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## Genetics and Breeding of the Genus *Mentha*: a Model for Other Polyploid Species with Secondary Constituents

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### Abstract

The greatest amount of research on the biochemical pathways and inheritance of the constituents of essential oils has been with the model systems of the genus *Mentha*. In particular, the genetic work of Dr. Merritt Murray and the biotechnological work of Dr. Rodney Croteau stand out for the amount of good, new data. However, new insights on previously published research in *Mentha* reveal that cytomixis provides a physical opportunity for complement fractionation, which, in turn, produces transgressive segregation in *Mentha*. Assimilating almost a century of breeding and biotechnological methods in *Mentha*, two approaches stand out: (1)  $\gamma$ -irradiation and (2) controlled hybridizations in the field. Are these methods applicable in other polyploid species with essential oils? Are they applicable for other plant constituents?

### Introduction

While disparaged until relatively recently, hybridization has been shown to be extremely important in evolution. Wissemann (2007) has written “Hybridization is important, because life on earth is predominantly a hybrid plant phenomenon.” We now know that post-hybridization events, such as genetic and epigenetic alterations and genome doubling, further propel hybridization and polyploidization as major phenomena in the evolution of plants (Paun *et al.*, 2007). Worldwide cultivation of

the genus *Mentha* centers around two primary constituents and four species, all of hybrid, polyploid origin. (-)-Menthol is the primary constituent of the essential oil of peppermint (*Mentha*  $\times$  *piperita* L. ‘Mitcham’ and derived cultivars) and Chinese cornmint or Japanese peppermint (*Mentha canadensis* L.). (-)-Carvone is the primary constituent of the essential oil of Scotch spearmint (*Mentha*  $\times$  *gracilis* Sole ‘Scotch’) and Native or “American” spearmint (*Mentha*  $\times$  *villosanervata* Opiz/*M. spicata* L.) (Tucker and Naczi, 2007).

‘Mitcham’ peppermint arose in England as a hybrid of *M. aquatica* L.  $\times$  *M. spicata*, prior to the 18<sup>th</sup> century. The plant has  $2n=72$  and is extremely sterile. Chinese cornmint is a naturally occurring hybrid of *M. arvensis* L.  $\times$  *M. longifolia* (L.) L. that probably arose in the Lower Tertiary; it has  $2n=96$  and is fertile, but gynodioecious. Scotch spearmint arose in Scotland as a hybrid of *M. arvensis*  $\times$  *M. spicata* prior to the 18<sup>th</sup> century, has  $2n=84$ , and is almost completely sterile. Native Spearmint arose as a hybrid of *M. spicata*  $\times$  *M. longifolia* or as a self of *M. spicata* prior to the 18<sup>th</sup> century, has  $2n=36$ , and is almost completely sterile (Tucker and Naczi, 2007).

Pioneer genetic work on the Mendelian inheritance of essential oil components in *Mentha* was published by Dr. Merritt Murray and his associates 1954-1986. Most of the work on the biosynthetic pathways of the essential oil components in *Mentha* has been published by Dr. Rodney Croteau and his associates 1971 to the present. These two

lines of research have been assimilated by Tucker and Kitto (in press). However, while the genetic work of Murray was current for its time, he failed to recognize some genetic phenomena in *Mentha* that influence the phenotypic expression of essential oil patterns.

### Transgressive Segregation

The most comprehensive, recent review of transgressive segregation (Rieseberg *et al.*, 1999) defines it as:

“the presence of phenotypes that are extreme relative to those of the parental line...a major mechanism by which extreme or novel adaptations observed in new hybrid ecotypes or species are thought to arise.”

This is not a rare phenomenon. From a survey of 171 studies that report phenotypic variation in segregating hybrid populations, the authors found 155 of the 171 studies (91%) reported at least one transgressive trait, and 44% of the 1229 traits examined were transgressive. They observed that transgression occurred most frequently in intraspecific crosses involving inbred, domesticated plant populations and least frequently in interspecific crosses between outbred, wild animal species. The primary cause was diagnosed as the action of complementary genes, although overdominance and epistasis also contribute. The overall conclusion is that “hybridization may provide the raw material for rapid adaptation and provide a simple explanation for niche divergence and phenotypic novelty often associated with hybrid lineages.”

