cpDNA of *Acer saccharum* and *Acer nigrum* are Very Similar[1]

ADAM P. SKEPNER AND DAN E. KRANE[2], Wright State University, Department of Biological Sciences, Dayton, OH 45435

ABSTRACT. **The number of species and sub-species that comprise North American sugar maples has remained in dispute since their first characterization over 100 years ago. The taxonomic distinctiveness of *Acer nigrum* (black maples) and *A. saccharum* (sugar maples) has been particularly controversial. An analysis of the nucleotide sequences from a non-coding region of the chloroplast genome of Ohio black and sugar maples suggests that these trees are genetically very similar and do not require separate taxonomic designations.**

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INTRODUCTION

Sugar maple (*Acer saccharum*) is important commercially in Ohio and throughout its range for its wood and sugar sap and also for its ornamental value. Previous studies of sugar maples (see Kriebel and Gabriel 1970 for a review) have been largely concerned with breeding and conservation programs that aim to find the best methods of obtaining high sugar sap content and superior wood quality. These early works revealed that sugar maple may actually be a group made of several distinguishable subspecies (*A. saccharum*, *A. nigrum*, *A. floridanum*, and *A. grandidentatum*).

The *nigrum* (black maples) designation has historically been the most controversial. In stands where the *A. nigrum* and *A. saccharum* grow alone, both have readily distinguished morphologies. The bark of *A. nigrum* is characteristically more deeply furrowed and darker in color than that of other members of the sugar maple group. *A. nigrum* is further distinguished by its three lobed leaves which stand in sharp contrast to the five lobed deeply-furrowed leaves of *A. saccharum*. However, the entire state of Ohio constitutes an overlap in the naturally occurring ranges of *A. nigrum* and *A. saccharum* and hybrids are abundant in this area.

A relatively limited (<15) number of morphological characteristics suitable for discrimination between the *A. nigrum* and *A. saccharum* morphologies has led to a lengthy debate regarding their relationship to each other as well as to other members of the sugar maple group. Authors of some tree guides recognize them as separate species (see the review of Little 1979) while other authors consider *A. nigrum* to be a subspecies of *A. saccharum* (Burns and Honkala 1990, Dirr 1990). Numerous studies (for example., Anderson and Hubricht 1938, Desmarias 1952, Kriebel 1957) have addressed but have been unable to resolve this classification question. Kriebel (1957) for example, recognized the subspecies designation out of convention but insisted that a final answer regarding the taxonomy of the trees could only come with further study. Others have both recognized the subspecies designation (Graves 1994a) and treated the trees as separate species in another study of the responses of the two morphologies to drought (Graves 1994b).

More recent molecular studies involving maples have increased the number of distinguishing characters available for these trees but have been concerned primarily with the conservation of *A. saccharum* in general and did not consider black and sugar maples as potentially distinct morphologies (Foré, Hickey, Guttman, and others 1992; Foré, Hickey, Vankat, and others 1992; Geburek 1993; Young and others 1993). Molecular markers generated with RAPD-PCR (random amplified polymorphic DNA – polymerase chain reaction; Skepner and Krane 1997) from the genomes of both black and sugar maples collected from throughout their ranges, however, indicate that the two morphologies are genetically very similar. However, while useful in discerning differences between very closely related groups of organisms, the anonymity and polymorphism levels of RAPD-PCR markers do not make them amenable for phylogenetic comparisons with less closely related species (Hillis and others 1996).

In contrast, nucleotide sequences from chloroplast genomes have increasingly proven to be useful in determining the relationships between more distantly related plant species (Palmer 1987, Gielly and Taberlet 1994). Taberlet and others (1991), in particular, have developed universal primers to cholorplast genomes that have been used to distinguish between other members of the genus *Acer* (Taberlet and others 1991) and other taxonomically difficult genera (Soltis and others 1990). In this study we have utilized PCR to amplify and sequence the region between the leucine (tRNA Leu) and phenylalanine (tRNA Phe) tRNA genes within the chloroplast genome to confirm the genetic similarity of *A. nigrum* and *A saccharum* and to place them phylogenetically with other members of the genus *Acer*.

