree estimated from the combined (concatenated) data may not track or may represent an oversimplification of the evolutionary history [21,22].

Various methods have been proposed to identify incongruence between phylogenies obtained from cpDNA and nrDNA data, such as tree-based comparisons [22], the parsimony-based incongruence length difference (ILD) test [23], compare-two permutation tests [24], Templeton's test [25], and the Shimodaira-Hasegawa (SH) test [26]. Recently, van der Niet & Linder [27] described a protocol as a guide on how to analyze cpDNA and nrDNA data, which included three steps: identifying incongruence and testing its significance, assessing the cause of incongruence, and reconstructing the species tree. However, it is sometimes extremely difficult to determine the cause of incongruence, particularly to distinguish hybridization from incomplete lineage sorting [28,29], which may result in similar phylogenetic patterns. Fortunately, several novel approaches have been developed for statistically distinguishing hybridization from incomplete lineage sorting [16,29].

In this paper, we first evaluate how empirical plant phylogenetic studies usually analyze cpDNA and nrDNA data at present. Surprisingly, we found that many studies directly combined cpDNA and nrDNA data sets without rigorously assessing and/or treating data combinability. We sequentially outline a procedure for correctly analyzing cpDNA and nrDNA data and list the possible methods involved in each step of the procedure. We hope that this will help raise awareness of the importance of dealing carefully with incongruence and that it may serve as a guide to help authors facing this problem.

1 Current status of cpDNA and nrDNA data analysis in plant phylogenetics

To investigate how empirical studies usually treat incongruence between cpDNA and nrDNA data sets and to explore the possible cause of incongruence between the two, we chose the journal *Systematic Botany* and examined its published articles. As an internationally famous and reputable journal that publishes research on plant molecular phylogenetics, *Systematic Botany* was considered to be representative to some extent of the present status of cpDNA and nrDNA methodology. Given that some statistical methods for distinguishing hybridization from incomplete lineage sorting have only been published relatively recently [16,27,29], we restricted our search to the period between 2011 and 2005. The articles investigated had to fulfill the following two criteria: (i) one of the goals of the paper was to reconstruct phylogenetic relationships among members of a group or to determine the systematic position of a taxon, and (ii) the paper employed both cpDNA and nrDNA data to construct phylogenetic trees for the same taxa. A paper was excluded if the author stated that they did not combine the cpDNA and nrDNA data sets because the separate phylogenies were adequate to answer their initial questions.

With regard to data collection, if a paper contained a statement that the phylogenetic signal from cpDNA and nrDNA data was significantly incongruent, we recorded it as "incongruent" regardless of the threshold for incongruence used.

If a paper noted that an ILD test was performed in the Materials and methods, and mentioned visual inspection of the two separate bootstrap consensus trees in the Results, we recorded it as "ILD test+tree-based comparisons".

If a paper used both the ILD test and tree-based comparisons, where the ILD test indicated the cpDNA and nrDNA data were significantly incongruent but the tree-based comparisons found that incongruence was weakly supported, we recorded it as "congruent".

We identified 138 articles that combined cpDNA and nrDNA data, of which 115 (83.3%) tested for combinability and 23 (16.7%) directly combined the datasets without any test of combinability. Detailed statistics are presented in Table 1. The most commonly used method of testing for combinability was the ILD test (53/115), followed by tree-based comparisons (34/115). Among the 20 papers that used both the ILD test and tree-based comparisons, the ILD test identified significant incongruence between cpDNA and nrDNA data in 16 studies, but tree-based comparisons identified incongruence occurred in only 10 of these 16 studies.

Among the 53 studies that only used the ILD test to examine the combinability of cpDNA and nrDNA data sets, significant incongruence was identified in 16 studies, but the cpDNA and nrDNA data were still combined in 14 of these 16 studies. Tree-based comparisons identified incongruence in 15 studies, of which the cpDNA and nrDNA data were still combined in seven studies. Ten studies were indicated to be incongruent by using both the ILD test and tree-based comparisons, of which the data were still combined in seven studies.