In *Mentha*, an example of transgressive segregation is the origin of ‘Mitcham’ peppermint. Murray *et al.* (1972) crossed *M. aquatica* ( $2n=96$ ) and *M. spicata* ( $2n=48$ ) to create 32,000 field-grown plants that survived from 120,000 seedlings. With a preliminary organoleptic analysis, followed by an essential oil analysis by gas chromatography of several selected hybrids with a peppermint aroma, only a few hybrids even came close to ‘Mitcham’ peppermint and none matched exactly. The closest match (#57-1577-191) had the morphology and major essential oil constituents of ‘Mitcham’ peppermint, but also had a strong “nasturtium” aroma, probably from an unidentified hydrocarbon. Other close matches in morphology and major essential oil constituents had “soapy, musty, fishy, or terpenic” aromas. However, enough evidence on morphology and essential oil

constituents was presented to support the hypothesis that *M. × piperita* is a hybrid of *M. aquatica* × *M. spicata*.

As another example, the accidental re-synthesis of *M. canadensis* by Tucker and Chambers (2002) resulted in some high menthol/isomenthol/menthone forms. Against three commercial standard clones with 57-73% menthol, only one hybrid (#23-41) had 31% menthol out of 39 hybrids which were analyzed by gas chromatography. However, enough evidence on morphology and essential oil constituents was presented to support the hypothesis that *M. canadensis* is a hybrid of *M. arvensis* × *M. longifolia*.

Menthol is only available at economically important levels in the genus *Mentha*, and its origin in peppermint and cornmint represents a major shift in ecological fitness. Menthol affects the TRPM8 channel in animals that results in a flux of ions similar to that produced by physical cold (McKemy, 2005).

Tucker and Chambers (2002) crossed two clones of *M. arvensis*, one high in pulegone and 1,8-cineole, the other high in linalool, with *M. longifolia*, which had high *trans*-piperitone oxide and germacrene D. Most of the  $F_1$  hybrids had the essential oil constituents of the parents. However, the authors also found hybrids with high levels (>10%) of isomenthone, menthone, *trans*-isopulegone, menthol, neomenthol, 3-octanol, *cis*-piperitone oxide, *trans*-piperitone oxide, carvone, limonene, piperitenone oxide, *trans*-carveol, *trans*-sabinene hydrate, 3-octanone, terpenin-3-ol, (*Z*)-beta-ocimene, geranyl acetate, citronellyl acetate and/or  $\beta$ -caryophyllene. The high levels of these constituents in the  $F_1$  hybrids were not predicted from the essential oil patterns in the parents, and probably a wider range could be generated with more hybrids.

As another example of transgressive segregation in *Mentha*, Tucker and Fairbrothers (1990) and Tucker *et al.* (1991) attempted to re-synthesize Scotch spearmint. Examining 20 cultivated and wild clones of *M. × gracilis* and 932  $F_1$  hybrids, only one hybrid (#27-19) matched one of the clones in morphology, essential oil constituents, and chromosome number. However, enough evidence was presented to support the hypothesis that *M. × gracilis* is a hybrid of *M. arvensis* × *M. spicata*.

### Cytomixis

Cytomixis was first observed in pollen mother cells of saffron (*Crocus sativus*) (Körnicke, 1902) and later defined by Gates (1911) as “an extrusion of chromatin from the nucleus of one mother-cell through cytoplasmic connections, into the cytoplasm of an adjacent mother-cell.” The definition of cytomixis now includes the cellular transfer of organelles or other cytoplasmic constituents, but there is still a substantial lack of understanding of the function of cytomixis (Guo and Zheng, 2004).

