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ASSESSING EVOLUTION AND BIODIVERSITY IN PLANTS AT THE MOLECULAR LEVEL

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ABSTRACT
DNA sequencing and fingerprinting methods for the analysis of plant evolution and diversity have been in use for over two decades. Thousands of research reports have been published which have provided insight not only into the evolutionary pathways of plant species but also into the evolution of several genetic loci. This review highlights several articles that have made use of nuclear and chloroplast loci to investigate plant phylogeny and the evolutionary processes that have led to the current distribution and diversity of plants.

INTRODUCTION
Biodiversity can most simply be defined as the variation that exists in the living world. On a planetary scale this variation is evident from an examination of the Earth's biomes and biogeographic realms. At the other extreme, variability can also be found within a sequence of DNA which is, of course, the fundamental source of all biodiversity.

The science of taxonomy has been developed to describe, identify, and classify the differences between organisms. From the time of Linnaeus, taxonomists have used the physical attributes of organisms to sort them into similar groups. As evolutionary theory developed, scientists attempted to reconstruct their evolutionary histories, and the science of systematics was born. However, because of the complex (and often cryptic) nature of evolution, the diversity of life could not always be neatly arranged into tidy phylogenies. The "Tree of Life" we have assembled still has many frayed, broken and missing branches.

When methods of DNA analysis – hybridization, amplification, fingerprinting and sequencing – became widely available in the 1980's, scientists were quick to apply these sensitive techniques to the study of systematics, especially to the difficult cases where physical comparisons alone provided insufficient or conflicting data unsuitable for the construction of conclusive phylogenies. Over the past 25 years, the number of studies using DNA analysis to clarify evolutionary relationships and classify species has proliferated. Genetic analysis is now an indispensable tool for systematists.

In recent years there has been the realization that DNA analysis, i.e., molecular systematics, does not always provide clean cut answers to every problem. The *gene tree* (the phylogeny estimated by DNA analysis) does not always match the *species tree* (the true phylogeny of organisms) (Lyons-Weiler and Milinkovitch 1997). The potential reasons for this incongruence are many, but the most common ones are caused by the evolutionary processes of rapid diversification, hybridization, introgression, incomplete lineage sorting, and inter-locus concerted evolution (Comes and Abbot 2001). In other words, not all genetic changes reflect the same type of evolutionary divergence that is measured by morphological characters.

No longer can only one genetic locus of a taxonomic group be sequenced and the reconstructed phylogenetic tree be assumed to reflect the true species tree. In response to this challenge, the relatively new field of *phylogeography* has been developed. This discipline, first conceived by John Avise (1986), takes into account not only standard molecular systematics, but also population genetics and the biogeographical history of organisms – the latter included because migration, reproductive isolation, pollen flow and seed dispersal are so dependent on geographic features. The past 10 years, in particular, has seen a tremendous number of phylogeographical studies published, especially in plants.

An in-depth review of the plant molecular systematics and phylogeography literature is beyond the scope of this paper. Instead, I will summarize representative studies which show the utility of DNA analysis in the assessment of plant classification, biodiversity and evolution. I hope to demonstrate the power of these methods – especially at the level of species and population genetics – in resolving taxonomic controversies, uncovering hidden diversity, and clarifying evolutionary mechanisms. But first, it is necessary to briefly review some basic theoretical and practical aspects of molecular systematics.

THE CONCEPT OF SPECIES

It is not coincidental that the world's biodiversity hotspots are also the centers of evolution for numerous species. Evolution produces biodiversity and, in turn, a more diverse biological environment creates more selection pressures which drive evolution.

The process of evolution is measured by the formation of new species (and the loss of old ones), and biodiversity is often measured by determining the number of species per unit area. Therefore, in order to get an accurate assessment of either for the purposes of conservation and management, it is of paramount importance that the term *species* be considered and methods devised to differentiate between species.

One would think that a century and a half after Darwin's 1859 publication of *On the Origin of Species* that there would be a consensus among biologists on what actually constitutes a species. However, there are a few dozen competing theoretical concepts of species (Mayden 1997) based on different reproductive, ecological, evolutionary and phylogenetic principals (de Queiroz 2007).

When two species have *fully* diverged from a common ancestor they will possess the properties commonly associated with independent species: reproductive incompatibility, distinctive morphology, reciprocal monophyly, and ecological uniqueness. But during the process of divergence, these properties are gradually acquired in a continuum spanning thousands of years. Therefore, when two lineages are in the *early* stages of speciation it is difficult for biologists holding different species concepts to agree on when there has been enough divergence to declare them as different species.

