Molecular Phylogenetics and Evolution 99 (2016) 16–33

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

The quest to resolve recent radiations: Plastid phylogenomics of extinct and endangered Hawaiian endemic mints (Lamiaceae) ☆

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ARTICLE INFO

Article history:
Received 30 November 2015
Revised 26 February 2016
Accepted 28 February 2016
Available online 5 March 2016

Keywords:
Hawaii
Lamiaceae
Phylogenomics
Plastid genomes
Radiation

ABSTRACT

The Hawaiian mints (Lamiaceae), one of the largest endemic plant lineages in the archipelago, provide an excellent system to study rapid diversification of a lineage with a remote, likely paleohybrid origin. Since their divergence from New World mints 4–5 million years ago the members of this lineage have diversified greatly and represent a remarkable array of vegetative and reproductive phenotypes. Today many members of this group are endangered or already extinct, and molecular phylogenetic work relies largely on herbarium samples collected during the last century. So far a gene-by-gene approach has been utilized, but the recent radiation of the Hawaiian mints has resulted in minimal sequence divergence and hence poor phylogenetic resolution. In our quest to trace the reticulate evolutionary history of the lineage, a resolved maternal phylogeny is necessary. We applied a high-throughput approach to sequence 12 complete or nearly complete plastid genomes from multiple Hawaiian mint species and relatives, including extinct and rare taxa. We also targeted 108 hypervariable regions from throughout the chloroplast genomes in nearly all of the remaining Hawaiian species, and relatives, using a next-generation amplicon sequencing approach. This procedure generated ~20 Kb of sequence data for each taxon and considerably increased the total number of variable sites over previous analyses. Our results demonstrate the potential of high-throughput sequencing of historic material for evolutionary studies in rapidly evolving lineages. Our study, however, also highlights the challenges of resolving relationships within recent radiations even at the genomic level.

1. Introduction

While molecular phylogenetic work over the last few decades has addressed relationships within and among major organismal groups, an on-going challenge is how to approach more recent evolutionary radiations. In particular, phylogenetic inference poses considerable limitations for resolving reticulate, recent relationships that may not exhibit a bifurcating, hierarchical evolutionary history. Remote island archipelagoes serve as a "natural laboratory" for studies of such recent evolutionary radiations. The isolation of these islands (both from the mainland and from one another), plus the wealth of different habitats available, provides ideal settings for rapid diversification and adaptation (Emerson, 2002). The Hawaiian archipelago, which lies greater than 3700 km from the nearest continental landmass, provides several famous examples of organismal radiations, including *Drosophila* (Kambysellis et al., 1995), spiders (Gillespie et al., 1994), birds (Lerner et al., 2011), and plants, such as the silversword alliance (Witter and Carr, 1988), and lobelioids (Givnish et al., 2013).

Another example is that of the endemic Hawaiian mints (Lamiaceae), one of the most species-rich plant lineages in the archipelago (Baldwin and Wagner, 2010). The ancestor of this monophyletic lineage arrived in the Hawaiian Islands 4–5 million years ago and rapidly diversified into three genera: *Haplostachys*, *Phyllostegia*, and *Stenogyne* (Roy et al., 2013). The 59 extant members of this lineage exhibit remarkable phenotypical and ecological diversity. For example, *Haplostachys* have fragrant white flowers associated with an insect-pollination syndrome and produce dry fruits, whereas both *Phyllostegia* and *Stenogyne* produce fleshy fruits, and *Stenogyne* have flowers associated with bird pollination.

☆ This paper was edited by the Associate Editor Stefanie M. Ickert-Bond.

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http://dx.doi.org/10.1016/j.ympev.2016.02.024
1055-7903/

Species are often endemic to one or two islands and can be found in a variety of habitats, ranging from xeric shrubland to wet forests, and at a wide range of elevations (Lindqvist and Albert, 2002). Similar to many other island endemics, however, many Hawaiian mint species have been impacted by anthropogenic influences. At least 13 species are extinct, and the majority of the extant taxa are threatened or critically imperiled, with several species having fewer than 100 individuals remaining (Wagner et al., 1999). Taxonomic knowledge for the majority of the species in this lineage comes from collections in herbaria and botanical gardens, and these collections represent primary sources of material in phylogenetic analyses of their evolutionary history (Lindqvist and Albert, 2002; Lindqvist et al., 2003).