Until the mid-20<sup>th</sup> century, however, cytomixis was considered an anomaly. Maheshwari (1950) wrote: “In some plants individual chromosomes, or groups of chromosomes, or even whole spindles are said to be carried from one cell into another. It is believed, however, that it is a pathological phenomenon, or that such appearances are caused by faulty fixation.”

In 1981, Tucker and Fairbrothers (1981) counted the chromosome numbers of crosses of *M. arvensis* ( $2n=72$ )  $\times$  *M. spicata* ( $2n=48$ ) and found  $2n=48$ , 60, 72, 84, and 96 in the  $F_1$  hybrids. At the time of publication, the mechanisms that produced this euploid series were unknown to the authors. These unexpected chromosome numbers in *Mentha* were later confirmed by Kundu and Sharma (1985), Tyagi and Ahmad (1989), and Tyagi (2003), and attributed to observed cytomixis. Tyagi (2003) wrote on *M. spicata*: “The phenomenon of cytomixis was observed in leptotene to pachytene states of the first meiotic prophase. The migration of nuclear material involved all of the chromosomes or part of the chromosomes of the donor cell. The occurrence of PMCs [pollen mother cells] with chromosome numbers deviating from the tetraploid number ( $n=48$ ), derived from the chromosome numbers deviating from the tetraploid number ( $n=48$ ), derived from the process of cytomixis indicated the possibility of aneuploid and polyploidy gamete production.”

Tucker and Chambers (2002) also observed unreduced gametes in their re-synthesis of *M. canadensis*. The expected chromosome number from crossing *M. arvensis* ( $2n=72$ ) with *M. longifolia* ( $2n=24$ ) should have been 48 ( $36+12$ ) with normal meiosis, but almost all the hybrids that were counted had  $2n=96$ .

Cytomixis, while routinely omitted from most textbooks on genetics and plant breeding, is not a relatively rare phenomenon. Cytomixis has been observed in both mitotic and meiotic cells, from mosses to flowering plants. A brief survey of papers that reported cytomixis in vascular plant families, obtained by the search term “cytomixis, Table 1”, and probably other existing papers have observed this phenomenon, but did not use this term in the key words or titles.

The natural causes of cytomixis are postulated to be: (1) genes, especially male-sterile genes, altered by environmental factors (pollution, fungal infection, and othes); (2) abnormal formation of the cell wall during premeiotic division; and/or (3) the microenvironment of the anthers. However, cytomixis can also be artificially induced by: (1) colchicine; (2) MMS (methylmethane sulfonate), EMS (ethyl methane sulfonate), rotenone, sodium azide, Trifluralin, and others, and/or (3)  $\gamma$ -irradiation (Bhat *et al.*, 2006, 2007a, 2007b; Bobak and Herich, 1978; Kumar and Tripathi, 2008; Narayana *et al.*, 2007; Sheidai *et al.*, 2002). Whether these could aid in plant breeding can be questioned, as these artificial agents are extremely toxic, and many precautions would have to be taken.

### Complement Fractionation

Complement fractionation was first coined as a term by Thompson (1962) while working with the genus *Rubus*: “I propose the term ‘complement fractionation’ for the general phenomenon wherein the chromosome complement is subdivided into independently operating groups within a cell. The consequence of this phenomenon will be cell-division products with variable chromosome numbers.” This term is suitable to explain the results of cytomixis in *Mentha* in which Tucker and Fairbrothers (1981) crossed *M. arvensis* ( $2n=72$ )  $\times$  *M. spicata* ( $2n=48$ ) and found  $2n=48$ , 60, 72, 84, and 96 in the  $F_1$  hybrids. In this instance, chromosomes migrated in multiples of the monoploid number,  $x=12$ . Normal meiosis would have produced progeny with  $2n=60$  ( $36+24$ ), and simple unreduced gametes would have produced progeny with  $2n=120$  ( $72+48$ ), 84 ( $36+48$ ), and 96 in the  $F_1$  hybrids. In this instance, chromosomes migrated in multiples of the monoploid number,  $x=12$ . Normal meiosis would have produced

Table 1. A brief survey of cytomixis in vascular plant families.

