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Mentha (Lamiaceae) Phylogenetic Analysis Using Chloroplast TRNL-TRNF and Nuclear Ribosomal DNA ITS Sequences

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MENTHA (LAMIACEAE) PHYLOGENETIC ANALYSIS USING CHLOROPLAST *TRNL-TRNF* AND NUCLEAR RIBOSOMAL DNA ITS SEQUENCES

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Mentha (Nepetoideae, Lamiaceae) is a taxonomically complex genus that includes economically important members such as spearmint and peppermint and species of global conservation interest. *Mentha* is considered challenging systematically due to its high incidence of polyploidy, diverse morphology, variation in base chromosome number, and frequent interspecific hybridization. Our objectives were to test the monophyly of *Mentha* and each of its traditionally recognized sections, assess phylogenetic relationships of the *Mentha* species, test hypotheses of hybridization for the putative stabilized allopolyploids (*M. spicata* and *M. canadensis*), and determine the ancestral base chromosome number using DNA sequence data from the chloroplast *trnL-trnF* and nuclear ribosomal internal transcribed spacer (ITS) regions. Based on *trnL-trnF* data, *Mentha* appears monophyletic. However, ITS data place the *Mentha* species into two distinct clades that include 12 other Mentheae genera. None of the sections with more than one species sampled form monophyletic groups based on either data set, and are therefore inconsistent with traditional classification. Cloned ITS sequences of *M. canadensis* and *M. spicata* support the hypothesis of hybridization as evidenced by nucleotide site polymorphism in ITS direct sequences and divergent clones cluster with different species. Moreover, our data indicate that *M. spicata*, rather than *M. longifolia*,

may be a parent of *M. canadensis*. Character optimization of base chromosome number on to the molecular phylogenies shows that $x = 12$ may be ancestral based on *trnL-trnF* data or possibly $x = 9$ or $x = 10$ based on ITS data.

INTRODUCTION

The plant genus *Mentha* L. is well known as a systematically complex group (Tutin et al., 1972; Harley and Brighton, 1977; Chambers and Hummer, 1994; Rösch, et al., 2002; Tucker, in manuscript). Taxonomic difficulty may be due to high incidence of polyploidy, variation in base chromosome number, diverse morphology, vegetative propagation, and frequent interspecific hybridization (Morton, 1956; Harley and Brighton, 1977; Tucker, in manuscript; Tucker and Chambers, in manuscript). Members in this genus include several economically important plants such as spearmint (*M. spicata*), peppermint (*M.* x *piperita*), and Japanese mint (*M. arvensis*) as well as two species of global conservation interest (*M. gattefossei* and *M. requienii*).

Geographic Distribution

Mentha is a member of the mint family (Lamiaceae; Labiatae A. L. de Jussieu), subfamily Nepetoideae, tribe Mentheae (Wagstaff, 1992; Wagstaff et al., 1995). Most *Mentha* species are widely distributed and occur primarily in Europe and Asia (Briquet, 1897; Tutin et al., 1972; Harley and Brighton, 1977; Gleason and Cronquist, 1991; Tucker, in manuscript). Moreover, three species are found in Australia (*M. australis, M. diemenica,* and *M. satureioide*s), and *M. cunninghamii* is a New Zealand endemic. *Mentha gattefossei* is restricted to Morocco, and *M. requienii* is found in Southern Europe, especially in Corsica (France), Sardinia, and Monte Cristo Island (Italy). In addition, the North American *M. canadensis* is the only species native to the new world (Harley and Brighton, 1977; Gleason and Cronquist, 1991). Members in this genus usually grow well in moist places, especially close to streams. Some species escape from

cultivation and have become naturalized in roadsides and fields (Tutin et al., 1972; Gleason and Cronquist, 1991).

Morphology

Mentha species are very diverse morphologically (Tutin et al., 1972; Harley and Brighton, 1977). For example, there are three different types of floral inflorescences: capitate, spicate, and verticillate. The capitate inflorescence consists of many sessile or subsessile florets clustered at the tip of a peduncle. The spicate inflorescence is elongated with sessile or subsessile flowers that bloom from the bottom upwards. Verticillate is another type of elongated inflorescence composed of three or more flowers at several nodes. Consequently, there is no single diagnostic trait, and thus a combination of characters must be used to define *Mentha*. Tucker (in manuscript, p. 1) defines *Mentha* based on the following characters: 'stamens 4, ± equal, filaments naked, anthers with parallel distinct thecae, ± actinomorphic calyx, weakly 2-lipped corolla, and subellipsoidal nutlets with rounded apex.' Gleason and Cronquist (1991, p. 443) use the following characters: 'stamens 4, exert beyond the corolla–throat, sometimes even surpassing the lips, corolla 4-lobed or 5-lobed or nearly regular, upper lip of the corolla well developed and manifest, formed by fusion of two, inflorescence essentially axillary, the verticils several to many, subtended by foliage leaves and separated by internodes, or the uppermost subtending leaves smaller and internodes shorter, calyx 10-13 nerved, regular or weakly 2-lipped, the lobes of the upper and lower lips similar or differing in shape and size.' Phylogenetic utility of these traits has not been tested.

Traditional Classifications

The history of *Mentha* systematics has been very confusing and even its current

status remains uncertain. Monophyly of *Mentha* is questionable because several members of *Mentha* have sometimes been placed in other, presumably closely related genera such as *Micromeria*, *Pulegium*, *Audibertia*, *Menthella*, *Thymus*, *Satureja* and *Preslia* (Briquet, 1897; Tucker, in manuscript). Circumscription of *Mentha* species is ambiguous. As a result, many new species (over 3,000 names) have been described (Tucker, in manuscript). For example, 113 new taxa were published for Hungary alone (Trautman, 1925). Moreover, many species (such as *M. longifolia*) are divided into multiple subspecies. Tucker (in manuscript) proposes 19 different subspecies of *M. longifolia* ranging from Western Europe to the Himalayas and three subspecies (*capensis, polyadena,* and *wissii*) in Southern Africa. European subspecies of *M. longifolia* generally correspond to political boundaries. Yet, taxonomic treatments for *Mentha* recognize only 13-18 species (see Table 1: Briquet, 1897; Harley and Brighton, 1977; Chambers and Hummer, 1994; Tucker, in manuscript).

Not only has circumscription of the *Mentha* species been problematic but also their infrageneric classification. For example, *Mentha* has been divided into two to six groups (Table 1). Briquet (1897) divided the *Mentha* species into five sections (sect. *Eupulegia*, sect. *Audibertiae,* sect. *Verticillatae*, sect. *Capitatae*, and sect. *Spicatae*) within two subgenera (subg. *Pulegium* and subg. *Menthastrum*), and *M. cervina* was placed in the genus *Preslia*. Harley and Brighton (1977) divided *Mentha* into five sections: sect. *Audibertia*, sect. *Eridontes*, sect. *Mentha*, sect. *Preslia*, and sect. *Pulegium*. Section *Eriodontes* included four Australasian species plus one Japanese species while the well-known economically important mints (e.g., spearmint) were placed in sect. *Mentha*, which is the largest and taxonomically most complex (Harley and Brighton,

1977). However, in Tucker's classification (in manuscript) based on morphology, base chromosome number, and major essential oil components, *Mentha* consists of 18 species divided into two sections: sect. *Mentha* and sect. *Pulegium*. This classification excludes *M. cunninghamii* from *Mentha*.

Debatable relationships occur not only at infrageneric levels but also at the suprageneric level. For instance, based on chloroplast DNA restriction site variation, *Mentha* is most closely related to *Thymbra* (Wagstaff et al., 1995). However, in an analysis of 10 Mentheae genera based on internal transcribed spacer (ITS) data, *Mentha* is closely related to *Thymus* and *Ziziphora* (Prather et al., 2002). Although monophyly of Nepetoideae, the largest subfamily in Lamiaceae, is strongly supported (Cantino and Sander, 1986; Cantino, 1992), phylogenetic relationships within the subfamily are unclear (Wagstaff et al., 1995). Parsimony analysis of chloroplast DNA restriction site variation shows that the tribe Mentheae sensu Bentham (1876) is polyphyletic, but the tribe Mentheae sensu Cantino et al. (1992) is monophyletic (Wagstaff et al., 1995)

Previous Studies in Characterization Techniques of Mints

Several studies have been done to assess relationships in *Mentha*. However, no robust phylogeny for the genus is available. For economic applications, it is important to identify and characterize mint species and named hybrids (Rösch et al., 2002). The use of only morphological features is insufficient when differentiating the commercial mint cultivars. Thus, many techniques have been applied to precisely identify mint taxa. For example, 11 accessions were analyzed by Khanuja et al. (2000) representing six *Mentha* taxa (*M. arvensis, M. spicata, M. spicata* cv. *viridis, M.* x *piperita, M.* x *piperita* cv. *citrata, and M.* x *gracilis*), and 17 accessions were analyzed by Fenwick and Ward

(2001) representing three *Mentha* taxa (*M. spicata, M.* x *piperita, and M.* x *gracilis*) using Randomly Amplified Polymorphic DNA (RAPD) markers demonstrating that this technique can be used to distinguish *Mentha* taxa. RAPDs were also useful in identifying somatic hybrids between *M. spicata* and *M.* x *piperita* (Krasnyanski et al., 1998). A combination of micro-Raman spectroscopy and hierarchical cluster analysis proved to be a rapid and easy characterization method for discriminating *Mentha* taxa (Rösch et al., 2002). Gas chromatogram profiles applied by Tucker et al. (1991) were also able to distinguish *Mentha* genotypes; however, this technique is slower and more expensive for screening larger numbers of plants (Fenwick and Ward, 2001).

Base Chromosome Number and Polyploidy

Four different base (haploid) chromosome numbers are found in *Mentha*: $x = 9$, $x = 10$, $x = 12$, and $x = 18$ (Harley and Brighton, 1977; Chambers and Hummer, 1994). Most species have a base chromosome number of $x = 12$. *Mentha requienii* is the only species with a base chromosome number of $x = 9$ while in *M. japonica, M. gattefossei*, and *M. pulegium* the base chromosome number is $x = 10$. In *M. cervina* the base chromosome number is $x = 12$; however, it has also been counted as $x = 18$ ($2n = 36$) by Makarov and Reznikova (1972) and Harley and Brighton (1977). If *Mentha* is monophyletic and the ancestral base chromosome number is $x = 9$, 10, 12, or 18, then the indication is that there have been at least three chromosome loss or gain events (e.g., one event from $x = 12$ to $x = 10$ by losing two chromosomes, or one event from $x = 9$ to $x = 10$ by gaining one chromosome). Until the ancestral base chromosome number has been determined, the pattern of chromosome evolution in *Mentha* cannot be examined.

According to Harley and Brighton (1977) and Chambers and Hummer (1994), only five *Mentha* species (*M. cervina, M. longifolia, M. pulegium, M. requienii,* and *M. suaveolens*) are diploid. However, some diploid species such as *M. longifolia* and *M. pulegium* have also been reported as tetraploids. Ploidy levels of the remaining species range from triploid to decaploid (Table 2). The most common ploidy levels are hexaploid (e.g., *M. arvensis*) and octaploid (e.g., *M. aquatica*).

Hybridization

Hybridization in *Mentha* occurs frequently in both wild and cultivated populations; however, only in sect. *Mentha* do hybrids occur naturally (Morton, 1956; Harley and Brighton, 1977; Tucker, 1990). Because hybrids often show morphological intermediacy and diversity as well as genetic variability (McDade, 1995, 2000), they create difficulties in species delimitation and also complicate problems in systematics and phylogenetics. Normally, hybrids are sterile due to unfavorable interactions between parental species' genomes (Rieseberg et al, 1996). However, some hybrids become stable species (Arnold, 1992). To reconstruct phylogenetic relationships, phylogeneticists assume that 'the evolutionary history of living organisms has been a series of divergent speciation events' (McDade, 2000; p. 147). Therefore, hybrids that have reticulating evolutionary histories may cause incorrect phylogenies.

In *Mentha*, there are 13 named hybrids (Harley and Brighton, 1977). These have resulted from breeding experiments. Frequent interspecific hybridization in *Mentha* may be due to gynodioecy: having carpellate and perfect flowers on separate plants (Harley and Brighton, 1977; Tucker, in manuscript). Some species such as *M. spicata* and

M. canadensis are thought to be ancient hybrids. *Mentha spicata* (Fig. 1b) has been hypothesized to be an ancient stabilized allopolyploid between *M. longifolia* (Fig. 1a) and *M. suaveolens* (Fig. 1c) (Harley and Brighton, 1977). *Mentha canadensis* (Fig. 2b) is thought to be an ancient stabilized allopolyploid between *M. longifolia* (Fig. 2a) and *M. arvensis* (Fig. 2c) (Tucker and Chambers, in manuscript). *Mentha canadensis* has the somatic chromosome number of 2n = 96 (octaploid) and a verticillate inflorescence. Some differences between the hypothesized parents of *M. canadensis* are as follows: *Mentha arvensis* has the somatic chromosome number of 2n = 72 or 96 (hexaploid/octaploid) and a verticillate inflorescence while *M. longifolia* has the somatic chromosome number of 2n = 24 or 48 (diploid/tetraploid) and a spicate inflorescence.