MATERIAL AND METHODS

Isolation of chloroplast DNA

Chloroplast DNA was isolated as described by Gantt (1980) from the leaves of three sugar and three black maples collected from the wooded preserve at Wright State University, Dayton, OH as well as one sugar maple

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[2]Address for Correspondence: Dan E. Krane, Department of Biological Sciences, Wright State University, Dayton, OH 45435. Phone: 937-775-2257, e-mail: dkrane@wright.edu

collected from the Missouri Botanical Garden in St. Louis, MO. Only leaves from indigenous trees that exhibited either strong *A. saccharum* or *A. nigrum* morphologies (described in Desmarias 1952) were selected. The resulting pellets of high molecular weight nucleic acids were resuspended in 20 µL of distilled water. The success of each DNA extraction was assessed by electrophoresis of 2 µL of the isolate on a 1.5% agarose gel.

Amplification and sequencing of chloroplast DNA

The region between the leucine (tRNA Leu) and phenylalanine (tRNA Phe) tRNA genes within the chloroplast genome was amplified by the polymerase chain reaction (PCR) using the following pairs of primers: 5'- GGTTCAAGTCCCTCTATCCC -3' and 5'- ATTTGAACTGGTGACACGAG -3' developed by Taberlet and others (1991). An MJ Research Mini-cycler thermocycler was used to perform the amplifications given the following parameters for 35 cycles: denaturation at 92° for 1 minute, annealing at 52° for 1 minute and extension at 68° for 1 minute. A final extension at 68° for 10 minutes was included at the completion of each PCR amplification. All reactions were performed in 50 µL volumes containing 2.0 mM TRIS *p*H 8.0, 0.25 mM $MgCl_2$, 1.6 mM $(NH_4)_2SO_4$, 15 µg/mL bovine serum albumin, 1 unit of KT1 KlenTaq polymerase (Wayne Barnes, Washington University, St. Louis, MO), 1 µL of 1:10 diluted chloroplast DNA and 60 µM dNTPs.

PCR products were cloned into a *p*NoTA/T7 double-stranded plasmid using the Prime PCR Cloning System (5 Prime - 3 Prime, Inc.; Boulder, CO) and the protocols provided by the manufacturer. Plasmids were recovered by alkaline lysis minipreps (Maniatis and others 1982). Sequencing was performed with Sequenase Ver. 2.0 (United States Biochemical, Cleveland, OH) using the manufacturer's protocol and determined with a 6% denaturing acrylamide gel or on a ABI 373 Automated DNA Sequencer. All sequences were confirmed with three independently isolated clones.

Nucleotide Sequence Analysis

Alignments of the nucleotide sequences were made using CLUSTAL W (Higgins and others 1992) and optimized by hand. Partial sequences from two European

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                                20                  40                  60                  80                 100
Acer saccharum          GGTTCAAGTCCCTCTATCCC CCCAAAAAGACCGATTTGAC --------TATTTCTCCCTA CCCTCTCTTTTTTTTAGTGG TTCAAAATTCGTTCTCTTTC
A. nigrum               .................... .................... .................... .................... ....................
A. platanoides                      .......... .....................-.. .................... ....................
A. pseudoplatanus                   .......... TTCCTAAC.T.......-.. .................... ..G.................
Echium simplex                        CCCAATG.........-.. -TA...A....CG....... ...C......C.CAC.....
Lobostemon fruticosus                 CCCAACG.........-.. -TA...A....CG....... ...C......C.CAC.....
Brunnera macrophylla                  CCCAACG.........-.C -TA...A....CG....... ..........C.CA.T....
Nicotiana tabacum       .................... .--.......-.T....C.. TCCCCAAC.....A...-G. ...C.----------....C.. ...C......C..A......

                               120                 140                 160                 180                 200
Acer saccharum          TCATTCATTCTACCTTTTTA CAAACGTAT---CAGAGCAG AATTTTTTTTTTTCTTTTAT CACAATCACAAGTGGTGTGG TATATATGATATACGTACAA
A. nigrum               .................... .................... .................... .................... ....................
A. platanoides          .................... .................... .....------..... ....................
A. pseudoplatanus       ...G.........T...... .................... .................... ....................
Echium simplex          .......C....TTC..... ......G......T.GC.G. ..ACGCC...CA....---- ------------------- -.C........G..A.....
Lobostemon fruticosus   .......C....TTC..... ......G......T.GC.G. ..ACGCC...CA....---- ----------ACACAA.T CT.G.......C..A.....
Brunnera macrophylla    .......C....TTC....A ....T.G..GAT.T.GC.G. ..ACACC....A....---- ----.A..AGTC.T..AA.A ..CG.......C..A.....
Nicotiana tabacum       .......C....TTC..... G...T.G.....TT....GT ..A.GGC...C-....---- ----.........CT....A ...........C..A..G..