DNA analysis has become a tremendously useful tool in helping systematists differentiate between and declare new plant species, characterize the evolutionary relationships between lineages, and even identify the early stages of speciation. Surprisingly, much of the research in this area has been accomplished by studying only a few regions of the plant genome.

WHERE IS GENETIC VARIATION FOUND IN PLANT DNA?

The smallest known haploid genome of a flowering plant still contains over 63 million base pairs of DNA, and some contain over 100 billion base pairs. So how does one decide which part of the genome (i.e., which *locus*) to examine when searching for genetic variation?

Regulatory and coding sequences tend to be highly conserved and will generally only show variability when comparing plants belonging to different genera, families, orders, classes and divisions – with more sequence variation apparent at the higher taxonomic levels. Examples of these loci include the *rbc*L chloroplast gene and the nuclear 28S rDNA gene. However, major controversies regarding plant classification above the family level are uncommon where enough morphological differences exist to place plants in the proper categories.

The greatest controversies in plant systematics occur at the lower taxonomic levels (genus and species) where morphological similarities can be so great as to confound proper classification. On the scale of geological time, these hierarchical units have only recently diverged from common ancestors and their DNA has had much less time to accumulate mutations. Therefore, when comparing plants at these levels it is necessary to study loci that can amass mutations very rapidly without having a deleterious effect on the organism. Formerly known as "junk" DNA, these regions are interspersed between the conserved structural genes.

The internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA), are non-structural in nature and have much higher mutation rates relative to the 18S, 5.8S and 28S rDNA genes which they separate (Fig. 1). These ITS regions are generally conserved within a species but show enough variation between species and genera to be useful in the construction of phylogenetic trees. These two loci, usually amplified and sequenced together along with the small 5.8S rDNA region, have been tremendously helpful in delimiting plant taxa.

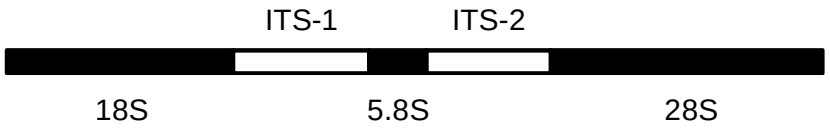

FIGURE 1. Diagram of the ribosomal DNA region in plants. The entire region is transcribed into precursor RNA which is then processed into 18S, 5.8S and 28S ribosomal RNA molecules by removal of the internal transcribed spacers (ITS-1 and ITS-2). Since the ITS sequences do not encode functional RNA, they accumulate mutations at a higher rate than most other regions of DNA and are therefore useful in assessing divergence between species. See Blattner (1999) for PCR primers that will amplify one or both ITS sequences.

Chloroplast DNA (cpDNA) is commonly used in studies assessing variation between populations, species, genera and sometimes even higher taxonomic levels. One of the most frequently used cpDNA locus for lower taxonomic comparisons is the intergenic spacer (IGS) found between the *trn*L and *trn*F genes which encode transfer RNAs for leucine and phenylalanine, respectively (Fig. 2). Interrupting the two *trn*L exons is an intron sequence that also shows significant sequence variation. Another chloroplast IGS locus used for phylogenetic assessment is found between the *rbc*L and *acc*D genes (Fig. 3). Since the chloroplast genome is haploid, unique sequences of cpDNA are referred to as *haplotypes*. Chloroplast haplotypes are usually inherited through the female in plants, although paternal inheritance (through pollen) is the norm in gymnosperms.

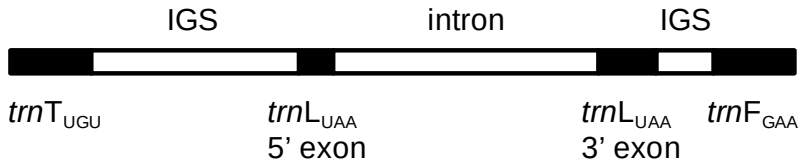

FIGURE 2. Diagram of the *trn*T-F region of chloroplast DNA. The transfer RNA (*trn*) genes are conserved while the *trn*T-L and *trn*L-F intergenic spacers (IGS) and the *trn*L intron are non-structural and therefore have more variable sequences that are phylogenetically useful. See Taberlet *et al.* (1991) for PCR primers that will amplify various sequences in the *trn*T-F region and Quandt *et al.* (2004) for a review of these loci.