Economically Important Mints

Many species and named hybrids of *Mentha* have considerable economic importance (Krasnyanski et al., 1998; Schulz et al., 1999; Mirzaie-Nodoushan et al., 2001; Tucker, in manuscript) such as *M. spicata* (spearmint), *M. aquatica*, *M. arvensis* (cornmint, Japanese mint, menthol mint), *M. canadensis*, *M. pulegium* (European pennyroyal), *M.* x *piperita* (peppermint), *M.* x *gracilis*, and *M.* x *villoso-nervata*. Shoots and leaves of mints are used as condiments in food, for example in Thai and Indian cooking. The major components of the peppermint and Japanese mint essential oils are *l*-menthol, menthone, isomenthone, and cineole (Schulz et al., 1999; Anon, 2001), whereas spearmint oil is composed primarily of carvone and *l*-limonene (Fujita and Nezu, 1980 in Imai et al., 2001; Pino et al., 2001). Their essential oils and derivatives are not only processed into flavorings for food, candy, chewing gum, chewing tobacco, and cigarettes but are also used in cosmetic formulations and perfumed products as fragrance

components in shampoo, cooling gel, shaving cream, bubble bath, deodorant, toothpaste, floss, mouthwash, and oral spray (Chambers and Hummer, 1994; Khanuja et al., 2000; Anon, 2001; Srivastava et al., 2002).

Mint essential oils also have medicinal properties. For example, both spearmint and peppermint oil show antibacterial and antifungal properties (Dikshit and Husain, 1994; Tassou et al. 1995; Adam et al. 1998; Imai et al., 2001). An extract of spearmint oil is also anticarcinogenic (Villaseñor et al., 1997) and insecticidal (Franzios et al., 1997). Peppermint oil may be used as antiallergic, antiinflammatory (Arakawa et al. 1992) and antispasmodic medicine (Foster et al., 1980). Moreover, peppermint oil has been shown to have animal feeding repellent activity (Ries et al., 2001) and has been evaluated for larvicidal activity against mosquitoes (Ansari et al., 2000). In addition, peppermint extract was found to contain an antimutagen against dietary carcinogen in human cancer (Samejima et al., 1995)

The world market for menthol mint essential oils is ~20,000 tons per year. Menthol mint is mainly grown in China, India, Brazil, Japan, France, and the United States. In India, about 145,000 ha of menthol mint are now cultivated (Srivastava, 2002). In the United States, peppermint, native spearmint, and Scotch spearmint (*M. x gracilis*) are also grown commercially (Krasnyanski et al. 1998; Johnson and Cummings, 2000). In 2001, The US harvested 19,500 and 78,500 acres of spearmint and peppermint, respectively (USDA-National Agricultural Statistics Service, 2002). Spearmint production is 2,052,000 lb and yields 105 lb spearmint oil. Peppermint production is higher (6,343,000 lb), but with a lower yield (81 lb).

Species of Global Conservation Interest

According to the IUCN (World Conservation Union) Red List of Threatened Plants, two *Mentha* species are listed among the ~33,000 rarest plants in the world (Walter and Gillett, 1998). In the IUCN Red List, there are five categories of extant threatened plants: '(1) **Extinct/Endangered** = taxa that are suspected of having recently become 'Extinct,' (2) **Endangered** = taxa in danger of extinction and whose survival is unlikely if the causal factors continue operating, (3) **Vulnerable** = taxa believed likely to move into the 'Endangered' category in the near future if the causal factors continue operating, (4) **Rare** = taxa with a small world population that are not at present 'Endangered' or 'Vulnerable,' but are at risk, and (5) **Indeterminate** = taxa that are known to be 'Endangered,' 'Vulnerable,' or 'Rare,' but where the information is inadequate for determining which of the three categories is appropriate.' *Mentha gattefossei* (Fig. 3a), an endemic species of the Atlas mountains of Morocco, is listed as 'vulnerable.' The other species, *M. requienii* (Fig. 3b), from Corsica, Sardinia, and Monte Cristo Island is listed as 'rare.'

Value of Phylogeny

The establishment of a robust organismal phylogeny is important so that we can understand 'how organisms, their traits, and interactions between species evolve' (Wiens, 2000). Phylogeny is also beneficial for plant breeders and breeding programs, molecular geneticists, pest and pathogen management, endangered species studies, and studies of systematics and biogeography. In the case of *Mentha*, knowledge of relationships may help molecular geneticists and plant breeders to improve species and hybrids leading to higher herbage and essential oil yields and quality (Khanuja 2000) as well as higher

disease-resistance (Krasnyanski et al., 1998). Understanding of phylogenetic relationships may lead to wild taxa that contain sources of novel genes and assist in conservation of germplasm (Kellogg et al., 1996). For *Mentha*, phylogeny reconstruction may help conservation efforts by identification of endangered *Mentha* species and closely related taxa. Furthermore, some authors have developed algorithms to evaluate the conservation priority of taxa using branch lengths of molecular phylogenies (Moritz, 1998). Topology of phylogeny can be used to estimate population trends (for example, we expect an expanding population from a star-like phylogeny based on DNA sequences).

Objectives

In this study, our objectives are to test the monophyly of *Mentha*, evaluate each traditional classification system, assess the relationships of the *Mentha* species, test hypotheses of reticulate ancestry for the putative stabilized allopolyploids (*M. spicata* and *M. canadensis*), and determine the ancestral base chromosome number using molecular data.

Molecular Data

Molecular data are a powerful source of information in studies of plant phylogeny and hybridization, especially in morphologically diverse groups (Baldwin et al., 1995). Because individual gene trees may not reflect the true organismal relationships (Doyle, 1992; Kellogg et al., 1996), we will generate DNA sequences from two cellular genomes: the chloroplast (cp) *trnL-trnF* region which is presumed to be maternally inherited (Dowling et al., 1996) and the nuclear ribosomal (nr) internal transcribed spacer (ITS) region which is biparentally inherited.

The *trnL-trnF* region includes the *trnL* intron and *trnL-trnF* intergenic spacer, both of which are noncoding (Fig. 4). This region is readily amplified using universal primers developed by Taberlet et al. (1991). Sequence data from this region have shown potential for investigating interspecific relationships in angiosperms (Harris and Ingram, 1991; Small et al., 1998). For example, *Salvia* (Lamiaceae) has been shown to be paraphyletic or polyphyletic using cpDNA *trnL-trnF* and *rbcL* data (Walker et al., 2002). For another example in Lamiaceae, *Dicerandra* may not be monophyletic based on *trnL-trnF*, ITS, and *matK* data (Oliveira et al., 2002).

The nrDNA ITS region consists of two transcriptional regions (ITS-1 and ITS-2) (Fig. 5) for which universal primers also exist (White et al., 1990). This region is part of the ribosomal multigene family that includes hundreds to thousands of copies at one or more chromosomal loci (Baldwin, 1992; Baldwin et al., 1995; Widmer and Baltisberger, 1999). Concerted evolution is an evolutionary process that maintains each copy of the same repetitive DNA families to be identical; otherwise random mutation would later increase differences among members of a family (Schopf, 1981). There are two main mechanisms for the occurrence of concerted evolution: **gene conversion**, a recombination process where one sequence of DNA acts like a template to convert another without changing itself and **unequal crossing over**, a recombination process between chromosomes that are not precisely paired, resulting in unequal length of chromosomes (Krieber and Rose,1986; Elder and Turner, 1995; Li, 1997). ITS is the most widely used nuclear DNA region in plant systematic studies (Baldwin et al., 1995) and is phylogenetically informative at low taxonomic levels. In Lamiaceae, ITS data strongly support the monophyly of *Monarda* (Prather et al., 2002), but show polyphyly of

Clerodendrum (Steane et al., 1999). In adddition, the combined analyses of ITS and cp *ndhF* data showed monophyly of *Trichostema* (Huang et al., 2002). The ITS region has also been shown to be highly useful in studies of hybridization (Campbell et al., 1997; Alice and Campbell, 1999; Widmer and Baltisberger, 1999; Alice et al., 2001) and provides more substantially phylogenetically informative characters than the *trnL-trnF* region (Small et al., 1998; Oliveira et al, 2002; Smedmark and Eriksson, 2002).

MATERIALS AND METHODS

Plant Samples

Plant samples used in this study (Table 2) include 15 *Mentha* species representing all five sections (*Audibertia, Eriodontes, Mentha, Preslia,* and *Pulegium*) and two named hybrids (*M.* x *piperita* and *M.* x *rotundifolia*). Samples were received as cuttings from the United States Department of Agriculture-Agricultural Research Service, National Clonal Germplasm Repository (USDA-ARS, NCGR) in Corvallis, OR and established as plants in the WKU Biology Department greenhouse. Morphological vouchers have been deposited in the Western Kentucky University herbarium (WKU) (Holmgren et al., 1990).

DNA Isolation and Polymerase Chain Reaction (PCR)

Total cellular DNAs were isolated from fresh young leaves stored at –80°C using a modified CTAB (hexadecyltrimethylammonium bromide) protocol (Doyle and Doyle, 1987). PCR amplification of *trnL-trnF* generally followed Taberlet et al. (1991). Target DNA was directly amplified using 15 µl of genomic DNA diluted 1:100 in a total volume of 25 µl, containing 0.4 µM of each oligonucleotide primer c and f, 200 µM of each dNTP, 1.9 mM $MgCl_2$ and 1 unit *Taq* DNA polymerase (Promega, Madison, WI). PCR was performed in a MJ research PTC-100 thermal cycler (MJ Research, Watertown, MA). After 40 cycles of amplification (1 min at 94°C, 1 min at 50°C, and 3 min at 72°C), the reaction temperature was held at 72°C for 20 min and then maintained at room temperature.

PCR amplification of ITS generally follows Baldwin (1992) using 11 µl of genomic DNA in a total volume of 25 µl, containing 12.5 µl 2x PCR master mix

(Promega, Madison, WI) and 0.3 μM of each oligonucleotide primer ITS5 and ITS4. After 40 cycles of amplification (1 min at 97°C, 1 min at 48°C, and 45 s + 4 s per cycle at 72°C), the reaction temperature was held at 72 °C for 7 min and then maintained at room temperature.

Agarose Gel Electrophoresis and Purification

PCR products were electrophoresed in 0.8% agarose gels (Fisher Scientific, Fair Lawn, NJ). Fragments corresponding in size to the target DNA were excised and purified using a QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions. Some PCR amplified ITS products were cloned (see below).

Cloning of ITS

In species that showed nucleotide site polymorphism in direct sequences (*Mentha arvensis*, *M. canadensis* and *M. spicata*), the ITS region was cloned. Fresh PCR products were ligated into a pCR II-TOPO vector using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). Potentially recombinant plasmids were chemically transformed into competent *E. coli* cells (20 min ice, 42°C for 30 s, and 2 min ice) and were then incubated in SOC medium for 1 hour at 37°C on a rotary shaker. After that they were plated onto LB agar with ampicillin containing 32 μl of 50 mg/ml X-gal and 40 μl of 0.1M IPTG and incubated overnight at 37°C. White colonies were selected for overnight growth in LB broth with 5 μl of 50 μg/ml ampicillin at 37°C. Plasmid DNAs were isolated using a QIAprep Spin Miniprep Kit (Qiagen, Inc., Valencia, CA). Potentially recombinant plasmids were restriction digested with *Eco*RI in a reaction volume of 10 μl, containing 1 μl of *Eco*RI 10X buffer, 10 units enzyme (New England Biolabs, Inc., Beverly, MA), and

2.0 µl plasmid DNA and incubated for 2-6 hours at 37°C. Digested DNA was electrophoresed in an 1.0% agarose gel to determine if the ITS insert was present.

DNA Sequencing

Target DNAs were sequenced using primers c, d, e, and f for the *trnL-trnF* region (Taberlet et al., 1991), primers ITS2, ITS3, ITS4, and ITS5 for the ITS region (Baldwin, 1992), and universal primers SP6 and T7 in clones with an ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and then electrophoresed in an ABI 310 Genetic Analyzer following the manufacturer's protocol (Applied Biosystems, Foster City, CA). The Advantage GC cDNA PCR kit (BD Biosciences, Palo Alto, CA) was applied for some species that were difficult to sequence (Greenwell et al., in manuscript).

Editing and Alignment of Sequences

DNA sequences were manually edited and aligned visually using the computer program Sequencher 4.1 (Gene Codes Cooperation, Ann Arbor, MI). Boundaries of ITS-1, 5.8S, ITS-2, *trnL* intron, and *trnL-trnF* spacer in *Mentha* were determined by comparison with other Euasterid sequences (Baldwin, 1992; Steane et al., 1999; McDade and Schwarzbach, 2002; Beardsley and Olmstead, 2002; Ronsted et al., 2002; Zimmer et al., 2002).

Outgroup Selection

We did preliminary phylogenetic analyses using sequences provided by Dr. Javier Francisco-Ortega (Florida International University). For the *trnL-trnF* data, we included 15 *Mentha* species, 2 named hybrids, and 30 Lamiaceae taxa. Preliminary results indicated that four Mentheae genera (*Acinos, Micromeria, Satureja,* and *Thymus*) are

closely related to *Mentha*. As a result, we selected these four genera for outgroups in the final analysis. For the ITS data, we included 12 *Mentha* species, four clones of *M. canadensis*, two clones of *M. arvensis*, two clones of *M. spicata*, and 29 Lamiaceae taxa. Preliminary results showed paraphyly of *Mentha*. Consequently, 13 Mentheae genera were included, and *Satureja* was selected as the outgroup in the final analysis.

Phylogenetic Analyses

The *trnL-trnF* and ITS sequence data of *Mentha* species were evaluated for length, GC content, pairwise sequence divergence, nucleotide site variation, and parsimony-informative sites. The *trnL-trnF* and ITS sequence data were analyzed separately using PAUP* 4.0b10 (Swofford, 1998). Gaps were coded as binary characters and included in the analysis. For the *trnL-trnF* data, a Branch-and-Bound search was performed for 21 total taxa. For the ITS data, a HEURISTIC search was executed for a total of 36 taxa using RANDOM (1,000 replicates) stepwise addition of taxa followed by TBR (tree bisection-reconnection) branch swapping. For each data set, a bootstrap analysis was performed with 500 replicates to assess support for each clade, and a decay analysis was also performed using AutoDecay 4.0 (Eriksson, 1998). Sets of equally parsimonious trees were summarized using strict consensus. Tree length, Consistency Index (CI), and Retention Index (RI), were also calculated (in PAUP) excluding uninformative characters.