The ILD test is the most extensively used method at present for assessing incongruence, but the threshold for incongruence (*P*-value) differs markedly among these studies, including 0.05, 0.01, 0.005, 0.002, and 0.001. Furthermore, the threshold for incongruence used in different studies for tree-based comparisons are also arbitrary, such as maximum parsimony bootstrap value (MP BS)>50%, MP BS⩾60%, MP BS⩾70%, MP BS>85%, MP BS>80% and/or posterior probabilities (PP)⩾0.95, MP BS>75% and PP>0.9, and maximum likelihood (ML) BS⩾70% or PP⩾0.95. Surprisingly, the cpDNA and nrDNA data were still combined in 29 of 46 studies in which significant incongruence was identified. The reasons usually given by the authors was that the ILD test is sensitive to differences in among-site rate variation between partitions, overall evolutionary rates, levels of noise, and the relative size of data partitions [30].

Thus, it may be problematic in that, among the clades that are in conflict between a pair of data sets, it does not differentiate between those that are weakly supported and those that potentially have different evolutionary histories. Furthermore, the ILD test may be insensitive to localized differences in the evolutionary histories of two data sets if many or several other clades are strongly supported and congruent [22]. In addition, authors also considered that total evidence could generate a phylogenetic tree with greater resolution and higher support [31–33]. Notably, statistical approaches were rarely used to explain incongruence.

2 A procedure for analyzing cpDNA and nrDNA data

It is surprising to note that many studies combining cpDNA and nrDNA DNA data sets did not rigorously assess and treat data combinability. We suggest researchers take more care in this important step. To correctly analyze cpDNA and nrDNA data, we here outline a procedure that includes the following four major steps: (i) identification of significant incongruence, (ii) determination of conflicting taxa, (iii) provision of possible interpretations for incongruence, and (iv) reconstruction of the phylogeny after treating incongruence.

2.1 Identification of significant incongruence

Tree-based comparisons can be used to visually identify incongruence between phylogenies obtained from cpDNA and nrDNA data sets [22,34]. The three most widely applied methods used in phylogenetic analyses are MP, ML and Bayesian inference (BI). The accurate alignment of each genetic region is a prerequisite for the three analytical methods. It is routine to exclude difficult-to-align regions from phylogenetic analysis. For model-based phylogenetic analysis methods (BI and ML), it is also crucial to select the best-fit model for each genetic region in the data sets. Generally, model-based phylogenetic analyses do not result in trees that substantially differ in topology from that of MP analysis [35]. In many studies, the majority of clades with MP BS⩾70% and/or ML BS⩾70% have PP>0.95 [36,37], and BS values of MP and ML analyses differ by less than 5% [36]. Given that different analysis methods are sometimes sensitive to different biases in the data set, Baum et al. [38] suggested that clades consistently supported in different analyses could be regarded as more robust than those supported strongly by one method but contradicted by a different method. At present, the majority of studies employ at least two phylogenetic analysis methods, most commonly MP and model-based methods (BI and/or ML) [27,35,37]. For tree-based comparisons, we propose the following thresholds as an indication of strongly supported incongruence between cpDNA and nrDNA data sets: MP BS⩾70% and PP⩾0.95 and/or ML⩾70%. The weakly supported clades are considered as potential conflicts [39] and need further examination by sampling additional molecular loci.

2.2 Determination of conflicting taxa in cpDNA and nrDNA trees

Given evidence of conflict, it is important to determine which taxa are involved. If only one or a few problematic taxa are involved, one can first run an MP-based ILD test with all taxa and obtain a *P*-value. The suspected problematic taxon is removed and an ILD test is re-run. If the *P*-value markedly increases, the removed taxon is considered to be in conflict between the cpDNA and nrDNA trees [36,37]. For data sets with numerous conflicting taxa, however, repeated cycles of tree comparisons, bootstrapping, pruning, and reanalysis may be impractical. In such cases, the Templeton test (also called the Wilcoxon signed-ranks (WSR) test) can be used to separately test each individual well-supported incongruent node [21]. In addition, Pelser et al. [16] designed a two-step approach to examine complex incongruence involving multiple lineages in which some also show internal incongruence. The largest mutually exclusive lineages in cpDNA and nrDNA trees are first identified by visual comparison. These lineages are then examined for the presence of strongly supported internal incongruence by evaluating branch support values and then subjecting them to ILD tests that only include the taxa of the lineage under investigation.

2.3 Provision of possible interpretations for incongruence

Incongruence between cpDNA and nrDNA gene trees may have a real or artificial basis, i.e., biological or artificial reasons. Artificial reasons can be easily identified, whereas biological reasons are usually complicated and need to be carefully inferred.