Family (genera)	References
Agavaceae ( <i>Chlorophytum</i> )	Lattoo <i>et al.</i> , 2006
Alliaceae ( <i>Allium</i> )	Bowes, 1973
Apiaceae ( <i>Centella, Tauschia</i> )	Bell, 1964; Consolaro and Pagliarini, 1995
Apocynaceae ( <i>Tabernaemontana</i> )	De and Sharma, 1983
Boraginaceae ( <i>Cordia</i> )	Bedi, 1990
Brassicaceae ( <i>Brassica, Diplotaxis</i> )	Malallah and Attia, 2003; Souza and Pagliarini, 1997
Cactaceae ( <i>Consolea</i> )	Negrón-Ortiz, 2007
Chenopodiaceae ( <i>Beta</i> )	Semyarkhina and Kuptsou, 1974
Fabaceae ( <i>Glycine, Lathyrus, Medicago, Ononis, Vicia, Vigna</i> )	Bellucci <i>et al.</i> , 2003; Bione, <i>et al.</i> , 2000; Haroun <i>et al.</i> , 2004; Morrisset, 1978; Seijo, 1996; Sen and Bhattacharya, 1988
Fagaceae ( <i>Quercus</i> )	Bedi, 1990
Hemerocallidaceae ( <i>Hemerocallis</i> )	Narain, 1979
Iridaceae ( <i>Crocus</i> )	Körnigke, 1902
Isoetaceae ( <i>Isoetes</i> )	Wang <i>et al.</i> , 2007
Lamiaceae ( <i>Caryopteris, Leonurus, Leucas, Mentha, Ocimum, Salvia</i> )	Bedi, 1990; Carlson and Stuart, 1936; Datta <i>et al.</i> , 2005; Kundu and Sharma, 1985, 1988; Tyagi, 2003; Tyagi and Ahmad, 1989
Liliaceae ( <i>Lilium</i> )	Zheng <i>et al.</i> , 1985
Malvaceae ( <i>Alcea, Gossypium</i> )	Mary, 1979; Mary and Suvarnalatha, 1981; Sarvella, 1958
Moraceae ( <i>Morus</i> )	Verma <i>et al.</i> , 1984
Oleaceae ( <i>Jasminum</i> )	George and Geethamma, 1985
Onagraceae ( <i>Oenothera</i> )	Davis, 1933; Gates, 1908, 1911
Orchidaceae ( <i>Ophrys</i> )	Feijó and Pais, 1989
Papaveraceae ( <i>Meconopsis, Papaver</i> )	Bahl and Tyagi, 1988; Singhal and Kumar, 2008a
Pinaceae ( <i>Picea</i> )	Guzicka and Wozny, 2005
Poaceae ( <i>Alopecurus, Avena, Brachiaria, Bromus, Coix, Dactylis, Elymus × Psathyrostachys, Lolium, Secale, Sorghum, Triticum, Urochloa, Zea</i> )	Basavaiah and Murthy, 1987; Boldrini <i>et al.</i> , 2006; Caetano-Pereira and Pagliarini, 1997; Cheng <i>et al.</i> , 1980; Fallistocco <i>et al.</i> , 1995; Ghaffari, 2006; Koul, 1990; Omara, 1976; Sapre and Deshpande, 1987; Sheidai and Fadaei, 2005; Sheidai <i>et al.</i> , 2003 Wang and Cheng, 1983; Yen <i>et al.</i> , 1993.
Polygonaceae ( <i>Polygonum</i> )	Haroun, 1995
Ranunculaceae ( <i>Caltha, Helleborus</i> )	Echlin and Godwin, 1968; Kumar and Singhal, 2008
Rosaceae ( <i>Prunus</i> )	Soodan and Waffai, 1987
Rubiaceae ( <i>Serissa</i> )	Bedi, 1990
Rutaceae ( <i>Citrus, Pilocarpus</i> )	Naithani and Raghuvanshi, 1958, 1963; Pagliarini and Pereira, 1992
Salicaceae ( <i>Salix</i> )	Bedi, 1990
Solanaceae ( <i>Datura, Nicotiana, Solanum, Withania</i> )	Cheng <i>et al.</i> , 1982; Datta <i>et al.</i> , 2005; Siddiqui <i>et al.</i> , 1979; Sicorchuk <i>et al.</i> , 2004; Singhal and Kumar, 2008b
Symplocaceae ( <i>Symplocos</i> )	Bedi, 1990