RESULTS

Length, GC Content, Sequence Divergence, Nucleotide Site Variation and Gaps in *Mentha*

cp *trnL-trnF* region

Length of the *trnL* intron varies from 477 (*M. aquatica*) to 490 (*M. gattefossei*) bp, and length of the *trnL-trnF* spacer ranges from 280 (*M. spicata*) to 291 (*M. aquatica*) bp in *Mentha*. Mean GC content in the *trnL* intron and *trnL-trnF* spacer is 32.7% and 37.4%, respectively. Mean pairwise divergence of sequences in the *trnL* intron and *trnL-trnF* spacer are 0.4% and 0.9%, respectively. Total number of aligned characters for the entire *trnL-trnF* region is 843, of which 502 are in the *trnL* intron, 50 are in the *trnL* 3′exon, and 291 are in the *trnL-trnF* spacer. Nucleotide site variability is 1.7% in the *trnL* intron and 4.1% in the *trnL-trnF* spacer. Of the 843 characters in the *trnL-trnF* data set, 2.0% are variable and 1.0% are parsimony-informative. One 4-bp deletion in the *trnL-trnF* spacer distinguishes *Mentha* from the outgroups. In *Mentha*, there are two parsimony-informative indels in the *trnL-trnF* region: a 1-bp insertion at position 252 in the *trnL* intron, and a 4-bp insertion at positions 146-149 in the *trnL-trnF* spacer(Fig. 6).

nrITS region

In the ITS region, length of ITS-1 ranges from 225 (*M. canadensis*) to 227 (*M. australis*) bp, and length of the ITS-2 ranges from 215 (*M. cervina*) to 232 (*M. canadensis*) bp. Length of the 5.8S region is constant (164 bp). Mean GC content in ITS-1, 5.8S, and ITS-2 are 68.0%, 55.1%, and 67.9%, respectively. Mean pairwise divergence of sequences in *Mentha* is 7.2% in ITS-1 and 6.4% in ITS-2. Aligned sequences of the ITS region yield 634 characters, of which 234 are in ITS-1, 164 are in

the 5.8S gene, and 236 are in ITS-2. Nucleotide site variability is 21.7% in ITS-1 and 19.3% in ITS-2. Nucleotide site variability in the 5.8S gene is 4.3%. In total, the ITS region contains 15.9% variable nucleotide sites and 9.7% parsimony-informative characters.

In *Mentha*, there are three parsimony-informative indels in ITS-1: two 1-bp insertions at positions 111 and 183 and a 1-bp deletion at position 112 (Fig. 7). In ITS-2, there are three parsimony-informative indels: two 1-bp deletions at positions 15 and 191, and a 1-bp insertion at position 151.

Phylogenetic Relationships of *Mentha*

cp *trnL-trnF* region

Parsimony analysis recovers 25 equally parsimonious trees of length 59 (strict consensus in Fig. 8). Excluding uninformative sites, the consistency index (CI) is 0.810 and the retention index (RI) is 0.917. All trees based on *trnL-trnF* data place the *Mentha* species and its named hybrids in the same clade with strong bootstrap (96%) and decay (4) support.

Within *Mentha*, there are six clades resolved in the *trnL-trnF* strict consensus tree. Four of the five species of sect. *Eriodontes*, (*M. australis*, *M. diemenica, M. satureoides, and M. cunninghamii*) form a well-supported clade (89% bootstrap and 2 decay) with *M. cunninghamii* sister to the others. However, the fifth member, *M. japonica*, nests within another clade with species from three other sections (*Mentha, Preslia,* and *Pulegium*). Four species (*M. japonica, M. aquatica, M. arvensis,* and *M. canadensis*) in this clade share a 4-bp insertion although this insertion cannot resolve these species as a clade. Another clade includes sect. *Mentha* taxa only (*M. longifolia, M. spicata, M.* x

rotundifolia, and *M.* x *piperita*). There is a close relationship between *M. pulegium* (sect. *Pulegium*) and *M. requienii* (sect. *Audibertia*) supported by an 86% bootstrap value and decay of 2.

nrITS region

Parsimony analysis of the ITS region yields 582 equally parsimonious trees of length 627 (strict consensus in Fig. 9) representing 13 different islands. Of the 582 trees found, 97.4% belong to a single island. Excluding uninformative sites, the CI is 0.451 and the RI is 0.638. All trees based on ITS data divide the *Mentha* species into two subclades (labeled A and B for purpose of discussion, Fig. 9) that are part of a large clade with 82% bootstrap support and decay of 4. Within this clade, 11 Mentheae genera are interspersed between the *Mentha* subclades, indicating that *Mentha* may not be monophyletic.

Within *Mentha* clade A, four (*M. australis, M. diemenica, M. satureoides, and M. cunninghamii*) of the five species of sect. *Eriodontes* form a clade (82% bootstrap value and decay of 2) that also includes *M. aquatica* of sect. *Mentha*. *Mentha japonica*, the fifth member of sect. *Eriodontes*, is one of the lineages of a trichotomy that also includes the other sect. *Eriodontes* species, and *M. arvensis* and two *M. canadensis* clones. Eight Mentheae genera are largely unresolved in a weakly supported clade that includes *Mentha* clade A. Three additional Mentheae genera (*Bystropogon*, *Acinos*, and *Ziziphora*) form a clade sister to *Mentha* clade B, again with little support. *Mentha* clade B contains species representing four of the *Mentha* sections. Four species of sect. *Mentha* (*M. suaveolens, M. spicata, M. longifolia*, and *M. canadensis*) in *Mentha* clade B

form a strongly supported subclade (100% bootstrap and decay of 9) referred to herein as the "*spicata*" clade.

Cloned ITS Region Sequences of *M. spicata* and *M. canadensis*

Based on our ITS phylogeny (Fig. 9), clones of *M. spicata* are included in the "*spicata*" clade with both of its hypothesized parents (*M. longifolia* and *M. arvensis*), plus two clones of *M. canadensis*. Comparisons of variable nucleotide sites in ITS-1, 5.8S, and ITS-2 for *M. longifolia, M. suaveolens, M. arvensis, M. spicata* clones, and *M. canadensis* clones are presented in Table 4. *Mentha spicata* is polymorphic at 13 nucleotide sites (positions 21, 54, 79, 83, 181, 196, 357, 363, 416, 449, 558, 572, and 606) based on the direct sequences. The sequences of *M. spicata* clones 1 and 9 are identical to each other and to *M. suaveolens* and *M. longifolia* except for two (positions 50 and 430) and five (positions 21, 50, 83, 363, and 416) sites, respectively. The sequences of *M. longifolia* and *M. suaveolens* differ at only six positions. At these six different nucleotide sites, *M. spicata* is polymorphic at only four (positions 21, 83, 363, and 416).

Based on the ITS phylogeny, divergent clones of *M. canadensis* occur in two distinct clades. Clones 7 and 15 group with *M. arvensis* (85% bootstrap and decay of 1) within *Mentha* clade A, and clones 8 and 25 strongly nest in the "*spicata*" clade within *Mentha* clade B. Nucleotide site polymorphisms found in the consensus sequence of *M. canadensis* are shown in Fig. 10. The sequences of *M. canadensis* clones 7 and 15 are identical to each other and to *M. arvensis* except for three sites (positions 49, 50, and 356) that are polymorphic in *M. arvensis*. The sequences of *M. canadensis* clones 8 and 25 differ at only two sites. *Mentha canadensis* clone 25 is identical to *M. spicata* clones

1 and 9, *M. suaveolens,* and *M. longifolia* except for one (position 558), three (positions 50, 430, and 558), and six (positions 21, 50, 83, 363, 416, and 558) sites, respectively. *Mentha canadensis* clone 8 is most similar to sequences of *M. spicata* clones 1 and 9, differing only at three sites (positions 558, 572, and 606). Three clones of *M. canadensis* (1, 5, and 16) not included in our analysis are chimeric (i.e., showing a mixture of nucleotides from their hypothesized parents).

Ancestral Base Chromosome Number

Based on the *trnL-trnF* region strict consensus phylogeny, the ancestral base chromosome number for *Mentha* appears to be $x = 12$. However, ITS data are less clear and imply that either $x = 9$ or $x = 10$ may be ancestral.

DISCUSSION

Phylogenetic Information of the *trnL-trnF* and ITS Sequences

In *Mentha*, the length of the *trnL-trnF* region is slightly shorter than those of previously reported Lamiales sequences, e.g., Acanthaceae (McDade and Moody, 1999; McDade et al., 2000), Phrymaceae (Beardsley and Olmstead, 2002), Plantaginaceae (Ronsted et. al., 2002), and Gesneriaceae (Zimmer et al. 2002) whereas the length of ITS-1 and ITS-2 are similar to other angiosperm sequences (Baldwin et al., 1995), and those of Lamiales, including *Clerodendrum* and *Monarda* (Lamiaceae) (Steane et al., 1999; Prather et al., 2002).

ITS region sequences in many angiosperms have a high GC content, normally ranging from ~50% in several groups to 75% in Poaceae (Baldwin et al., 1995). Mean GC content of ITS in *Mentha* is 63.7% which is within the range mentioned above and close to plants in Acanthaceae (McDade et al., 2000). Mean GC content in ITS-1 and ITS-2 is even higher (68%). This high GC content may cause difficulties in ITS region sequencing due to the formation of ITS secondary structures (Baldwin et al., 1995; McDade et al., 2000). Although all four primers (ITS2, ITS3, ITS4, and ITS5) were used in sequencing, some *Mentha* species could not be completely sequenced initially for both strands. However, Greenwell et al. (in manuscript) resolved this problem using an Advantage-GC Genomic PCR kit from BD Biosciences Company (Palo Alto, CA). This kit contains a proprietary GC Melt reagent that helps in sequencing through regions with high GC content. Although the mean GC content in the *trnL* intron and *trnL-trnF* spacer is lower (35.1%) than in ITS, we also had to use four primers (c, d, e, and f) in sequencing due to the longer length of the *trnL-trnF* region.

In *Mentha*, the *trnL-trnF* spacer has more variable nucleotide sites and more parsimony-informative characters than the *trnL* intron (Table 3) and is similar to plants in Acanthaceae (McDade and Moody, 1999). The *trnL-trnF* spacer is less conserved than the *trnL* intron due to stronger functional constraints on introns (Gielly and Taberlet, 1996; Gielly et al., 1996; McDade and Moody, 1999). However, compared to the ITS region sequences, the *trnL-trnF* region sequences are substantially less variable. Variable nucleotide positions and parsimony-informative sites of ITS-1 and ITS-2 in *Mentha* are slightly higher than in *Monarda*. The outcome that ITS-2 is less variable than ITS-1 in *Mentha* is consistent with results in other Lamiales (McDade et. al., 2000; Prather et al., 2002), Gentianales (Gielly et al., 1996) studies and angiosperms in general (Baldwin et al., 1995; Hershkovitz and Zimmer, 1996). The ITS data (CI = 0.451) show much higher levels of homoplasy than the *trnL-trnF* data (CI = 0.810). This trend is similar to data from Phrymaceae and Acanthaceae (McDade and Moody, 1999; McDade et al., 2000; Beardsley and Olmstead, 2002), and might be responsible for the overall lack of resolution and low support values in the ITS phylogeny.

Monophyly of *Mentha* and Sister Groups

The *trnL-trnF* data are useful in assessing relationships among Mentheae genera and for resolution of some *Mentha* lineages (Fig. 8). Based on these data, monophyly of *Mentha* is well supported and is therefore consistent with the classification of Harley and Brighton (1977). Conversely, this result conflicts with the classifications of both Briquet (1897) and Tucker (in manuscript). However, paraphyly of *Mentha* based on ITS data shows disagreement with all traditional classifications. Briquet (1897) treated *Mentha cervina* as *Preslia cervina*, but in our study there is no support for placing *M. cervina* in a

separate genus. According to Tucker's analysis (in manuscript) based on morphology, base chromosome number, and major essential oil components, *M. cunninghamii* was not included in the clade with other *Mentha* species. However, only one outgroup (*Micromeria brownie* var. *pilosiuscula* A. Gray) was used. Moreover, there are no support values; thus, support for the monophyly of *Mentha* is not known. In contrast, both of our data sets indicate that *M. cunninghamii* is strongly nested in a clade along with other *Mentha* sect. *Eriodontes* species, thus demonstrating that *M. cunninghamii* is closely related to the Australasian species and appropriately included in *Mentha.*

Based on our *trnL-trnF* phylogeny, the sister group of *Mentha* appears to be *Acinos, Micromeria* and/or *Thymus*. Based on our ITS phylogeny, two of the Mentheae genera (*Thymus* and *Ziziphora*) are closely related to *Mentha.* This result is consistent with ITS data from Prather et al. (2002). In a study by Wagstaff et al. (1995) using cpDNA restriction site variation, they found that *Thymbra* is the sister group of *Mentha.* These different outcomes may be due to sampling differences.

Traditional Classification and Phylogenetic Relationships among *Mentha* Species

In considering the interspecific relationships within *Mentha*, none of the sections with more than one species sampled form monophyletic groups based on either *trnL-trnF* or ITS region sequences (Figs. 8, 9), and is therefore inconsistent with existing traditional classification schemes. Some parts of our *trnL-trnF* and ITS phylogenies lack resolution, yet several clades have good support. The lack of resolution may be due to insufficient information in the *trnL-trnF* region sequences and possibly too much variation in ITS region sequences. Each section based on the classification of Harley and Brighton (1977) is discussed below.