                               220                 240                 260                 280                 300
Acer saccharum          ATGAACATCTTTGAACAAAG AATCCC---------------- -----GATTCACAATTCATA TCATTGCTCATACTGAAACT TATAAATTATTCTATTCAAG
A. nigrum               .................... .................... .................... .................... ....................
Echium simplex          ....C.............G. ...GAATCGCCTTTTGAATT TGAAT..........TG... .....T....G..... ..C...G.CG...TA.TG.A
Lobostemon fruticosus   ....C.............G. ...GAATCGCCTTTTGAATT TGAAT..........GG... .T...T....G..... ..C...G.CG...TA.TG.A
Brunnera macrophylla    ..................-- --.AAATTTCCTTGTGAATT TGAAT..........GG... .....T....G..... ..C...G.TG...TA.TG.A
Nicotiana tabacum       ......G....-..G...G. ......T----------AGT TGAAT.....C.T..CA... .....A.............. -.C...G.CA...T..TG.A

                               320                 340                 360                 380                 400
Acer saccharum          AA---ATCGAATTCCACGCT CAAGA--CTTTTAATACTTT TTTTATCTTTTTTTTAATTG ACATAGACCCCAGTCATCTA GTAAAATGAGGATGATGCGC
A. nigrum               .................... .................... .................... .................... ....................
Echium simplex          G.TCC.AGA...-A..GTAC .CG----AAAG.TGGGAA.C ..C.T..A.AC.......... ..............
Lobostemon fruticosus   G.TCC.AGA....A..GTAC .CG----AAAG.TGGGAA.C ..C.T..A.AC.......... .................... A......A....
Brunnera macrophylla    G.TCC.AGA....A..GTAC .CG..TAAAAG.TGGTAA.C ..C.T..A.AC.......... .................... A......A....
Nicotiana tabacum       G.TCG.AGA......C..GC TTT...GAAAA.TT.TAA.C A-C.T.TG.C...G..... ..............TC.... A............AC.A.AT
Nicotiana tabacum       GATCGAAGAAATTCCCCGGC TTTGA-GAAAATTTTTAATC A-CTTTTGTCCTTGTAATTG ACATAGACCCCAGTTCTCTA ATAAAATGAGGATACTACAT

                               420                 440                 460                 468
Acer saccharum          CGGTAATGGTCGGGATAGCT CAGCTGGTAGAGCAGAGGAC TGAAAATCCTCGTGTCACCA GTTCAAAT
A. nigrum               .................... .................... .................... ........
Nicotiana tabacum       -..G...A.C.......... ...T..............  .................... ........
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FIGURE 1. Alignment of non-coding region between tRNA Leu and tRNA Phe. *Acer saccharum* (sugar maple), *A. nigrum* (black maple), and currently available homologous sequence from other *Acer* species and four other plant species are aligned. Nucleotides identical to those found in *A. saccharum* sequences are shown as periods (.). Primer sequences used for amplification by PCR are underlined. Gaps inserted to improve alignments are indicated by dashes (-).

species *Acer platanoides* (Acc. X60209) and *A. pseudoplatanus* (Acc. X60208), in which the same region of the chloroplast genome was available, were also aligned with the black and sugar maples from this study. Sequences available in GenBank release 102.0+ (September, 1997) that were assigned high scores of homology by the BLASTN search program (Altschul and others 1990) were also aligned with the *Acer* sequences. A phylogenetic tree was constructed using an exhaustive search and then bootstrapped for 5000 replications using PAUP 3.0 (Swofford 1991).

RESULTS

The amplification of the region between the tRNA Leu and tRNA Phe genes within *A. nigrum* and *A. saccharum* chloroplast DNA resulted in products 433 bp in length. No variation was found in the nucleotide sequence of any of the three sugar and three black maples collected from the wooded preserve at Wright State University (Dayton, OH) or the sugar maple collected from the Missouri Botanical Gardens (St. Louis, MO). However, differences were found between black and sugar maples and their homologs in other *Acer* species, as well as other, more distantly related plant species (Fig. 1).

Three equally parsimonious phylogenetic trees were constructed using PAUP 3.0 (Swofford 1991) (Fig. 2). Each of the trees differed only in their relative grouping of *A. saccharum*, *A. nigrum*, and *A. platanoides* within the *Acer* clade which was consistently found to be separate from the other organisms in the alignment.