progeny with  $2n=60$  (36+24), and simple unreduced gametes would have produced progeny with  $2n=120$  (72+48), 84 (36+48), and 96 (72+48); the progeny with  $2n=48$  could only have arisen with the phenomenon of complement fractionation as described by Thompson.

Complement fractionation, like cytomixis, is routinely omitted in textbooks on genetics and plant breeding, but is not relatively rare. A number of papers on vascular plant families and genera have reported complement fractionation (Table 2). This current list was simply generated from the search term “complement fractionation,” and a larger list probably could be created by looking for papers that reported unusual chromosome numbers in polyploids, but did not use the term complement fractionation in their keywords or titles. Tucker and Fairbrothers (1981), for example, observed complement fractionation, but did not use this term. A number of observations on aneuploids have also been made and might be included in a broader discussion of this phenomenon. For example, Darlington and Mather (1944) observed a variety of numbers between  $2x$  and  $4x$  in *Hyacinthus* due to loss of chromosomes during meiosis. Similar phenomena of irregular meiosis and aneuploids also exist in *Fragaria* (East, 1934; Yarnell, 1931), *Malus* (Hegwood and Hough, 1958), *Primula* (Upcott, 1940), *Rosa*, etc. (Lim *et al.*, 2005; Wissemann *et al.*, 2007; Werlemark, 2003).

The meaning of complement fractionation explored by Murray and others (Murray, *et al.*, 1972) in the phenotypic expression of the genes for essential oil constituents remains unresolved. The “Reitsema rule” (Reitsema, 1958) states that 3-oxygenated monoterpenes (*e.g.*, menthol) and 2-oxygenated monoterpenes (*e.g.*, carvone) are biosynthesized on mutually exclusive pathways, controlled by mutually exclusive genes, and cannot be in the same plant, making “doublemints” impossible. Tucker *et al.* (1991) reported, however, on a clone of *M. × gracilis* with 40% carvone/dihydrocarveol, 22% menthol, and 13% limonene. This clone also had  $2n=96$  (Tucker and Fairbrothers, 1990), and we can speculate that multiple copies of recessive and dominant genes cause a breakdown of normal Mendelian genetics.

The clones of *M. spicata* designated by Murray (Murray, *et al.*, 1972) as  $2n$  Cr and  $2n$  line 1 were

postulated to have genotypes of *AaCciilmPPrr*, standard workhorse clones, and used in the creation of thousands of hybrids (Tucker and Kitto, 2011). These genes, however, were determined by an organoleptic analysis by trained panels, not by gas chromatography. A selfing Murray’s  $2n$  Cr in our laboratory and analysis by gas chromatography/mass spectrometry indicated the phenotypes with constituents greater than 10% of the oil were: 40 carvone, 5 pulegone, 4 menthol, and 1 piperitone, a distribution that agrees with the genotype postulated by Murray. Selfing of  $2n$  Line 1 and analysis by GC/MS, however, revealed the following phenotypes with constituents greater than 10% of the oil: 21 carvone; 1 carvone/dihydrocarvone; 3 carvone/limonene; 6 carvone/limonene/1,8-cineole; 4 carvone/1,8-cineole; 4 menthone/piperitone oxide; 3 pulegone; 2 pulegone/menthone; 3 pulegone/menthone/isomenthone/1,8-cineole; 1 pulegone/piperitone; 1 menthone/isomenthone/1,8-cineole; 1 menthone/isomenthone/piperitone. Obviously, either the Mendelian genetics of *Mentha* are more complex than envisioned by Murray or cytomixis aids complement fractionation, which in turn is reflected phenotypically as transgressive segregation.