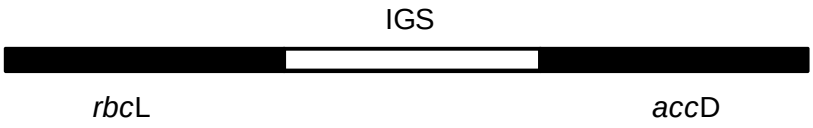

FIGURE 3. Digram of the *rbc*L-*acc*D gene region of chloroplast DNA including the intergenic spacer (IGS). The *rbc*L gene sequence has been used to differentiate between genera within families while the IGS region has been more useful for population and phylogeographical studies.

When analyzing intra-specific genetic variation, i.e., within a population or between populations of a species, another type of variable region, called a microsatellite, is often studied (Fig. 4). Microsatellites, also referred to as Short Tandem Repeats (STRs), provide the highest resolution since their alleles can mutate from one generation to the next. Because of their abundance in nuclear DNA and the fact that they are inherited in a Mendelian fashion, microsatellite analysis (a type of DNA fingerprinting) is useful in determining kinship and gene flow and is most powerful when applied at the intra-specific level.

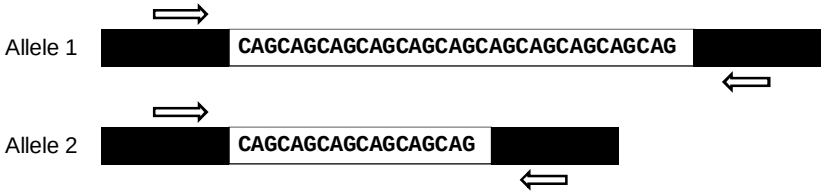

FIGURE 4. Diagram of two alleles at a hypothetical microsatellite locus. Due to replication errors by DNA polymerase during meiotic division, short tandem repeat sequences (such as this CAG trinucleotide region) can become shorter or longer. In this example, the individual is heterozygous, having a $(CAG)_{11}$ allele and a $(CAG)_6$ allele. PCR amplification using primers complementary to conserved flanking sequences (arrows) will produce products that differ in size by 15 base pairs. The large number of microsatellite loci and abundance of Mendelian inherited alleles make these loci ideal for studying biodiversity and gene flow in populations. See Weising *et al.* (2005) for a review of microsatellite analysis in plants.

The loci described above are by no means the only variable regions used to investigate plant biodiversity and evolution at the genetic level, but they are used in the vast majority of cases and will serve as good examples for the purposes of this review. For excellent reviews of how to

choose plant loci for specific phylogenetic goals see Soltis and Soltis (1998) and Ouborg *et al.* (1999).

MOLECULAR SYSTEMATICS AND PLANT CLASSIFICATION

There are several examples in the literature where DNA analysis has led to the resolution of long-standing controversies in plant classification.

Brunsfeld *et al.* (1994) showed that species in the Taxodiaceae (redwood) family had only minor sequence differences in their chloroplast *rbc*L genes compared to species in the Cupressaceae (cypress) family. The result of this study led to the unusual abolishment of a family designation and the incorporation of most Taxodiaceae species into Cupressaceae. Although unpopular with redwood loyalists, many taxonomists had been calling for this revision for years based on certain morphological and developmental similarities between the two groups, but it was the DNA analysis that finally convinced the botanical community to accept the revision.

The buckwheat family (Polygonaceae) is widely recognized as a monophyletic group, but the membership of genera within its two subfamilies (Erigonoideae and Polygonoideae) has been controversial due to incongruencies in morphological character phylogenies. Sequence data from the chloroplast genes *rbc*L, *mat*K and *ndh*F helped to redefine the tribes and genera within these subfamilies (Lamb Frye and Kron 2003; Sanchez and Kron 2008).

By far, the nuclear ITS regions have proven most useful in deducing the correct phylogenies of plant groups. An ITS-derived phylogeny resolved the evolutionary relationships between genera in the Nyctagineae tribe of Nyctaginaceae while the *rbc*L-*acc*D IGS sequence provided relative few informative sites (Levin 2000). A highly resolved phylogeny of 115 species of the widespread genus *Astragalus* was constructed from ITS sequence data and showed a distinct monophyly of New World *Astragalus* species (Wojchiechowski *et al.* 1999).

Although ITS sequences are most useful in resolving taxa at the level of genus and species, Kawase *et al.* (2007) found an unusually high number of ITS sequence types in subspecies and varieties of *Erigeron thunbergii* in Japan. These sequence types separated into two clades with Clade I originating from Kamchatka Peninsula and Clade II from the Chinese mainland. They also found a correlation between ITS sequence type and plants found growing in serpentine soils. In addition, the investigators looked at the chloroplast *trn*L-F region but mainly found indels (insertions and deletions) which are less phylogenetically informative than nucleotide substitutions.