Section *Eriodontes*

In our *trnL-trnF* and ITS phylogenies, four species (*M. australis, M. diemenica, M. satureioides,* and *M. cunninghamii*) of sect. *Eriodontes* from Australia, Tasmania, and New Zealand group together with strong bootstrap and decay support. This result is consistent with a biogeographic pattern for the Australasian species. In the *trnL-trnF* phylogeny, the last member (*M. japonica*) groups with other species in other sections. In the ITS phylogeny, although *M. japonica* forms a lineage next to other sect. *Eriodontes* species, 82% bootstrap and 2 decay values exclude it from this group. This outcome suggests that *M. japonica* should not be included in sect. *Eriodontes*.

Section *Mentha*

Although sect. *Mentha* is not monophyletic based on our *trnL-trnF* phylogeny, this result has only 51% bootstrap support. The cultivated hybrids are closely related to their hypothesized parents as expected; *M.* x *piperita* close to *M. spicata* and *M.* x *rotundifolia* close to *M. longifolia*. Based on the ITS phylogeny, members of sect. *Mentha* are separated into two distinct clades, and *M. aquatica* seems to be more closely related to species in sect. *Eriodontes* rather than species in sect. *Mentha*. Taxonomic revision of this section should be considered.

Section *Audibertia, Preslia*, and *Pulegium*,

From the *trnL-trnF* tree, *Mentha pulegium* appears more closely related to *M. requienii* from sect. *Audibertia* rather than *M.gattefossei* from sect. *Pulegium*. However, *M.gattefossei* appears closely related to *M. cervina* from sect. *Preslia* based on the ITS tree. This result supports the suggestion of Harley and Brighton (1977) of a possible close relationship between *M.gattefossei* and *M. cervina* based on morphological and

ecological similarity of these two species. Moreover, Chambers and Hummer (1994) found that *M.gattefossei* had an intermediate morphology between *M. pulegium* and *M. cervina*. Sampling the last member (*M. micrantha*) in sect. *Pulegium* may provide more information regarding these relationships.

Putative Allopolyploid Origin of *M. spicata* and *M. canadensis*

Based on our *trnL-trnF* phylogeny (Fig. 8), *M. spicata* forms a clade with *M. longifolia*. Because the *trnL-trnF* region is presumably maternally inherited (Dowling et al., 1996), *M. longifolia* may be the maternal parent of *M. spicata*. Based on the ITS phylogeny (Fig. 9), clones of *M. spicata* form a clade with both of its hypothesized parents, *M. longifolia* and *M. suaveolens*, and some clones of *M. canadensis* in the "*spicata*" clade. The ITS region sequence comparison of all *Mentha* species sampled indicates that the sequences of *M. spicata* clones are similar to both of its hypothesized parents. The sequence of *M. longifolia* is identical to *M. suaveolens* except for only six sites. Therefore, our result is consistent with the suggestion of Harley and Brighton (1977) that "*M. spicata* arose by chromosome doubling of hybrids between the two closely related and interfertile diploids *M. longifolia* and *M. suaveolens*." However, in considering the polymorphism found in *M. spicata*, only four of 13 polymorphic sites (positions 21, 83, 363, and 416) support that *M. spicata* is a hybrid between *M. longifolia* and *M. suaveolens*. The other nine polymorphic sties may have originated by independent mutation or possibly *Taq* error during PCR and sequencing. Another possibility is due to the single *M. longifolia* accession that we sampled. *Mentha longifolia* has considerable morphological diversity, and consequently is divided into multiple subspecies (Harley and Brighton, 1977; Tucker, in manuscript). Therefore, our

sample of *M. longifolia* may not adequately represent the within-species variation present in this species. Sampling more clones of *M. spicata* and more individuals of *M. spicata* as well as *M. longifolia* would help to clarify this problem.

According to Tucker and Chambers (in manuscript), *M. canadensis* is an ancient stabilized allopolyploid between *M. longifolia* and *M. arvensis*. Based on our *trnL-trnF* phylogeny (Fig. 8), *M. canadensis* groups in the same clade with one hypothesized parent (*M. arvensis*) along with four other species. Therefore, *M. arvensis* may be the maternal parent of *M. canadensis*. However, greater resolution is needed in the cpDNA phylogeny to increase confidence of this result. The outcome based on the *trnL-trnF* phylogeny is consistent with the ITS result (Fig. 9) where two clones of *M. canadensis* (clones 7 and 15) form a clade with *M. arvensis*. Moreover, according to ITS sequence comparison (Table 4), the sequences of *M. canadensis* clones 7 and 15 are identical to *M. arvensis* except for one site that is unique in *M. arvensis*. Thus, it seems quite probable that *M. arvensis* may be a parent of *M. canadensis*. Based on our ITS phylogeny, the other parent of *M. canadensis* is likely one of the three species in the "*spicata*" clade because the other two clones of *M. canadensis* (clones 8 and 25) form the "*spicata*" clade along with *M. longifolia, M. suaveolens,* and *M. spicata*. However, sequence comparison among all *Mentha* species sampled suggests that either *M. suaveolens* or *M. spicata*, which has a *M. suaveolens* allele, has the highest possibility to be the other parent of *M. canadensis* rather than *M. longifolia* as proposed by Tucker and Chambers (in manuscript) because the sequence of *M. canadensis* clone 25 is identical to *M. spicata* clones 1 and 9 and *M. suaveolens* except for one and three sites, respectively.

Some clones of *M. canadensis* show chimeric ITS repeats, a mixture of nucleotides from its hypothesized parents. Theses chimeric patterns may be the result of PCR-mediated recombination (Cronn et al., 2002), or alternatively could represent transitional stages in the concerted evolution process (Campbell et al., 1997; Alice et al., 2001)

Ancestral Base Chromosome Number in *Mentha*

Based on our *trnL-trnF* phylogeny, the ancestral base chromosome number in *Mentha* may be $x = 12$. Assuming *Mentha* is monophyletic as implied, the suggestion is that there have been at least three chromosome losses. For example, in *M. pulegium* and *M. requienii*, two losses from $x = 12$ to either $x = 10$ or $x = 9$. A third loss must have occurred in the ancestor(s) of *M. gattefossei* and *M. japonica*. However, based on our ITS phylogeny, either $x = 9$ in *Mentha* clade A or $x = 10$ in *Mentha* clade B may be the ancestral base chromosome number in *Mentha* -- thereby suggesting that there have been at least three chromosome gains. For example, a gain of two chromosomes from $x = 10$ to $x = 12$ in the "*spicata*" clade, a one-chromosome gain from $x = 9$ to $x = 10$ in *M. japonica*, and a three-chromosome gain from $x = 9$ to $x = 12$ in *M. australis*. Determination of ancestral base chromosome number in *Mentha* is complicated because base chromosome number is extremely variable both within and among Mentheae genera. For example, *Satureja* includes species with base chromosome numbers of $x = 9$, 10, 11, and 15. Increased taxon sampling of Mentheae and greater resolution of relationships would substantially aid in assessing patterns of chromosome evolution in *Mentha* and Mentheae.

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Table 1. Traditional classification schemes of *Mentha* species.

Briquet (1897)	**Harley & Brighton (1977)**	**Tucker (in manuscript)**
Genus *Mentha* L.	Genus *Mentha*	Genus *Mentha*
Subgenus *Pulegium* (Mill.) Lamk. Et DC. Sect. *Eupulegia* Briq. *M. pulegium* L. Sect. *Audibertiae* Briq. *M. requienii* Benth. Subgenus *Menthastrum* Coss. Sect. *Verticillatae* L. *Eriodontes* Benth. *M. cunninghamii* Benth. *M. satureioides* Br. *M. repens* (Hook.) Briq. *M. serpyllifolia* Benth. *Tubulosae* Briq. *M. diemenica* Spreng. *M. australis* Benth. *Grandiflorae* Briq. *M. grandiflora* Benth. *Laxiflorae* Briq. *M. laxiflora* Benth. *Arvensis* Benth. *M. arvensis* L. Sect. *Capitatae* L. *M. aquatica* L. Sect. *Spicatae* L. *Silvestres* Malinv. *M. viridis* L. *M. longifolia* Huds. *Rotundifoliae* Malinv. *M. microphylla* C. Koch *M. rotundifolia* L. Genus *Preslia* Opiz *P. cervina* (L.) Fres.	Sect. *Audibertia* (Benth.) Briq. *M. requienii* Benth. Sect. *Eriodontes* Benth. *M. cunninghamii* Benth. *M. satureioides* R.Br. Sect. *Mentha* *M. aquatica* L. *M. arvensis* L. *M. longifolia* (L.) Huds. *M. microphylla* C. Koch *M. spicata* L. *M. suaveolens* Ehrh. Sect. *Preslia* (Opiz) Harley *M. cervina* L. Sect. *Pulegium* (Miller) DC. *M. gattefossei* Maire *M. pulegium* L. *M. micrantha* (Benth.) Schost-Desjat.	Sect. *Pulegium* (Mill.) Lam. & DC. *M. australis* R.Br. *M. cervina* L. *M. diemenica* Spreng. *M. gattefossei* Maire *M. grandiflora* Benth. *M. requienii* Benth. *M. pulegium* L. *M. repens* (Hook. f.) Briq. Sect. *Mentha* *M. aquatica* L. *M. arvensis* L. *M. canadensis* L. *M. dahurica* Fisch. ex Benth *M. japonica* (Miq.) Makino *M. laxiflora* Benth. *M. longifolia* (L.) L. *M. satereioides* R.Br. *M. spicata* L. *M. suaveolens* Ehrh.

Table 2. *Mentha* accessions and outgroups used in this study. Classification of *Mentha* follows Harley and Brighton (1997). Source = United States Department of Agriculture-Agricultural Research Service National Clonal Germplasm Repository. Chromosome counts from Chambers and Hummer (1994), Tucker (in manuscript), Index to Plant Chromosome Numbers (IPCN) from Missouri Botanical Garden (www.mobot.org/W3T/Search/ipcn.html). Morphological vouchers have been deposited in the Western Kentucky University herbarium (WKU) (Holmgren et al., 1990). Outgroup sequences provided by Dr. Javier Francisco-Ortega. All sequences generated in this study will be deposited in GenBank (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health).

Taxon	2n (*x*) =	Origin	Source	Voucher
Section *Audibertia*				
M. requienii	18 (9)	England	PI 557781	Bunsawat 2002-20
Section *Eriodontes*				
M. australis	72 (12)	Australia	PI 617498	Bunsawat 2002-43
M. cunninghamii	72 (12)	New Zealand	PI 617481	Bunsawat 2002-19
M. diemenica	120 (12)	Australia	PI 617482	Bunsawat 2002-23
M. japonica	50 (10)	Japan	PI 617475	-
M. satureioides	144 (12)	Australia	PI 617500	-
Section *Mentha*				
M. aquatica	96 (12)	Germany	PI 557572	Bunsawat 2002-57
M. arvensis	72/96 (12)	Europe	PI 557918	Bunsawat 2002-25
M. canadensis	96 (12)	Brazil	PI 277803	Bunsawat 2002-58
M. longifolia	24/48 (12)	Europe	PI 557755	Bunsawat 2002-38
M. spicata	36/48 (12)	unknown	PI 557885	Bunsawat 2002-40
M. suaveolens	24 (12)	France	PI 557911	Bunsawat 2002-49
Section *Pulegium*				
M. gattefossei	40 (10)	Morocco	PI 557639	Bunsawat 2002-45
M. pulegium	20 (10)	OR-U.S.A.	PI 557771	Bunsawat 2002-36
Section *Preslia*				
M. cervina	36 (12/18)	Southern Europe	PI 557634	Bunsawat 2002-05

Taxon	2n (x)	Origin	Source	Voucher
Named Hybrids				
M. x *piperita* cv. 'Todd Mitcham'	36/42/72/120	DC-USA	PI 557973	Bunsawat 2002-55
M. x *rotundifolia*	24 (12)	France	PI 557638	Bunsawat 2002-39
Lamiaceae Genera				
Acinos	NA	NA		
Agastache	NA	NA		
Blephilia	NA	NA		
Bystropogon odo	NA	NA		
Bystropogon punctata	NA	NA		
Calamintha	NA	NA		
Cedronella	NA	NA		
Conradina	NA	NA		
Cuminia	NA	NA		
Dicerandra	NA	NA		
Dracocephalum	NA	NA		
Hesperozygis	NA	NA		
Horminum	NA	NA		
Hyptis	NA	NA		
Hyssopus	NA	NA		
Lallemantia	NA	NA		
Lamium	NA	NA		
Lavandula	NA	NA		
Lepechinia	NA	NA		
Lycopus	NA	NA		
Micromeria	NA	NA		
Minthostachys	NA	NA		
Monarda	NA	NA		
Monardella	NA	NA		

Taxon	2n (x)	Origin	Source	Voucher
Nepeta	NA	NA		
Origanum	NA	NA		
Orthosiphon	NA	NA		
Perovskia	NA	NA		
Piloblephis	NA	NA		
Plectranth	NA	NA		
Prunella	NA	NA		
Pycnanthemun	NA	NA		
Rosmarinus	NA	NA		
Salvia	NA	NA		
Satureja	NA	NA		
Thymus	NA	NA		

Table 3. ITS and *trnL-trnF* region sequence characteristics in *Mentha*.

	trnL intron	*trnL-trnF* spacer	ITS-1	5.8S	ITS-2
Length (in base pairs)	477 - 490	280 - 291	225 - 227	164	215 - 232
% Mean GC content	32.7 ± 0.0	37.4 ± 0.0	68.0 ± 0.01	55.1 ± 0.0	67.9 ± 0.01
% Mean sequence divergence	0.4	0.9	7.2	1.0	6.4
Variable nucleotide sites (%)	9 (1.7)	12 (4.1)	51 (21.7)	7 (4.3)	47 (19.3)
Parsimony-informative sites (%)	4 (0.8)	6 (2.0)	31 (13.2)	3 (1.8)	30 (12.3)

Table 4. Variable ITS region nucleotide sites for the "*spicata*" clade species (*M. longifolia*, *M. suaveolens*, and *M. spicata*) and *M. arvensis*. Nucleotides found in "*spicata*" clade species are in uppercase and bold, and nucleotides present in *M. arvensis* are in lowercase. Corresponding states in the putative hybrids, *M. canadensis* and *M. spicata*, are similarly indicated. The lowercase "c" indicates a clone.