DISCUSSION

The cholorplast genome (cpDNA) has been used extensively to resolve questions of plant systematics at a wide variety of levels because of its conservation of gene content and lack of major structural rearrangements (reviewed in Palmer 1987). Analyses of coding regions such as *rbc*L (Chase and others 1993) within cpDNA have been commonly employed to clarify relationships at the family-level due to their relatively slow evolutionary rates (Geilly and Taberlet 1994). The dramatically higher rate of substitution within non-coding regions (Gielly and Taberlet 1994) of the cholorplast genome, however, make them very suitable for intergeneric comparisons (Palmer 1987).

The non-coding region between the chloroplast tRNA Leu and tRNA Phe genes used in this study has proven to be hypervariable between and even within other plant genera (Gielly and Taberlet 1994). The total invariance of sequence data obtained from 3 native Ohio black and sugar maples as well as 1 Missouri sugar maple, however, provides additional strong evidence that trees of these morphologies are genetically very similar (Fig. 1). This conclusion is consistent with those drawn from an analysis of RAPD-PCR generated anonymous markers obtained from a larger sampling of these trees collected throughout their ranges (Skepner and Krane 1997).

Characteristic differences at the nucleotide sequence level were found between 4 representative *A. saccharum* and 3 representative *A. nigrum*, 2 European *Acer* species and several other plants (Fig. 1). These differences not only confirmed the general utility of this non-coding region in distinguishing between both closely and distantly related species, but also allowed a molecular based phylogenetic tree to be constructed that illustrated the taxonomic relationship between *A. nigrum* and *A. saccharum* and other plant species (Fig. 2).

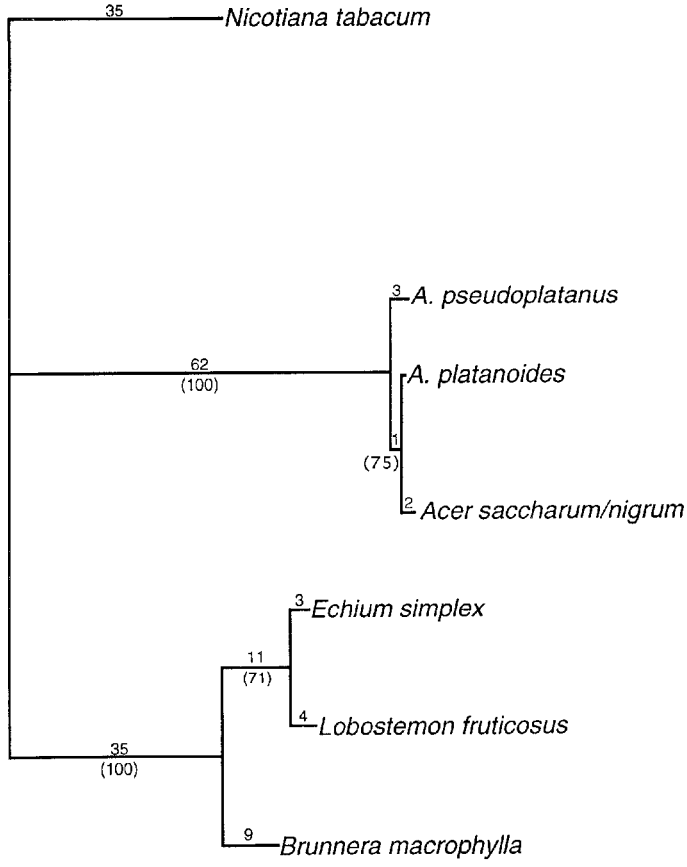

FIGURE 2. Phylogenetic tree generated from multiple alignment of *Acer* species and other closely related sequences. Numbers above horizontal branches indicate relative branch length and numbers in parentheses indicate bootstrap values (5000 replications).

The extent to which *A. nigrum* and *A. saccharum* are genetically similar at a large number of selectively unconstrained sequences (ranging from the anonymous RAPD-loci [Skepner and Krane 1997] to the intergenic chloroplast sequences described in this study) implies that extensive gene flow has occurred between their respective populations. Indeed, the morphological features that distinguish them are likely to be attributable to a relatively small number of polymorphisms which do not contribute to a natural reproductive barrier between them. As such there is little taxonomic basis for separate designations for *A. saccharum* and *A. nigrum*.

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