Morpho-meiotic study in *Mentha longifolia* from cold desert regions of Lahaul-Spiti and adjoining areas of Himachal Pradesh (India)

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**ABSTRACT** A morpho-meiotic study of wild *Mentha longifolia* (L.) L. (Lamiaceae) is presented from the nine populations (Kukumsari, Zero-point, Kishori, Tosh, Kasol, Key, Tiling, Mudh and Darcha) in and around the cold desert regions of Lahaul-Spiti of Himachal Pradesh. Present work is needful effort to fill the gap of morpho-meiotic (morphological and cytological) knowledge in *M. longifolia* growing in high altitude regions. Meiotic study revealed the different chromosome counts in these populations as n = 12, n = 12 + 0-3B and n = 9. Presence of B-chromosome in the species is reported for the first time from the study area and it reflects inter-population variation in five important descriptors (such as a nature of whole plant, stem, leaves, inflorescences and pollen) with 17 sub-descriptor states and occurrence of B-chromosomes. Present study reflects the existence of *M. longifolia* at diploid (2x) level based on base numbers x = 12 and x = 9.

**KEY WORDS**
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**INTRODUCTION**

*Mentha longifolia* (L.) L. (Lamiaceae), known well as Habek mint or Horse mint, is an aromatic perennial and tomentose herb that grows mostly in semi-shady places on moist soils (Ahmad et al. 2011; Shinwari et al. 2011). It is indigenous to Eastern Europe, Middle East, South and North Africa, Saudi Arabia and it grows wild in cold desert regions of Himachal Pradesh and in North West Himalayan belt of India. Fresh leaves and stems are mostly used for flavouring in 'salads' and cooked foods (Facciola 1990). Like other members of the genus *Mentha* L., it is used in domestic herbal remedy, being valued especially for its antisepsic properties and beneficial effects on digestion (Karoussou et al. 2007). Leaves are the chief source of essential oils, enriched in compounds like menthol, menthone, pulegone, piperitone oxides, certain monoterpenes, carvone, flavonoids, limonene and dipentene (Gulluce et al. 2007; Al-Rawashdeh 2011; Ayshath et al. 2016); and are widely used in food, beverages, flavour, cosmetics and pharmaceutical industries (Džamić et al. 2010; Bhargava 2016; Mahmoudi et al. 2016).

The species is an extremely variable taxon as large number of varieties and sub-species are reported in it (Sobti 1962) and highly variable with respect to habit, plant size, shape, denticulation and hairiness of leaves, spike length and flower colour (Aswal and Mehrotra 1994; Šarić-Kundalić et al. 2009). The information collected from the online data bases (IPCN of the w*TROPICOS* database of Missouri Botanical Garden, Chromosome Web Watch of Japan, PhytoKaryon of University of Patras, Chromosome Counts Database, etc.), research journals and literatures (Darlington and Wylie 1955; Kumar and Subramanian 1986; Khatoon and Ali 1993; Rice et al. 2015, etc.) indicate that the species is equally variable in terms of chromosome number as 2n = 18 to 144 (Srivastava 2012; Malik et al. 2017). So far, morpho-meiotically, *M. longifolia* has not been studied from the India. Thus, present study deals with morpho-meiotic investigation of *M. longifolia* collected from the high-altitude location in and around the cold desert regions of Lahaul-Spiti of Himachal Pradesh (India). Present work is needful effort to fill the gap of morpho-meiotic (morphological and cytological) knowledge in *M. longifolia* growing in high altitude regions.

**MATERIALS AND METHODS**

Plant material for the present study comprised of nine populations of *M. longifolia* from Lahaul-Spiti and adjoining area of Himachal Pradesh. Plants were studied and collected in Kukumsari, Zero-point, Kishori, Tosh, Kasol,
Key, Tiling, Mudh and Darcha localities. Morphological characters were noted in the field and data were recorded at 50% flowering stage. Voucher specimens are deposited in the herbarium, Department of Botany, Punjabi University, Patiala, India (PUN). For evaluation, five important descriptors were considered, i.e. plant, stem, leaf, inflorescence and pollen. Each descriptor was further studied under different sub-descriptor states which were accessed statistically. The sub-descriptor states included were plant habit (HB), height (PH), habitat (HBT), stem hairiness (SLH), stem branching (SB), intermodal length (INL), petiole length (PL), leaf shape (LSp), dorsal (DP) and ventral pubescence (VP), presence or absence of leaf denticulation (DLP), intensity of denticulation in leaf margin (DLM), spike length (SL), flower colour (FC), flowering period (FP), pollen size (PS) and pollen fertility percentage (PF%).