	ITS-1																												
Taxon	21	28	34	39	47	49	50	54	55	60	61	68	74	75	79	83	86	93	96	106	107	112	142	181	193	196	207	211
M. longifolia	**C**	**A**	**T**	**T**	**C**	**A**	**T**	**A**	**A**	**G**	**C**	**T**	**T**	**G**	**T**	**T**	**C**	**G**	**T**	**T**	-	**T**	**T**	**C**	**A**	**G**	**C**	**C**
M. suaveolens	**T**	**A**	**T**	**T**	**C**	**A**	**A**	**A**	**A**	**G**	**C**	**T**	**T**	**G**	**T**	**C**	**C**	**G**	**T**	**T**	-	**T**	**T**	**C**	**A**	**G**	**C**	**C**
M. spicata c1, c9	**T**	**A**	**T**	**T**	**C**	**A**	**C**	**A**	**A**	**G**	**C**	**T**	**T**	**G**	**T**	**C**	**C**	**G**	**T**	**T**	-	**T**	**T**	**C**	**A**	**G**	**C**	**C**
M. spicata c10	**T**	**A**	**T**	**T**	**C**	**A**	**C**	**G**	**A**	**A**	**C**	**T**	**T**	**G**	**C**	**T**	**C**	**G**	**T**	**T**	-	**T**	**T**	**T**	**A**	**T**	**C**	**C**
M. spicata c20	**C**	**A**	**T**	**T**	**C**	**A**	**C**	**G**	**A**	**G**	**C**	**T**	**T**	**G**	**C**	**T**	**C**	**G**	**T**	**T**	-	**T**	**T**	**T**	**A**	**T**	**C**	**C**
M. spicata c5	**C**	**A**	**T**	**T**	**C**	**A**	**C**	**G**	**A**	**G**	**C**	**T**	**T**	**G**	**C**	**T**	**C**	**G**	**T**	**T**	-	**T**	**C**	**T**	**A**	**T**	**C**	**C**
M. canadensis c25	**T**	**A**	**T**	**T**	**C**	**A**	**C**	**A**	**A**	**G**	**C**	**T**	**T**	**G**	**T**	**C**	**C**	**G**	**T**	**T**	-	**T**	**T**	**C**	**A**	**G**	**C**	**C**
M. canadensis c8	**T**	**A**	**T**	**T**	**C**	**A**	**C**	**A**	**A**	**G**	**C**	**T**	**T**	**G**	**T**	**C**	**C**	**G**	**T**	**T**	-	**T**	**T**	**C**	**A**	**G**	**C**	**C**
M. canadensis c1	**T**	**A**	**T**	**T**	**C**	**A**	**C**	**A**	**A**	**G**	**C**	**T**	**T**	**G**	**T**	**C**	**C**	**G**	**T**	**T**	-	**T**	**T**	**C**	**A**	**G**	**C**	**C**
M. canadensis c16	c	t	c	g	g	**A**	**C**	**A**	c	**G**	a	c	c	a	c	t	t	t	c	g	g	c	c	t	**A**	**G**	**C**	**C**
M. canadensis c5	c	t	c	g	g	**A**	**C**	**A**	c	**G**	a	c	c	a	c	t	t	t	c	g	g	c	c	t	g	**G**	t	g
M. canadensis c7, c15	c	t	c	g	g	**A**	**C**	**A**	c	**G**	a	c	c	a	c	t	t	t	c	g	g	c	c	t	g	**G**	t	g
M. arvensis	c	t	c	g	g	r	y	a	c	g	a	c	c	a	c	t	t	t	c	g	g	c	c	t	g	g	t	g

	5.8S				ITS-2																							
	3	3	3	3	4	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5	6
	5	5	6	7	0	1	1	2	3	4	4	4	6	6	8	0	3	4	4	4	5	7	7	7	7	9	9	9	0
	6	7	3	3	7	6	9	0	0	1	3	9	4	6	3	5	7	2	3	5	8	2	6	7	8	6	7	9	6
M. longifolia	**A**	**C**	**C**	**C**	**T**	**G**	**C**	**A**	**T**	**G**	**T**	**T**	**T**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**T**	**A**	**C**	**A**	**C**	**A**	**C**	**T**	**T**
M. suaveolens	**A**	**C**	**T**	**C**	**T**	**A**	**C**	**A**	**C**	**G**	**T**	**T**	**T**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**T**	**A**	**C**	**A**	**C**	**A**	**C**	**T**	**T**
M. spicata c1, 9	**A**	**C**	**T**	**C**	**T**	**A**	**C**	**A**	**T**	**G**	**T**	**T**	**T**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**T**	**A**	**C**	**A**	**C**	**A**	**C**	**T**	**T**
M. spicata c10	**A**	**T**	**C**	**C**	**T**	**G**	**C**	**A**	**T**	**G**	**T**	**C**	**T**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**T**	**A**	**C**	**A**	**C**	**A**	**C**	**T**	**T**
M. spicata c20	**A**	**C**	**T**	**C**	**T**	**A**	**C**	**A**	**T**	**G**	**T**	**T**	**T**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**T**	**A**	**C**	**A**	**C**	**A**	**C**	**T**	**T**
M. spicata c5	**A**	**C**	**T**	**C**	**T**	**A**	**C**	**A**	**T**	**G**	**T**	**T**	**T**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**C**	**C**	**C**	**A**	**C**	**A**	**C**	**T**	**C**
M. canadensis c25	**A**	**C**	**T**	**C**	**T**	**A**	**C**	**A**	**T**	**G**	**T**	**T**	**T**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**A**	**C**	**A**	**C**	**T**	**T**
M. canadensis c8	**A**	**C**	**T**	**C**	**T**	**A**	**C**	**A**	**T**	**G**	**T**	**T**	**T**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**C**	**C**	**C**	**A**	**C**	**A**	**C**	**T**	c
M. canadensis c1	**A**	**C**	**T**	**C**	**T**	**A**	**C**	**A**	**T**	**G**	**T**	**T**	**T**	**C**	a	g	-	t	g	t	t	**A**	-	g	a	t	a	g	c
M. canadensis c16	**A**	**C**	**T**	**C**	c	g	t	c	**T**	c	c	**T**	c	t	a	g	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**A**	**C**	**A**	**C**	**T**	**T**
M. canadensis c5	**A**	t	c	**C**	**T**	**A**	**C**	**A**	**T**	**G**	**T**	**T**	**T**	**C**	a	g	-	t	g	t	t	**A**	-	g	a	t	a	g	c
M. canadensis c7, c15	**A**	t	c	t	c	g	t	c	**T**	c	c	**T**	c	t	a	g	-	t	g	t	t	**A**	-	g	a	t	a	g	c
M. arvensis	r	t	c	t	c	g	t	c	t	c	c	t	c	t	a	g	-	t	g	t	t	a	-	g	a	t	a	g	c

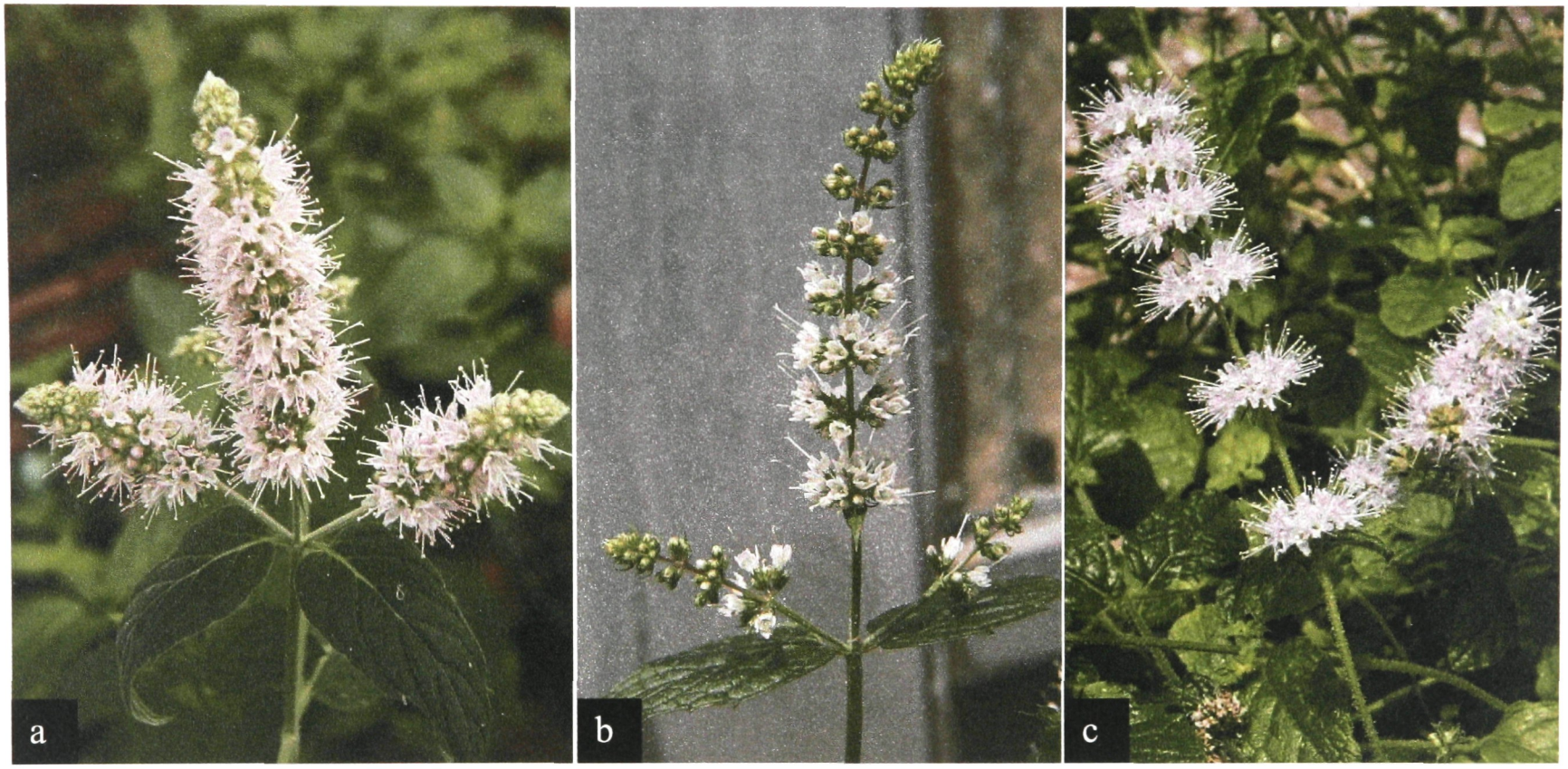

Figure 1. Photographs of putative hybrid, *Mentha spicata* (b) and its hypothesized parents, *M. longifolia* (a) and *M. suaveolens* (c).

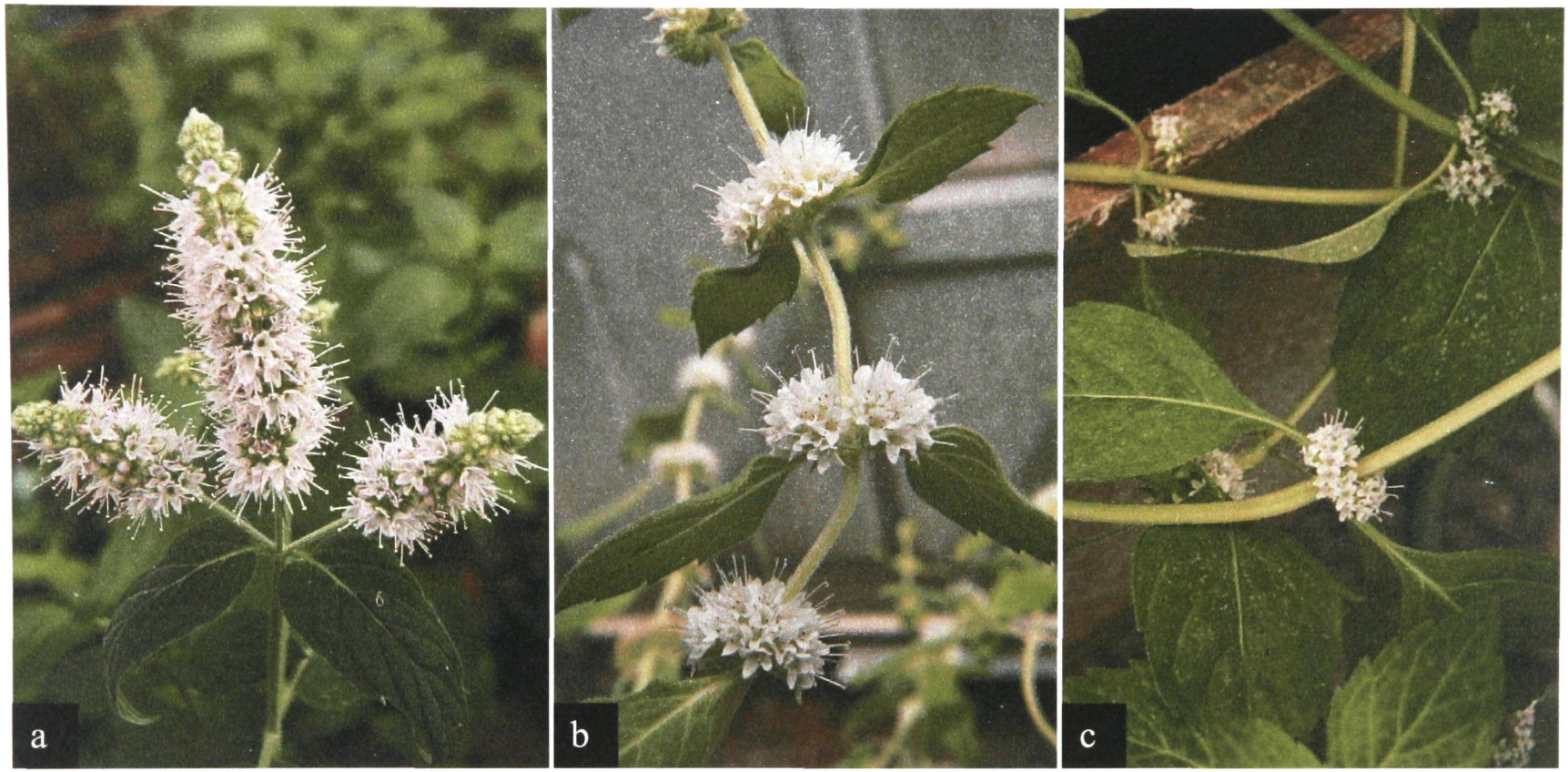

Figure 2. Photographs of putative hybrid, *Mentha canadensis* (b) and its hypothesized parents, *M. longifolia* (a) and *M. arvensis* (c).