The subsection *Pungentes* within the genus *Chorizanthe* (Polygonaceae) is composed of seven species (Reveal and Hardham 1989). However, a recent study by this author's laboratory (Brinegar and Baron 2008) using ITS sequence data, showed that *C. diffusa* had a considerable sequence divergence relative to the other six species and should be removed from the subsection. Furthermore, the ITS phylogeny of the four varieties in the *C. pungens-C. robusta* complex indicated that all varieties might need to be reclassified within *C. pungens* with the *C. robusta* species designation abolished.

DISCORDANCE BETWEEN NUCLEAR AND PLASTID PHYLOGENIES

In some of the previous examples chloroplast DNA showed very little variation between taxa – not enough to provide a well resolved phylogeny. Low genetic differentiation of organelle DNAs between closely related species is not unusual (Wolf *et al.* 1997; Tsumura and Suyama, 1998; Domulin-Lapegue *et al.* 1999). However, even when a chloroplast locus shows significant

sequence variation, it may not provide a similar gene trees as a nuclear locus. In fact, such discordance between nuclear and plastid phylogenies is fairly common.
One of the most common reasons for this discordance is the phenomenon of incomplete lineage sorting. Ancestral chloroplast sequence haplotypes, sometimes more than a million years old, can survive in descendents through speciation events so that different species of a genus may carry the same haplotype. In a section of the genus *Senecio* where 502 individuals of 18 species were analyzed, only a few *trn*K haplotypes were found to be species specific (Comes and Abbott 2001). In their *trn*L-F analysis of 875 individuals in 31 species of *Hordeum*, Jakob and Blattner (2006) found up to 18 haplotypes per single species with some haplotypes estimated to have survived for 4 million years. In both cases, chloroplast DNA phylogenies were determined to be unreliable compared to species trees estimated by ITS sequence data.

However, according to a modeling study by Maddison and Knowles (2006), by choosing the appropriate number of loci to examine *and* the appropriate number of individuals to analyze, one can still make reasonable phylogenetic inferences even in the presence of significant incomplete lineage sorting. In fact, it is unreasonable to expect reciprocal monophyly between recently derived species since another modeling study showed that it can take up to a million years after divergence before reciprocal monophyly is reached at 15 loci (Knowles and Carstens 2007).

Another evolutionary process that confounds phylogenetic analysis in plants is chloroplast capture. Hybridization between two closely related species followed by repeated backcrossing of the hybrid with one of those species can result in the "capture" of another species' chloroplast genome. This mechanism was suggested to be responsible for several points of conflict between chloroplast restriction site data and phylogenies constructed from ITS, allozyme and morphological data in the *Heuchera* group (Saxifragaceae) (Soltis and Kuzoff 1995).

In many situations it is not possible to determine the exact cause of nuclear and chloroplast phylogeny discordance. Sometimes, however, the fact that there are conflicting molecular data can often help resolve taxonomic controversies. Kimball *et al.* (2003), in their phylogenetic study of *Coreocarpus* and closely related genera, noted some incongruencies between their nuclear (ITS) and chloroplast (*rpl*16) trees. Although they could not differentiate between lineage sorting or chloroplast capture as the cause, they concluded that *C. hintonii* and *C. cronquistii* probably belonged in the genus *Bidens*.

PHYLOGEOGRAPHY AND GLACIAL REFUGIA

Plant phylogeographers have made good use of chloroplast DNA loci to study the evolutionary and biogeographical forces that have resulted in the current distribution and diversity of plant species. Since the maternal inheritance of organellar DNA, such as the chloroplast genome, can be affected by population size, isolation and migration (Hoelzer *et al.* 1998), chloroplast DNA loci are obvious choices for phylogeographic studies – although the results are not always straightforward due to some of the confounding mechanisms mentioned in the previous section.

The last glacial period had a tremendous impact on the distribution of plant species in the northern latitudes of the Northern Hemisphere. There have been many phylogeographical investigations which have attempted to identify glacial refugia for particular species and then reconstruct post-glacial recolonization. The study of Fjellheim *et al.* (2006) was concerned with the question of whether meadow fescue (*Festuca pratensis* Huds.) recolonized Europe naturally from southern glacial refugia or was spread by agriculture from the Fertile Crescent like perennial ryegrass (*Lolium perrene* L.) (Balfourier *et al.* 2000). Their results, from three chloroplast DNA loci, indicated that a Western European haplotype had its origin from the northward post-glacial expansion from an Iberian refugium while two Eastern European haplotypes were derived from

southern and eastern refugia. Agriculture appeared not to play a role in the recent expansion of this species.