Complement fractionation may also restore some fertility to normally completely sterile hybrids. Table 3 presents The fertility of 18 natural clones of *M. × gracilis* is almost complete sterility in the expected chromosome number  $2n=60$  (36+24) from a cross of *M. arvensis* ( $2n=72$ )  $\times$  *M. spicata* ( $2n=48$ ) (Table 3). The clones with  $2n=72$  and 84, however, have pollen fertility of 0-14% and seed fertility of 0-0.2%. The clone with  $2n=96$  is essentially complexly sterile.

### Successful Breeding Methods in *Mentha*, Past & Future

During almost a century of conventional breeding and biotechnological methods in the genus *Mentha*, only two methods have resulted in any release of significantly new germplasm that has benefitted the farmer:  $\gamma$ -irradiation and controlled hybridization in the field. In view of what we now know about the importance of cytomixis, complement fractionation, and transgressive segregation in mints, this is not too surprising.

In 1955-1959, A. M. Todd  $\gamma$ -irradiated 100,000

Table 2. A brief survey of complement fractionation in vascular plant families.

Family (genera)	References
Clusiaceae ( <i>Hypericum</i> )	Qu <i>et al.</i> , 2010
Lamiaceae ( <i>Mentha</i> )	Kundu and Sharma, 1985, 1988; Tyagi, 2003; Tyagi and Ahmad, 1989; Tucker and Fairbrothers, 1981
Malvaceae ( <i>Gossypium</i> )	Menzel and Brown, 1952
Orchidaceae ( <i>Aranda</i> , <i>Phaius</i> )	Teoh, 1981; Teoh and Ong, 1982
Poaceae ( <i>Hordeum</i> , <i>Secale</i> , <i>Triticum</i> )	Geng <i>et al.</i> , 1979; Finch <i>et al.</i> , 1981
Rosaceae ( <i>Rubus</i> )	Bammi, 1965; Jennings <i>et al.</i> , 1967; Thompson, 1962
Scrophulariaceae ( <i>Mimulus</i> )	Tai and Vickery, 1970

Table 3. Fertility of 18 natural clones of *M. × gracilis* (Tucker and Fairbrothers, 1990).

2n = (# of clones)	60 (6 clones)	72 (5 clones)	84 (5 clones)	96 (2 clones)
Average fertile pollen	0%	0-4%	0-14%	0%
Average fertile seeds	0-0.1%	0-0.2%	0-0.2%	0%

plants of 'Mitcham' peppermint at Brookhaven National Laboratory (Murray and Todd, 1972; Todd *et al.*, 1977). This resulted in the formal release of two verticillium-wilt resistant clones, 'Todd Mitcham' and 'Murray Mitcham.' Additional clones currently recognized by the Mint Industry Research Council (MIRC) include M-83-7, B-90-9, and 'Roberts Mitcham,' of which all were essentially derived from 'Mitcham' through mutation breeding (Morris, 2007).

Controlled hybridization in the field resulted in the release of *M. canadensis* 'Himalaya' (U.S. Plant Patent 10935) and 'Kosi' (Kumar *et al.*, 1997, 1999). Alternate rows of 'Kalka' and 'Gomti' were planted and allowed to open-pollinate. The subsequent progeny was evaluated for yield and disease resistance.

Both these methods,  $\gamma$ -irradiation and controlled hybridization in the field, hinge upon two factors, large populations of hybrids and ease of evaluation. With essential oils in *Mentha*, training organoleptic panels for preliminary evaluation is relatively easy,

and later confirmation can be done in the laboratory by gas chromatography. This methodology could be beneficial to research in other genera with sufficient labor and a quick and easy method of preliminary evaluation of the constituents.

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