Figure 3. Photographs of species of global conservation interest, *Mentha gattefossei* (a) and *M. requienii* (b).

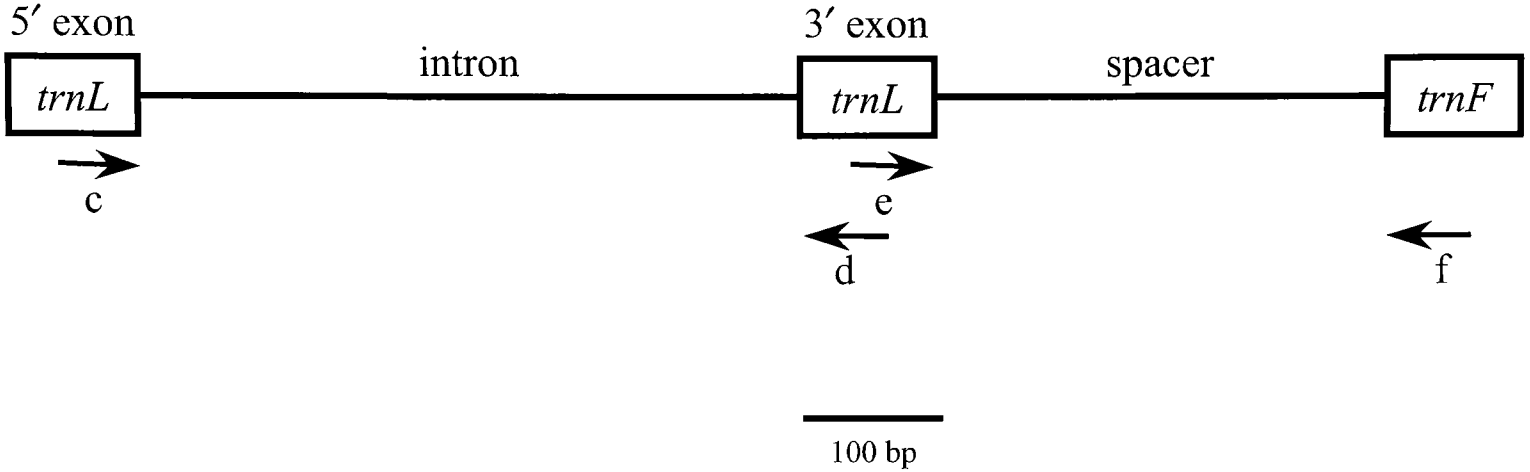

Figure 4. Organization of *trnL-trnF* region modified from Taberlet et al. (1991). Coding regions are shown as the boxes. Arrows represent orientation and position of primers (c, d, e, and f).

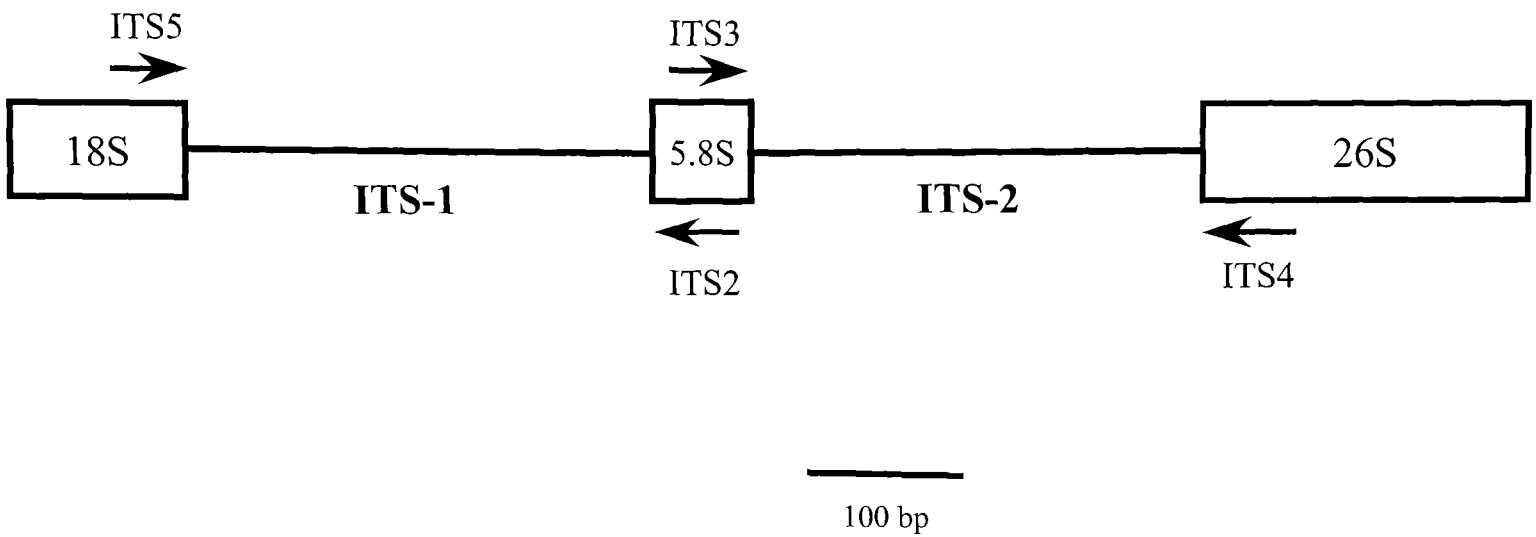

Figure 5. Organization of ITS region modified from Baldwin (1992). Coding regions are shown as the boxes. Arrows represent orientation and position of primers (ITS2, ITS3, ITS4, and ITS5).

***trnL* intron**

```
                        1         2         3         4         5         6         7         8
                        0         0         0         0         0         0         0         0
M. aquatica     ATGGAAACTT ACTAAGTGAT AACTTTCAAA TTCAGAGAAA CCCCGGAATT AATAAAAATG GGCAATCCTG AGCCAAATCC
M. arvensis     .......... .......... .......... .......... .......... .......... .......... ..........
M. australis    .......... .......... .......... .......... .......... .......... .......... ..........
M. cervina      .......... .......... .......... .......... .......... .......... .......... ..........
M. gattefossei  .......... .......... .......... .......... .......... .......... .......... ..........
M. japonica     .......... .......... .......... .......... .......... .......... .......... ..........
M. longifolia   .......... .......... .......... .......... .......... .......... .......... ..........
M. suaveolens   .......... .......... .......... .......... .......... .......... .......... ..........

                                  1         1         1         1         1         1         1
                        9         0         1         2         3         4         5         6
                        0         0         0         0         0         0         0         0
M. aquatica     TGTTTTCCCA AAACAAAGGT TTCAAAAAAC GAAAAAAA-- ----GGATAG GTGCAGAGAC TCAATGGAAG CTGTTCTAAC
M. arvensis     .......... .......... .......... ........-- ----...... .......... .......... ..........
M. australis    .......T.. .......... .......... A.......A- ----...... .......... .......... ..........
M. cervina      .......... .......... .......... ........AA AAAA...... .......... .......... ..........
M. gattefossei  .......... .......... .......... ........AA ----...... .......... .......... ..........
M. japonica     .......... .......... .......... ........-- ----...... .......... .......... ..........
M. longifolia   .......... .......... .......... ........A- ----...... .......... .......... ..........
M. suaveolens   .......... .......... .......... ........AA ----...... .......... .......... ..........

                        1         1         1         2         2         2         2         2
                        7         8         9         0         1         2         3         4
                        0         0         0         0         0         0         0         0
M. aquatica     AAATGGAGTT GACTGCGTCG GTAGAGGAAT CTTTCCATGG AAACTTTATT TTATAAAGGA TGAAAGATAA ACGCATCTAT
M. arvensis     .......... .......... .......... .......... .......... .......... .......... ..........
M. australis    .......... .......... .......... .......... .......... .......... .......... ..........
M. cervina      .......... .......... .......... .......... .......... .......... .......... ..........
M. gattefossei  .......... .......... .......... .......... .......... .......... .......... ..........
M. japonica     .......... .......... .......... .......... .......... .......... .......... ..........
M. longifolia   .......... .......... .......... .......... .......... .......... .......... .....C....
M. suaveolens   .......... .......... .......... .......... .......... .......... .......... ..........
```

Figure 6. Aligned sequences of the *trnL* intron and *trnL-trnF* spacer in eight *Mentha* species illustrating parsimony-informative indels. Dots represent the same nucleotide present in *M. aquatica* and dashes represent gaps.

```
                    2          2          2          2          2          3          3          3
                    5          6          7          8          9          0          1          2
                    0          0          0          0          0          0          0          0
M. aquatica         TGAATACTAT A-TCAAATGA TTAATGTTGG CCCGAATCTA TTTTTTTAAT ATGAAAATGA AAAAATGGAA AAATCGGTGT
M. arvensis         .......... .-........ .......... .......... .......... .......... .......... ..........
M. australis        .......... .-........ .......... .......... .......... .......... .......... ..........
M. cervina          .......... .A........ .......... .......... .......... .......... .......... ..........
M. gattefossei      .......... .A........ .......... .......... .......... .......... .......... ..........
M. japonica         .......... .-........ .......... .......... .......... .......... .......... ..........
M. longifolia       .......... .-........ .......... .......... .......... .......... .......... ..........
M. suaveolens       .......... .-........ .......... .......... .......... .......... .......... ..........

                    3          3          3          3          3          3          3          4
                    3          4          5          6          7          8          9          0
                    0          0          0          0          0          0          0          0
M. aquatica         GAATTTTTTT CACGTTGAAG AAAAAATAGA ATATTCATCA ACTCATTCAC TCCGTAGTCC GATAGATCTT TT-----AAA
M. arvensis         .......... .......... .......... .......... .......... .......... .......... ..-----...
M. australis        .......... .......... .......... .......... .......... .......... .......... ..-----...
M. cervina          .......... .......... .......... .......... .......... .......... .......... ..-----...
M. gattefossei      .......... .......... .......... .......... .......... ......A... .......... ..ATTTT...
M. japonica         .......... .......... .......... .......... .......... .......... .......... ..-----...
M. longifolia       .......... .......... .......... .......... .......... .......... .......... ..-----...
M. suaveolens       .......... .......... .......... .......... .......... .......... .......... ..-----...

                    4          4          4          4          4          4          4          4
                    1          2          3          4          5          6          7          8
                    0          0          0          0          0          0          0          0
M. aquatica         GAACTTATT- ----AATCGG ACGAGAATAA AGATAGAGTC CCATTCTACA TGCTACATAT CAATACCGGC AACAATGAAA
M. arvensis         .........- ----...... .......... .......... .......... .......... .......... ..........
M. australis        .........- ----...... .......... .......... .......... .......... .......... ..........
M. cervina          .........- ----...... .......... .......... .......... .......... .......... ..........
M. gattefossei      .........T TATT...... .......... .......... .......... .......... .......... ..........
M. japonica         .........- ----...... .......... .......... .......... .......... .......... ..........
M. longifolia       .........- ----...... .......... .......... .......... .......... .......... ..........
M. suaveolens       .........- ----...... .......... .......... .......... .......... .......... ..........
```

Figure 6. continued

```
                          4
                          9
                          0
M. aquatica       TTTATAGTAA GAGG
M. arvensis       .......... ....
M. australis      .......... ....
M. cervina        .......... ....
M. gattefossei    .......... ....
M. japonica       .......... ....
M. longifolia     .......... ....
M. suaveolens     .......... ....
```