With failure to reconstruct the correct cpDNA or nrDNA trees, artificial reasons are responsible for incongruence, such as laboratory errors, long-branch attraction, and evolutionary saturation.

Laboratory errors can generate incorrect sequences and thereby result in incongruence. The simultaneous processing of multiple samples at one laboratory table can lead to contamination of the samples (personal observation). To identify potential contaminants, sequences from each gene can be initially analyzed using MP and/or subjected to a BLAST search against the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). If different species have identical sequences, contamination may have occurred [40].

Incongruence can also be caused by long-branch attraction in one of the two data sets [27], although a long branch observed on a phylogenetic tree may not always obscure phylogenetic signal [41]. The taxa having potential long-branch problems can first be identified by visual inspection of the phylograms with strongly incongruent lineages [16], or by using relative apparent synapomorphy analysis (RASA; http://test1.bio.psu.edu/LW/list.htm). The taxa subject to long-branch attraction are then removed and the data set is reanalyzed. If the arrangement of other taxa in the tree is changed, this indicates that long-branch attraction has occurred [42]. Increasing the number of characters may overcome some of the problems due to long-branch attraction [43]. The cpDNA and nrDNA data sets can subsequently be combined if the incongruence is caused by long-branch attraction in individual data sets. In addition, MP analysis is sensitive to long-branch attraction [44–46], whereas ML and BI analyses that implement appropriate substitution model(s) are able to largely overcome the problem [41,46,47].

When substitution rates are particularly high, phylogenetic signal may be decreased because of multiple changes that mask evolutionary history and create homoplasy [48], which may result in phylogenetic incongruence. Given that such saturation is apparent only for comparisons of highly divergent taxa, such incongruence is confined primarily to early-diverging taxa in the phylogeny and is not caused by different phylogenetic histories. In this case, data sets can subsequently be combined [49].

When cpDNA and nrDNA data may have different underlying phylogenetic histories, one must use biological reasons to explain incongruence, such as paralogy, hybridization, and incomplete lineage sorting.

If a data set includes paralogous gene copies, the actual phylogeny will partly reflect the duplication history of the gene, which is incongruent with the species divergence history. Plastid DNA markers usually lack paralogous copies because the plastid genome is a single, non-recombining locus [50–52]. Paralogy is usual for multiple-copy nuclear loci. In cases where PCR products of nuclear loci form more than one band, or double peaks or ambiguous base calls are observed in electropherograms, cloning should be attempted. Pseudogenes observed in nrDNA sequences of some taxa should first be identified and then excluded from phylogenetic analyses. Pseudogenes can be detected by searching the well-defined conserved sequence motifs and by pairwise comparison of substitution rates and need to be deleted from phylogenetic analyses [53]. Subsequently, phylogenetic analyses can be combined to obtain a preliminary tree using all sequences. If several clones of one accession occur in the same clade, one representative clone can be selected [20] or a consensus sequence is generated in which polymorphisms are coded as ambiguous characters, and this consensus sequence is used for subsequent phylogenetic analyses [16,54]. The distribution of several clones of one accession in different clades results in paralogy.

Introgression is the result of repeated backcrossing of a hybrid with one or both of its parents. Incomplete lineage sorting can occur when an ancestral species undergoes several speciation events within a short period of time [22]. If, for a given gene, the ancestral polymorphism is not fully resolved into two clades when the second speciation event takes place, the gene tree will likely differ from the species tree [55,56]. Incomplete lineage sorting is notorious for producing patterns similar to those caused by hybridization and introgression. Several methods have been proposed to distinguish between hybridization and incomplete lineage sorting:

(i) Morphological intermediacy. Hybrids contain a combination of different genotypes and accordingly may display some phenotypic traits that are intermediate between their parental taxa [57]. Thus, morphological intermediacy is widely used as evidence for hybridization [36,58,59]. However, intermediate characters can also arise from convergent morphological evolution or from the existence of ancestral populations from which the two species diverged [60]. Moreover, gene expression in hybrid genotypes may be complicated and the resulting phenotypes may show intermediacy, resemble either parental species, or even exhibit novel character states. Morphology alone can therefore be misleading as evidence of hybridization, as has been demonstrated in recent molecular studies [61,62]. Morphological evidence for hybridization represents only a probable hypothesis. Nevertheless, if the hybrid nature of a species is supported by artificial and/or field experiments, this can be considered as robust evidence for hybridization [39,63,64].