Species of ivy (*Hedera*) showed similar patterns in that they survived the glacial period in the southern refugia of Spain and the Balkans (Grivet and Petit 2002). The diversity of chloroplast haplotypes decreased from southern to northern Europe indicating that there was a leading edge effect of a few haplotypes which were more effective in recolonization. This study showed that 13 *trn*L intron haplotypes were shared across species, indicating once again that incomplete lineage sorting is common in organellar inheritance.

Glaciation also changed the distribution of plant life in North America. The forest understory plant *Trillium cuneatum* retreated to six non-overlapping refugia in the Southeastern part of the United States as detected by haplotype analysis of the *trn*L intron and *trn*L-F IGS regions (Gonzales *et al.* 2007). One haplotype was found only to the west of the Appalachian Mountains and appeared to be a separate lineage compared to the other eastern haplotypes.

Soltis *et al.* (1997) were interested in the post-glacial history of plant species in the Pacific Northwest and found many inter-species similarities in the geographical distribution of chloroplast DNA haplotypes. However, they were unable to discern whether the recolonization originated from separate northern and southern refugia or from a southern refugium with a leading edge effect from a dominant haplotype.

MICROSATELLITE ANALYSIS OF SPECIES AND POPULATIONS

Owing to their high mutation rates and overlapping size classes, microsatellite loci are generally not used for phylogenetic studies. However, chloroplast microsatellites may mutate at lower rates than nuclear microsatellites and therefore be more useful in distinguishing between taxa at the species and varietal levels. Bucci *et al.* (1998) and Gugerli *et al.* (2001) were able to get phylogenetic resolution between several species of European pines (*Pinus*) using chloroplast microsatellite analysis.

Chloroplast microsatellites have found more utility in the assessment of genetic diversity within and among populations. Seven populations covering the entire range of the endangered Brazilian plant *Caesalpinia echinata* were analyzed at seven chloroplast microsatellite loci (Lira *et al.* 2003). Isolation and genetic drift resulted in the fixation of haplotypes in five of those populations. A chloroplast microsatellite locus was used to compare haplotype frequencies between redwood (*Sequoia sempervirens*) populations in the southern and northern parts of their range (Brinegar *et al.* 2007). Southern populations had less genetic diversity, perhaps due to more of a dependence on clonal rather than sexual reproduction in the drier part of the range.

Nuclear microsatellites, being biparentally inherited and codominant, are ideal single-locus markers for population genetics, gene flow, hybridization, introgression, breeding and cultivar identification studies. Bredemeijer *et al.* (1998) developed a semi-automated detection method for tomato cultivar identification using microsatellite markers. Pollen dispersal was found to be more than seven times greater over pasture land compared to undisturbed forest in the tropical tree *Dizinia excels*a (Dick *et al.* 2003). Comparison of microsatellite loci of the sunflower species *Helianthus deserticola* with its presumptive parents provided strong evidence of its hybrid origin (Gross *et al.* 2003). Genetic maps of nuclear microsatellite loci in some of the world's most important crops, such as wheat (Roder *et al.* 1998), have proved to be extremely useful in breeding programs.

As valuable and versatile as microsatellites are, developing a panel of microsatellite loci can be a long and expensive proposition (Squirrel *et al.* 2003). Genomic libraries must be constructed and probed using hybridization methods. Positive clones are then confirmed by sequencing. PCR primers must then be designed and tested on a population of individuals to determine if there is sufficient variability at those loci to make them useful. Attrition is significant at each of these steps, and it is not unusual to screen tens of thousands of clones in order to obtain only a few useful microsatellite markers. Despite these challenges, microsatellites are gaining in popularity due to their tremendous resolving power in distinguishing between individual plants.

CONCLUSIONS

This review has considered the contributions of single-locus DNA sequencing and fingerprinting to the fields of plant phylogenetics, phylogeography, population genetics and breeding. There is an equally large set of literature dedicated to multi-locus techniques such as Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), and Inter-simple Sequence Repeat (ISSR) which I have not been able to touch on. For an introduction to those methods see Weising *et al.* (2005).

As more and more examples appear in the literature, it becomes obvious that multiple evolutionary processes can have variable effects on the loci we choose to analyze for phylogenetic inference or population studies. However, by analyzing more loci, more individuals and more populations, the evolutionary secrets locked in the genomes of plant species begin to unveil themselves. Although the loci we have used for years have served us well, there is a need to develop new molecular markers such as the nuclear long-terminal repeat (LTR) retrotransposons of Cornman and Arnold (2007). There is no doubt, with the advent of DNA microarray technology and whole genome sequencing, that the near future will bring even more tools which we can use to investigate the evolutionary histories and biodiversity of plants.

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