***trnL-trnF* spacer**

```
                          1          2          3          4          5          6          7          8
                          0          0          0          0          0          0          0          0
M. aquatica       AAAAGCCTAT TTGACCCCTA AAATATTTAC CCTATCCCCC TTCTTTTTCG TTAGCGGTTC CAAATTCCCT TATCCTTCTG
M. arvensis       .......... .......... .......... .......... .......... .......... .......... ..........
M. australis      .......... .......... .......... .......... .......... .......... .......... ..........
M. cervina        .......... .......... .......... .......... .......... .....A.... .......... ..........
M. gattefossei    .......... .......... .......... .......... .......... .......... .......... ..........
M. japonica       .......... .......... .......... .......... .......... .......... .......... ..........
M. longifolia     .......... .......... .......... .......... C......... .......... .......... ..........
M. suaveolens     .......... .......... .......... .......... .......... .......... .......... ..........

                                    1          1          1          1          1          1          1
                          9         0          1          2          3          4          5          6
                          0         0          0          0          0          0          0          0
M. aquatica       ATTCTTTGAC AAACGTATTT GGGCATAAAT GACTTTCTCT TATCACATGT GATATAGAAT ACACACACAT TCCAAATGAA
M. arvensis       .......... .......... ....G..... .......... .......... .......... .......... ..........
M. australis      .......C.. .......... ....G..... .......... .......... .......... .....----. ..........
M. cervina        .......... .......... .......... .......... .......... .......... .....----. ..........
M. gattefossei    .......... .......... ....G..... .......... .......... .......... .....----. ..........
M. japonica       .......... .......... ....G..... .......... .......... .......... .......... ..........
M. longifolia     .......... .......... ....G..... .......... .......... .......... .....----. ..........
M. suaveolens     .......... .......... ....G..... .......... .......... .......... .....----. ..........
```

Figure 6. continued

```
                          1          1          1          2          2          2          2          2
                          7          8          9          0          1          2          3          4
                          0          0          0          0          0          0          0          0
M. aquatica       GCAATGAATG CCGATATGAA TGAATAGCAT TGAAATTACA GGACTCGGAG AAAACTTTGT AATCCCCCGC CTTTAATTGA
M. arvensis       .......... .......... .......... .......... .......... .......... .......... ..........
M. australis      .......... .......... .......... .......... .......... .......... .........T ..........
M. cervina        .......... .......... .......... .......... .......... .......... .......... ..........
M. gattefossei    .......... .......... .......... .......... .......... .......... .......... ..........
M. japonica       .......... .......... .......... .......... .......... .......... .......... ..........
M. longifolia     .......... .......... .......... .......... .......... .......... .........T ..........
M. suaveolens     .......... .......... .......... .......... .......... .........C .........T ..........

                          2          2          2          2          2
                          5          6          7          8          9
                          0          0          0          0          0
M. aquatica       CATCGACTCC AGTCATCTAA TAAAATGAGG GTGGGATGCT ACATTGGAAA T
M. arvensis       .......... .......... .......... .......... .......... .
M. australis      .......... .......... .......... .......A.. .......... .
M. cervina        .......... .......... .......... .......... .......... .
M. gattefossei    .......... .......... .......... .......... .......... .
M. japonica       .......... .......... .......... .......... .......... .
M. longifolia     .......... .......... .......... .......... .......... .
M. suaveolens     .......... .......... .......... .......... .......... .
```

Figure 6. continued

ITS-1

```
                          1          2          3          4          5          6          7          8
                          0          0          0          0          0          0          0          0
M. australis      TCGAAACCTG CAAAGCAGAC CGCGAACTCG TAACTAACGC CGCGGGGCGC GGCACGGGGG AG---ACCCC CT-GCMGCGT
M. arvensis       .......... .......... .......... .......... ........A. .......... ..---..... ..-..C..A.
M. cervina        ....G..... .......... .......A.. ........A. ......A... .......... C.---..... ..T-.C....
M. gattefossei    ....T..... .......... .......A.. ....C...AT ......C.A. ...G.....T C.ATT...G. ---..C....
M. longifolia     .......... .......... .......A.. ...T....T. ......C.A. ....A..... C.---..... T.-..C.T..
M. pulegium       .......... .......... .......A.. ...T....A. ......C... .......... C.---..... ..-..C....
M. requienii      .......... .......... .......A.. ........A. ......C... ...G...... C.---..... ..-..C...G

                                    1          1          1          1          1          1          1
                          9         0          1          2          3          4          5          6
                          0         0          0          0          0          0          0          0
M. australis      CCCGTCTCCT GCCGGCGTGC CCCCTCGGGG GCACGCCGTG CGGGCTAACT AA-CCCCGGC GCGGAACGCG CCAAGGAAAA
M. arvensis       .......... ......T... T......... .......... .........G ..-....... .......... ..........
M. cervina        ....C....C .........T .........T -.G.....C. .........G ..-....... .......... ..........
M. gattefossei    .........C .........T T.........T -....T..C. .........G ..-....... .......... ..........
M. longifolia     ..T......C .........T T.........T -....T.... .........G ..-....... ......T... ..........
M. pulegium       A........C .....T.CT. T........A .-...T.... .........G ..A....... ......T... ..........
M. requienii      .........C ........T. T........C .-...T.... .........G ..-....... ......T... ..........

                          1          1          1          2          2          2          2
                          7          8          9          0          1          2          3
                          0          0          0          0          0          0          0
M. australis      CCAAA-CGTA GCGTCCGCCC CC-GGCATCC CGTTCGCGGG GCGTGCCGTG GGATCGGGCG TCTATCAAAA TGTC
M. arvensis       .....-..A. .....T.... ..-....... .......... .......... .......... .........- ....
M. cervina        .....A-.G. ...C...... ..C....... .......... .T......C. ...C...... .........- ....
M. gattefossei    .A...-..G. .......... .GCT...C.. .........A ........C. ...C...C.. .........- ....
M. longifolia     .....-..A. .......... ..-....C.. .........A .......... ...C...C.. .........- ....
M. pulegium       .....-..A. .......... ..-....C.. .........T ........C. .......C.. .........- ....
M. requienii      .....-..A. .......... ..-....... .........A .......... ....T..C.. .........- ....
```

Figure 7. Aligned sequences of ITS-1 and ITS-2 in seven *Mentha* species illustrating parsimony-informative indels. Dots represent the same nucleotide present in *Mentha australis* and dashes represent gaps.

ITS-2

```
                          1          2          3          4          5          6          7          8
                          0          0          0          0          0          0          0          0
M. australis     ATCGCGTCGC CCCC-A-TCC CCGCGCACAT CGC-CGGGCG GTCGGGGG-C GGACACTGGC CTCCCGTGCG CCTCGGCGTG
M. arvensis      .......... ....C.-C.. ......G... ...-...... ..T.....-. .......... .......... ..........
M. cervina       .........T ....C.-C.. ......G..C T..-...... ..T.....G. ...G...... .C........ ........C.
M. gattefossei   .......... ....-.-... ......T..A A..C....T. ..T.....-. ...G.T.... .......... ...GT.....
M. longifolia    .......... ....C.-... ......G..C A..-...... ..T.....-. ...G.T.... .......... ......T.C.
M. pulegium      .......... ....C.A... .......A.. ...-...... ..G.....-T ...G.T.... .......... ........C.
M. requienii     .......... ....C.-C.. ......G... ...-...... ..T.....-. .......... .......... ..........

                                    1          1          1          1          1          1          1
                          9         0          1          2          3          4          5          6
                          0         0          0          0          0          0          0          0
M. australis     CGGCCGGCCC AAATGGGATC CCCGGGCGAC TGGCGTCGCG ACAAGTGGTG GTTGAACGTC TCAATCTCTC -TCGTGGTCG
M. arvensis      .......... .....A.... .......... .......... .......... .......A.. .......... -.........
M. cervina       .......... ...A.C.... ........T. .......A.. .......... .......A.. .......... C...C.....
M. gattefossei   .......... .....C.... ........T. .......A.. .......... .......A.A .......... C...C.....
M. longifolia    .......... .....C.... .......... .......A.. .......... .......A.. .......... C...CA.C..
M. pulegium      .......... .....C.... ........T. .......A.. .......... .......A.. .......... C...CA....
M. requienii     .......... .....A.... .......... .......... .......... .......A.. .......... -.........

                          1          1          1          2          2          2          2
                          7          8          9          0          1          2          3
                          0          0          0          0          0          0          0
M. australis     TGCCRCCGTG TCGTCCCGTA CGGGAATCGA -AAATGACCC AACGGTGCTC GGCGCGAATA GCGTCTCACC TTCGA
M. arvensis      ....G..... .......... .......... -...C..... .........A .........C. .......... .....
M. cervina       ....G....A C.......A. G.......A. CG..A..... ......---- ---------- ----T.T... .....
M. gattefossei   ....G....A C.......C. A.......C. CG........ .......T-- -......GC. ...C...... .....
M. longifolia    ....G..... .......... ........C. C...C..... .........A. .T........ .......... .....
M. pulegium      ....G..... C..-...... ........CG A...C..... .........G. .C.....GCG .......... .....
M. requienii     ....G..... .......... .........- A...C..... .........A .........C. .......... .....
```

Figure 7. continued

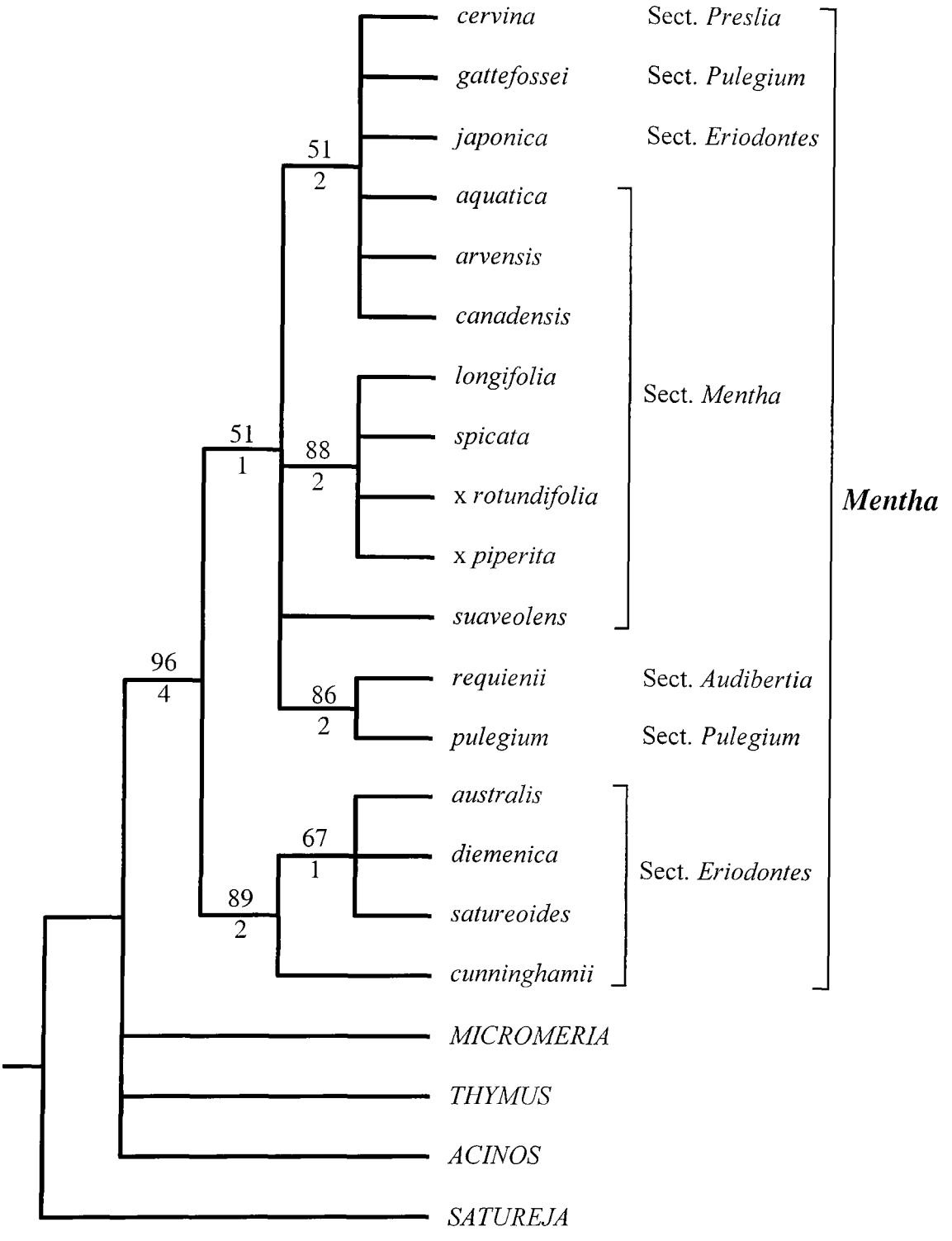

Figure 8. Strict consensus phylogeny of 25 equally parsimonious trees based on chloroplast *trnL-trnF* sequences. CI = 0.810 and RI = 0.917. *Mentha* taxa are shown in lowercase and outgroups are shown in uppercase. Numbers above and below branches are bootstrap and decay values, respectively.