For meiotic analysis the appropriate stage of young inflorescences were collected between 9.00 to 11.00 a.m. and fixed in Carnoy’s fixative (6:3:1 = ethanol:chloroform:glacial acetic acid, v/v) for 24 h., after which they were transferred to 70% alcohol and stored at 4 °C. Pollen mother cells (PMCs) were obtained through standard squash technique in 1% acetocarmine. Several freshly prepared and permanent slides were carefully examined from each population to determine the chromosome number at different stages and meiotic abnormalities. Pollen stainability in glycerol: acetocarmine (1:1) was used to estimate pollen viability. For micro-photography an Eclipse 80i microscope system (Nikon) was used.

**RESULTS**

Presently, morpho-meiotic study was carried out in nine population of *M. longifolia* L. growing in cold desert regions of Himachal Pradesh. Morphologically, populations of *M. longifolia* were recorded with large variation in their field characters (sub-descriptor states) and clearly revealed the existence of four morphotypes viz. morphotype α, β, γ and δ. (Table 1) Scoring for the morphological characters, meiotic chromosome number (2n) and ploidy in each population is given in Table 1. Chromosome number (2n) records in *M. longifolia* and cytological information of genus *Mentha* L. from India and rest of the world is provided in Table 2 and Table 3.

**Morphological study**

Among the studied populations of four morphotypes in *M. longifolia* L., two morphotypes, i.e. α and β were erect

| Characters | Morphotype α | Morphotype β | Morphotype γ | Morphotypes δ |
|------------|--------------|--------------|--------------|---------------|
| **Populations** | Kukumsari | Zero-point, Kishori | - | Tosh, Kasol | Key, Tiling, Mudh | Darcha |
| **Plant** | | | | | |
| HB | Erect | Erect | Erect | Erect | Semi-erect | Semi-erect |
| PH (cm) | 60-150 | 70-150 | 60-150 | 40-80 | 35-50 | 45-70 |
| HBT | Moist & shady places | Moist & open slopes | Moist places | Moist places & road sides | Dry slopes & banks of Spiti river | Dry Slope near-Bhaga tributary |
| **Stem** | | | | | |
| SLH | Non-hairy | Non-hairy | Non-hairy | Less hairy | Densely hairy | Densely hairy |
| SB | Rarely 3 to 4 branched | Rarely 2 to 4 branched | Branch | Highly branched | Unbranched | Unbranched |
| INL (cm) | 4.6-7.7 | 4.2-8.1 | 5.72 | 3.4-5.2 | 4.4-8.2 | 4.5-7.6 |
| **Leaf** | | | | | |
| PL (cm) | 1.3-3.2 | 1.4-2.9 | 1.73 | 4.2-5.0 | 0.6-0.8 | 1.8 |
| LSp | Lanceolate | Lanceolate | Lanceolate | Oblong | Oblong | Oblong |
| DP | (+) low | (+) low | (+) low | (+) high | (+) high | (+) low |
| VP | - | - | - | - | - | - |
| DLP | + | + | + | + | + | ± |
| DLM | Conspicuously toothed | Conspicuously toothed | Conspicuously toothed | Moderately toothed | Moderately toothed | Wavy |
| **Inflorescence** | | | | | |
| SL | 9-18 | 7-15 | 12.11 | 6-14 | 2.5-4.5 | 2.5-4.1 |
| FC | Purple | Purple | Purple | Purple | Purple | Cream white |
| FP | July-Sept. | July-Sept. | July-Sept. | June-Aug. | July-Aug | July-Aug |
| **Pollen** | | | | | |
| PS (μm) | 25.80 x 25.56 | 26.11 x 25.80 | 26.35 x 25.82 | 24.85 x 24.74 | 25.06 x 24.88 |
| PF (mean %) | 100 | 73.89 | 94.25 | 93.04 | 93.33 |
| 2n Count | 24 | 24 + (1/2-3) B | 24 | 24 | 24 | 18 |
| Ploidy | 2x | 2x | 2x | 2x | 2x |