Recent molecular phylogenetic work in the subfamily Lamioideae has established the phylogenetic position of the Hawaiian lineage within tribe Stachydeae, a largely heterogeneous tribe spanning a wide array of morphological, cytological, and biogeographical diversity comprising of at least 12 genera, including the subcosmopolitan genus *Stachys* (Bendiksby et al., 2011; Lindqvist and Albert, 2002; Scheen et al., 2010). The Hawaiian genera are nested within a strongly supported lineage predominantly composed of New World *Stachys*. Intriguingly, molecular phylogenetic studies show that while chloroplast DNA (cpDNA) sequence data group the monophyletic Hawaiian mints with Meso-South American (SA) *Stachys* (Fig. 1a), nuclear ribosomal DNA (nrDNA) sequences suggest that they are most closely related to temperate North American (NA) *Stachys* (Fig. 1b) (Lindqvist and Albert, 2002; Lindqvist et al., 2003; Roy et al., 2013). Interestingly, the Californian-Pacific northwestern species *Stachys chamissonis* is closely related to the Hawaiian clade in both cases. This cyto-nuclear phylogenetic incongruence is corroborated with low-copy nuclear loci that indicate a polyphyletic placement of the Hawaiian species, with some members grouping with temperate NA *Stachys* and others grouping with Meso-SA *Stachys* (Fig. 1c) (Roy et al., 2015). These results, in combination with karyotypic and morphological data and multi-locus coalescence species tree reconstruction, convey a scenario for the Hawaiian mint lineage that is best explained by a hybrid origin involving at least two separate American *Stachys* lineages, descendants of which colonized the Hawaiian Islands (Lindqvist and Albert, 2002; Roy et al., 2013, 2015). A number of other Hawaiian angiosperm radiations of American origin appear to have a hybrid ancestry, which may have been an important factor in insular colonizations and diversification of species-rich lineages (Baldwin and Wagner, 2010).

Paradoxically, in contrast to their extensive morphological and ecological diversity, DNA sequence variation among radiating species is often remarkably low. Phylogenies of the Hawaiian mints have been reconstructed from datasets composed of commonly used DNA sequence markers, including cpDNA and nuclear ribosomal 5S non-transcribed spacer and external transcribed spacer regions (Lindqvist and Albert, 2002; Roy et al., 2013), which are rapidly evolving in many other species (Baldwin and Markos, 1998; Shaw et al., 2005; Small et al., 1998), but they fail to resolve relationships among the Hawaiian mints. A similar pattern of low genetic diversity has been noted in other recent radiations of Hawaiian plants (Appelhans et al., 2014; Baldwin and Sanderson, 1998; Cronk et al., 2005; Givnish et al., 2009, 2013). Given this low diversity in recent radiations, large amounts of data are clearly required to increase resolution. Moreover, to resolve reticulate relationships, robust phylogenies based on both uniparental and biparental markers are required. Since standard cpDNA markers commonly used in plant molecular phylogenetics are insufficient to trace the ancestry of the Hawaiian lineage, we undertook a phylogenomic approach.

With the development of next-generation sequencing technologies (NGS) it is now possible to generate large amounts of sequence data for non-model taxa (Ekblom and Galindo, 2011). Sequences of complete chloroplast genomes are now becoming available for many contemporary species (Moore et al., 2006), and phylogenetic analyses of complete chloroplast genomes have been found to substantially increase resolution in taxa that have undergone rapid radiations (Parks et al., 2009), bypassing previous trade-offs between the number of taxa and the number of nucleotides utilized (Martin et al., 2005; Soltis et al., 2004). However, while generating large amounts of data has become relatively easy, sequencing and assembling complete chloroplast genomes, particularly from historical and ancient material, can still be challenging. DNA obtained from herbarium samples is highly degraded (Staats et al., 2011; Weiss et al., 2015). While its fragmented nature is well suited for the relatively short read lengths produced by second generation sequencing technologies (Knapp and Hofreiter, 2010; Staats et al., 2013), it can be difficult to work with. For example, it has been demonstrated that the decay rate in herbarium samples is about ten times faster than the rate in bones (Weiss et al., 2015), and that 96–99% of endogenous DNA may be lost during long term tissue storage (Staats et al., 2011). In addition, ancient material often contains DNA from environmental contaminants with genomic sequencing typically limited to low fractions of endogenous DNA. For example, in 70 year-old herbarium material, less than 5% of reads were from the target species (Staats et al., 2013). This can complicate high quality assembly of complete genomes, unless extensive sequencing is conducted to recover as much of the endogenous genome as possible. Furthermore, mapping reads to a reference genome can be complicated by divergence between the reference and species of interest (Ruffalo et al., 2011). *De novo* assembly, on the other hand, can be time consuming and computationally intensive (Miller et al., 2010a), and may provide only limited success for herbarium samples (Staats et al., 2013). Comparative, reference-guided assemblers (Ratan, 2009) take an intermediate strategy, and can provide a powerful approach for taxa like the Hawaiian mints, for which no close reference sequence is available.

The aims of this study were to (1) test the application of NGS to Hawaiian mint herbarium material and sequence the first complete chloroplast genome of an Hawaiian mint to facilitate resequencing and assembly of additional chloroplast genomes from

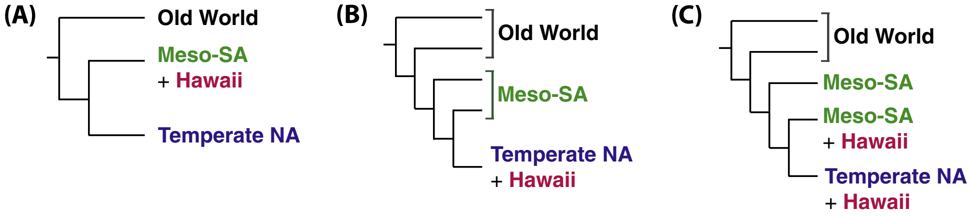

Fig. 1. Schematic phylogenetic trees demonstrating the position of the Hawaiian mints among close continental *Stachys* relatives in the Americas based on (A) chloroplast DNA, (B) nuclear ribosomal DNA, and (C) low-copy nuclear DNA. SA = South America; NA = North America.