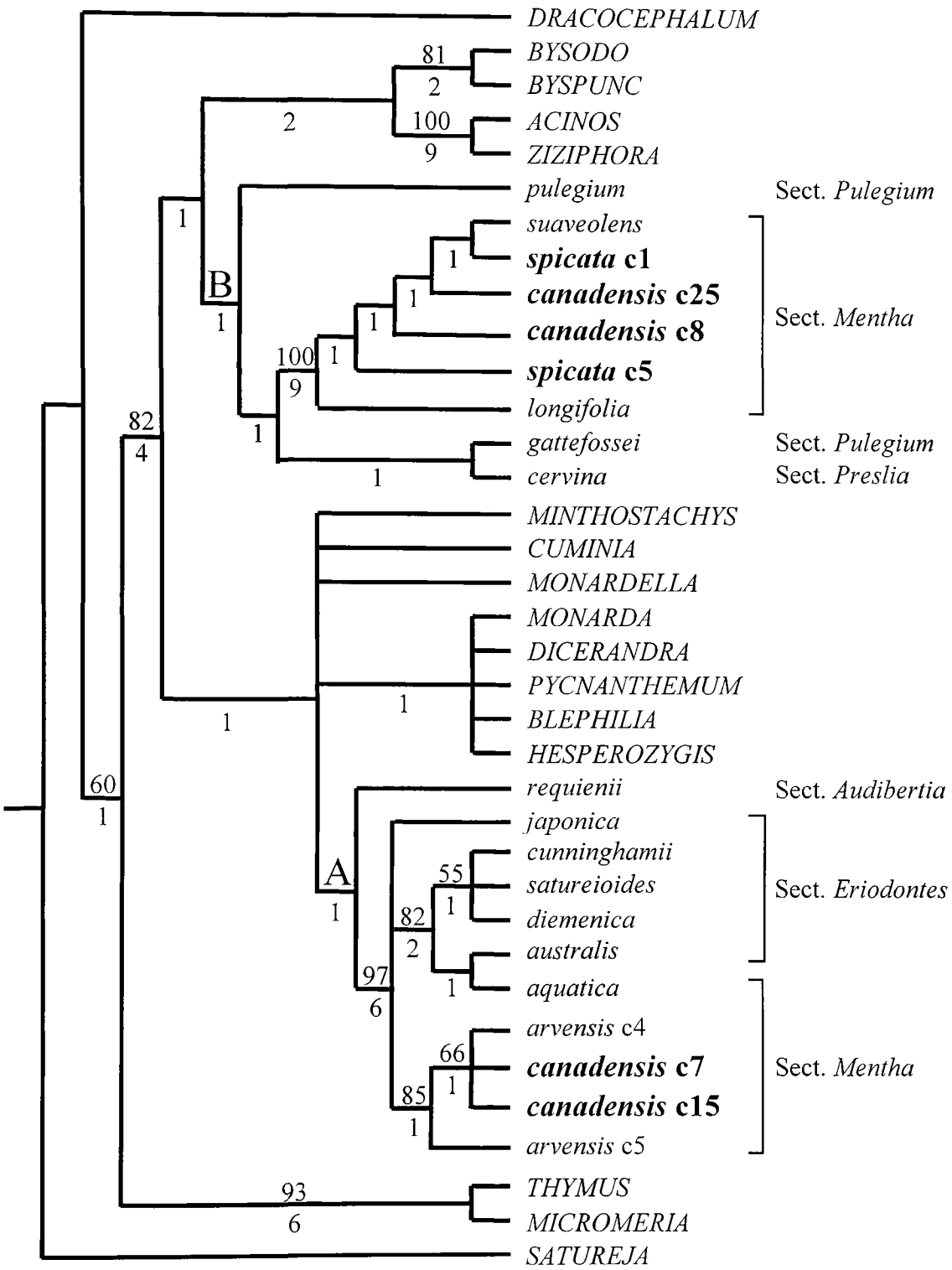

Figure 9. Strict consensus phylogeny of 582 equally parsimonious trees based on nuclear ribosomal ITS sequences. CI = 0.451 and RI = 0.638. *Mentha* taxa are shown in lowercase and outgroups are shown in uppercase. Numbers above and below branches are bootstrap and decay values, respectively. Cloned sequences of *M. canadensis* and *M. spicata* are highlight in bold.

Figure 10. *Mentha canadensis* consensus sequence. Boundaries for ITS-1, 5.8S gene, and ITS-2 were determined from Baldwin (1992). Fifty-seven nucleotide site polymorphisms in *M. canadensis* are bolded and underlined. (R = A/G, Y = C/T, K = G/T, M = A/C, S = G/C, and W = A/T) The "*" at site 526 represents a gap/thymine polymorphism and the "+" at sites 537 and 575 represents a gap/cytosine polymorphism.

ITS-1 →

1 TCGAAACCTG CAAAGCAGAC **Y**GCGAAC**W**CG TAA**Y**TAAC**K**C CGCGGG**S**CAC GGCA**M**GGGGG

61 **M**GACCCC**Y**TG CCG**YR**TCC**Y**G TC**Y**CC**Y**GCCG GC**K**TG**Y**TCCC TCGGG**KG**CAC G**Y**CGTGCGGG

121 CTAACGAACC CCGGCGCGGA A**Y**GCGCCAAG GAAAACCAAA CGAAGCGTCC GCCCCCGGC**R**

5.8S →

181 **Y**CCCGTTCGC GG**R**GCGTGC**Y** GTGGGA**Y**CGG **S**CGTCTATCA AATGTCAAAA CGACTCTCGG

241 CAACGGATAT CTCGGCTCTC GCATCGATGA AGAACGTAGC GAAATGCGAT ACTTGGTGTG

301 AATTGCAGAA TCCCGTGAAC CATCGAGTCT TTGAACGCAA GTTGCGCCCG AAGCCA**Y**TAG

ITS-2 →

361 GC**Y**GA**R**GGCA CG**Y**CTGCCTG GGC**R**TCACGC ATCGCGTCGC CCCCCA**Y**CCC CGCGC**R**CA**YM**

421 GCCGGGC**R**GT TGGGGGCGGA **S**A**Y**TGGCCTC CCGTGCGCCT CGG**Y**G**Y**GCGG CCGGCCCAAA

481 TG**M**GATCCC**Y** GGGCG**R**CTGG CGTC**R**CGACA AGTGGTGGTT GAACA*****CTCA ATCTCT**+**CTC

541 G**YR**G**Y**CGTGC CGCC**K**TG**Y**CG TCCCGTACGG G**M**AT**+**C**RM**AA ACGACCCAAC GGTGC**WM**G**K**C

601 GCGAACAGCG TCTCACCTTC GA

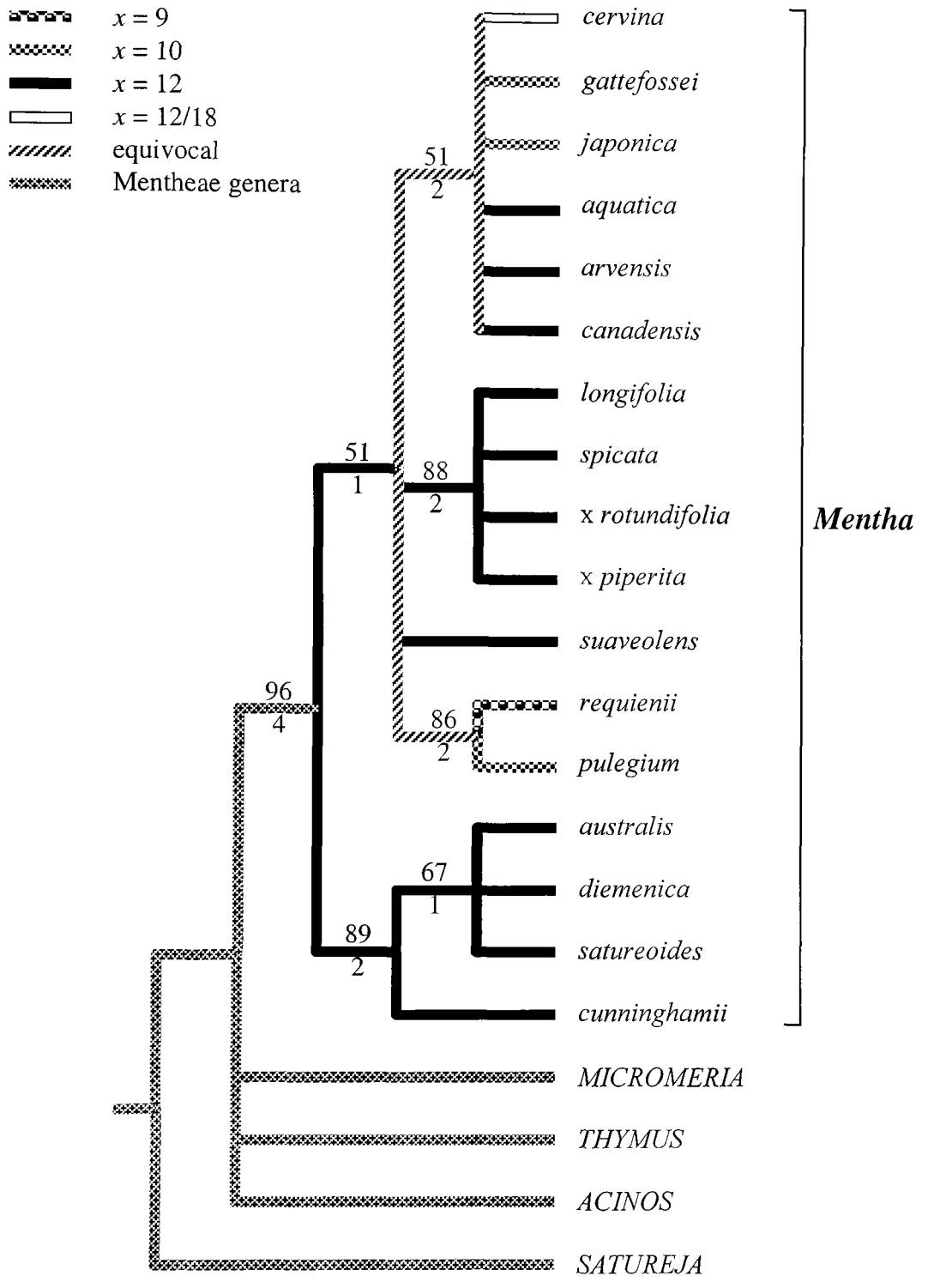

Figure 11. Character optimization of base chromosome number based on our *trnL-trnF* strict consensus phylogeny. See Fig. 8 caption for details.

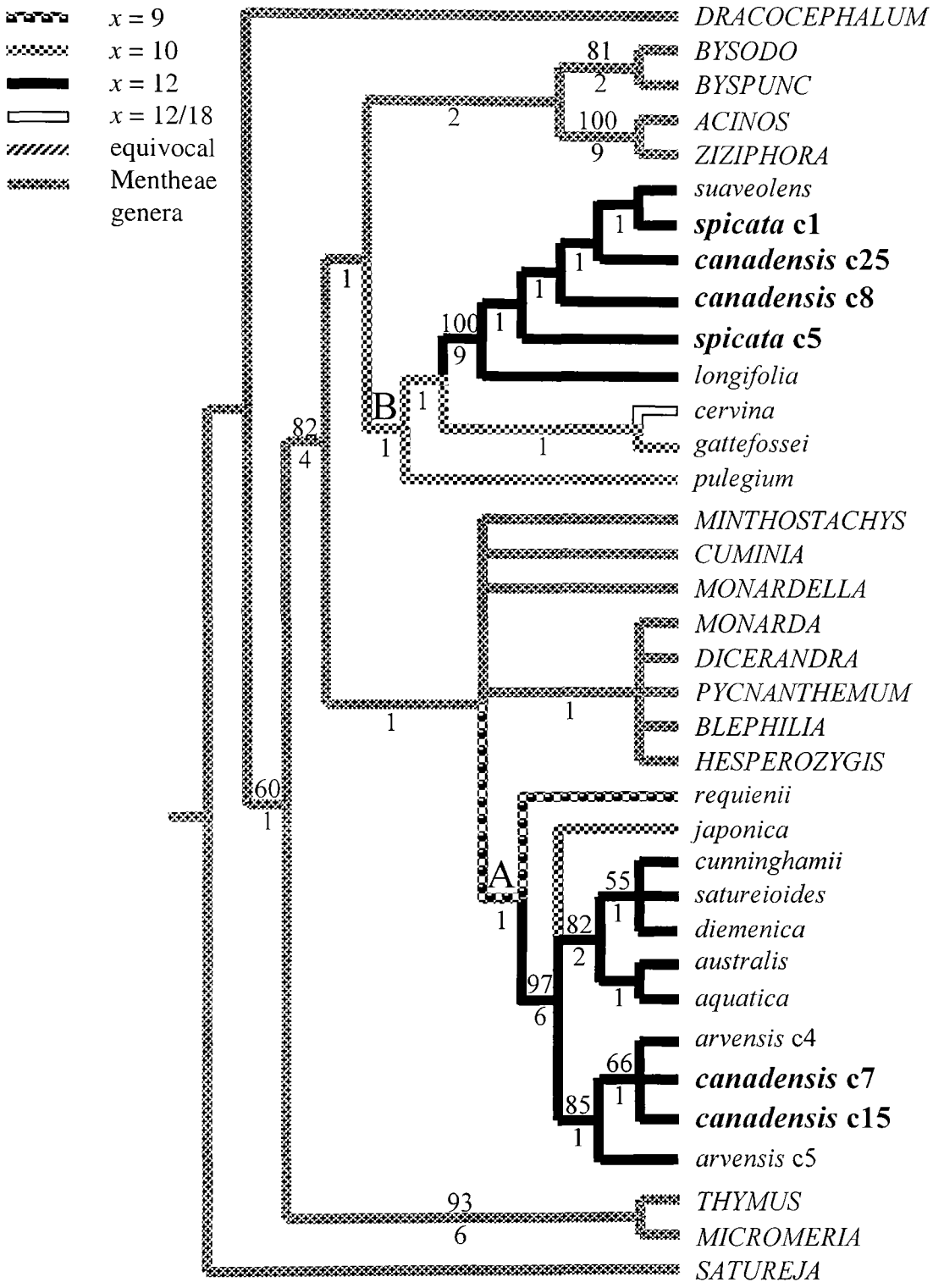

Figure 12. Character optimization of base chromosome number based on our ITS strict consensus phylogeny. See Fig. 9 caption for details.