Xᵢ = congruent mean value for morphotype α.
while other two (γ and δ) were semi-erect in habit (HB) (Table 1). Average value of plant height (PH) varies from 60 to 150 cm in the populations of morphotype α (X₁ = 60-150 cm; PUP55023, Kasol = PUP54937), morphotype δ (45-70 cm; Darcha = PUP55031) and morphotype γ (Key = PUP54936, Tiling = PUP55033, Muīth = PUP55004). Plants of all the morphotypes except that of morphotype δ (which were growing on dry slope near Bhaga River tributary), were reported from moist and open shady conditions. Stem hairiness (SLH) was absent in the population of morphotype α while presents in others morphotype populations. Branching habit (SB) was observed as entire in the population of morphotype β and it is moderate in morphotype α with 2/3-4 branches per plants, while two morphotypes, i.e. γ and δ were unbranched (Table 1). Average (X₁) of variation values in inter-nodal length (INL) was less differentiable among the populations of the studied morphotypes α (X₁ = 5.72 cm; ranged b/w 4.6-7.7 & 4.2-8.1), γ (4.4-8.2 cm) and δ (4.5-7.6 cm), but it was lesser in range for the plant population of morphotype β (3.4-5.2 cm).

For the character of petiole length (PL) higher value was reported in the plants of morphotype α (X₁ = 1.73 cm), while it was lower in range for the plants of morphotype γ (0.6-0.8 cm) and intermediate in plant population of morphotype β (1.4-2.5 cm) and δ (1.1-1.8 cm). Leaves in all the morphotypes were oblong in shape (LSp) except in morphotype α were they were lanceolate. Leaf margin varies in denticulation (DLM) as sparse (more or less wavy in outline) in morphotype δ, moderate in morphotype β and γ to conspicuously toothed in morphotype α. For sub-descriptor state of leaf pubescence, the character of pubescence was almost present on dorsal surface (DP) of leaves of all the available morphotypes (dense and high in γ and δ morphotypes), but absent from the ventral surface (VP) in plant populations of morphotype α and γ. Among the studied accessions of morphotypes, range of flower spike lengths (inflorescence; SL) varies as 2.5-4.1 cm, 2.5-4.5 cm, 6-14 cm, 7-15 cm and 9-18 cm in morphotype δ, γ, β, morphotype α (plants with B-chromosomes) and morphotype α (plants without B-chromosomes), respectively; whereas flower colour (FC) was either light purple (morphotype α, β and γ) or cream white (morphotype δ) in colour. Flowering period (FP) varies marginally in all the available morphotypes with the difference of one month only and it starts in month of June (morphotype β) or July (morphotype α, γ and δ) and remains in blooming condition between mid-July to August. No major differences were observed in pollen size (PS) measurement within the studied morphotypes (Table 1).

**Cytological study**

*Morphotype α*: Meiotic studies in the plants of these morphotype revealed the presence of two different chromosome numbers. Plants belonging to Kukumsari population were reported with 2n = 24 at M-I (Fig. 1a), while PMCs...
in the plants of Zero-point and Kishori populations were observed to show B-chromosomes along with haploid chromosome count of n = 12 (Fig. 1b-g). The plants with and without B-chromosomes were indistinguishable in this morphotype (i.e. a). The number of B-chromosomes was observed to vary from 0 to 3 in the plants of population of Zero-point area (Fig. 1b-f), while it varied between 1 to 2 in Kishori population (Fig. 1g-i).

PMCs in the plants of Zero-point population were observed with inter-bivalent connections (37.50%) and chromosome stickiness (13.20%; Fig. 1c-f). Inter-bivalent connections (18.33%) and chromosome stickiness (7.50%) was also reported in few PMCs of the plants collected from Kishori population (Fig. 1j). Nearly 100% pollen fertility (Fig. 1k) was recorded in Kukumsari population while in plants of Zero-point and Kishori population the average pollen fertility was below 76.30%.

**Morphotype β:** Numerous PMCs in both the populations of this morphotype revealed the chromosome count of 2n = 24 at M-I (Fig. 1l). Meiotically the plants were normal and were recorded with high (above 90%) pollen fertility.

**Morphotype γ:** Meiotic analysis in the plants collected from the Key, Tiling and Mudh populations confirms the presence of 2n = 24 chromosome at M-I (Fig. 1m). Meiotic course in these plants was normal and pollens grains were cent-percent fertile (>90%).

**Morphotype δ:** The plants belonging to Darcha population exhibited a different meiotic count. Several PMCs at different stages of meiosis were observed with chromosome count of n = 9 (Fig. 1n). The plants of this population were perfectly normal in their meiotic course (Fig. 1o-q) and were recorded with high (>90%) pollen fertility (Fig. 1r).