(ii) Comparison of distribution, phenology, and habitats. From inspection of cpDNA and nrDNA trees, one can identify two hypothetical parents if hybridization is responsible for incongruence. Plastid capture is much more likely to take place than nuclear capture, owing to maternal inheritance [65,66], lack of linkage relative to nuclear gene selection [67], and smaller effective population size because of clonal inheritance [68]. If the putative hybrid grows sympatrically with its hypothetical mother, hybridization can be considered to be a factor [53,63]. This is, however, not always the case, as has been shown in *Cornus* [69]. Instead, phenology and habitat can be used to identify possible hybridization events. For example, *Cornus eydeana* QY Xiang & YM Shui was sister to the *Cornus mas* L.-*Cornus officinalis* Seib. & Zucc. clade in the cpDNA tree, whereas *Cornus eydeana* and *Cornus chinensis* Wangerin formed a clade in the nrDNA tree; Xiang et al. [69] hypothesized that the conflict in the position of *Cornus eydeana* in the cpDNA and nrDNA trees was due to cpDNA lineage sorting because the flowering time and habitats of *Cornus eydeana* and the *Cornus mas*-*Cornus officinalis* clade are non-overlapping, although both are distributed in eastern Asia. However, dispersal in combination with extinction in the parental distribution area, and/or novel phenology or a novel habitat may invalidate this approach.

(iii) Counting the minimum number of evolution events. Lineage sorting can be distinguished from hybridization by comparing the minimum numbers of evolution events presumed necessary to attain the observed pattern of incongruence [27]. For lineage sorting, the minimum number of multiple lineages that survive through any particular branch segment of a tree has been assessed using GeneTree [70,71]. For hybridization, the minimum number of dispersal and/or extinction events needs to be postulated for probable hybridization between putative parental ancestors based on their present distribution ranges.

(iv) Minimum genetic distance. If incomplete lineage sorting is responsible for incongruence, the similar sequences will have coalesced before the speciation event. If hybridization is responsible for incongruence, the similar sequences from different species could have coalesced either before or after the speciation event. Joly et al. [29] describe a parametric approach for statistically distinguishing some hybridization events from incomplete lineage sorting scenarios based on minimum genetic distances.

(v) Coalescence-based methods. Based on coalescent theory, ancestral polymorphisms are likely to coalesce within approximately 5 N_e generations (N_e being the effective population size) [72,73]. Thus, congruence between gene trees and species trees is highly probable. If incongruence is to be explained by incomplete lineage sorting, one can calculate the assumed minimum N_e. If the assumed N_e is much higher than that observed in nature, then incomplete lineage sorting can be excluded, and hybridization is supported as the most likely explanation for the observed incongruence [16].

2.4 Reconstruction of the phylogeny after treating incongruence

After conflicting taxa are identified and possible interpretations for incongruence are given, taxa responsible for the conflict are usually removed before the combined analysis of cpDNA and nrDNA data sets is carried out [36,37,74]. This method may be problematic, however, in that the placement of the conflicting taxa cannot be indicated in the larger tree [22]. If hybridization is responsible for the incongruence, van der Niet & Linder grafted subsequently the incongruent taxa onto the tree obtained from the combined analysis [27], whereas Pelser et al. performed the combined analysis of cpDNA and nrDNA data sets by recoding the incongruent taxa twice: once as a cpDNA-only accession (nrDNA characters were scored as missing) and once as a nrDNA-only accession (cpDNA characters were scored as missing) [16]. If nrDNA lineage sorting is responsible for the incongruence, the combined analysis of cpDNA and nrDNA data sets can be carried out by recoding nrDNA characters as missing.

3 Conclusion

If cpDNA and nrDNA data do indeed reflect different evolutionary histories, their data sets may result in different topologies, and a phylogenetic tree estimated from the simple combined data set would produce an incorrect estimate of the phylogeny or may sometimes represent an oversimplified version of the genetic history. To more accurately reconstruct the phylogeny and evolutionary history of plant groups, the combined analysis of cpDNA and nrDNA data sets must be done with caution, and if incongruence between the data sets exists, its possible causes should be addressed in detail.

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