**DISCUSSION**

**Morphological variation**

Morphological variation within a species is inductive of taxonomic heterogeneity and is considered as one of the fundamental factors in the process of evolutionary changes (Blinova 2012). These variations are not only helpful in establishing the connectivity of populations but also associated with the adaptability and evolution ability of taxa. It is well known fact that intraspecific morphological variations are more common in the widespread species than in the local or endemic ones (Darwin 1839; Whittakar and Fernandez-Palacios 2007). Species growing in different types of habitats also shows variations in morphological characters that are accounted for by differences in ecological conditions (Valen 1965; Stout et al. 2015). Morphological adaptations among plants to different kind of climatic and environmental conditions make them more diverse and evolved. Such plasticity in the genetic and morphological characters is more prominent among the individuals of different populations than among the members of the same population (Svensson 1983; Španiel et al. 2008). In general, morphological variations could happen due to the differences in the environmental conditions (Jones and Geber 1999; Petru et al. 2006; Stout et al. 2015), geographical boundaries (White 1971), selection and genetic drift (Abdelkrim 2005; Stuessy et al. 2006).

Variations of morphological characters among different members (i.e. species) of genus Mentha L. are quite common. In the presently studied species, intraspecific morphological variation was reported in different cytotypes of M. longifolia L. (n = 9, 12). Intraspecific morphological variation in the populations of M. longifolia (Table 1) might be attributed to the variation in chromosome numbers (n = 9, 12) and abnormal meiotic course, as it has been reported earlier in large number of flowering plants, e.g., Centaurea phrygia (Koutecký 2007), Centaurea stoeb (Španiel et al. 2008), Ranunculus pannassifolius (Cires et al. 2009), Agrimonia sp., Geranium wallachianum, Ranunculus hirtellus and Vicia rigidula (Kaur and Singhal 2010a). The presence or absence of B-chromosomes did not affect the morphological characteristics as the plants of M. longifolia with or without B-chromosome are indistinguishable in most of their descriptor states.

Morphological diversity among the species of genus Mentha is great. Due to high polymorphism, the number of species in the genus has been a matter of speculation for many years. Several features have been used in the past to examine the diversity of species using morphological (Malinvaud 1880) and cytological (Harley and Brighton 1977; Singh and Sharma 1986) aspects. Natural interspecific hybridization occurs with high frequency in Mentha species which may also led to morphological variation within the species. For example, cytological studies in M. spicata (= M. laveagata) shows two cytotypes with 2n = 36 (x = 9) and 48 (x = 12) chromosomes, which differ only (except molecular) on the basis two aspects, i.e. presence or absence of non-secreting trichomes and chemical data (Gobert et al. 2002). Based on cytological and karyological data Harley and Brighton (1977) suggested the M. spicata (n = 24) as a hybrid of M. suaveolens (n = 12) and M. longifolia (n = 12).

Several varieties have been proposed by different authors in M. longifolia based on morphological and/or cytological differences, e.g., M. spicata var. longifolia, M. sylvestris var. royleana and M. longifolia var. royleana, M. longifolia ssp. capensis, M. longifolia ssp. longifolia, etc. (Mukerjee 1940; Harley and Brighton 1977; Raizada and Saxena 1978; Chambers and Hummer 1994; Tarimcilar and Kaynak 2004). But currently most of these varieties
Table 2. Chromosome number (2n) count in M. longifolia (L.) L. from India and rest of the world (outside India).

| Chromosome number (2n) | India | Outside India |
|------------------------|-------|---------------|
| 2n = 18:               | (a) Sobti 1962; Bala & Gupta 2012. | |
| 2n = 24:               | (a,b) Sobti 1965; (a,b) Sobti 1971a,b; Gohil et al. 1981; | (a, d) Saggoo 1983; Bhat et al. 2002; Kaur & Singhal 2010b; (g) Malik et al. 2017. |
| 2n = 27:               | (a) Sobti 1962. | |
| 2n = 36:               | (a,b) Sobti 1971b. | (a) Arora 1960; (a) Sobti 1965. |
| 2n = 48:               | (a,b) Sobti 1965. | |

*Mentha longifolia* (L.) L.

| Synonyms: | |
|-----------|---|
| M. longifolia (L.) Huds.; | M. longifolia (L.) Nath.; |
| M. longifolia (L.) L. subsp. longifolia; | M. sylvestris Linn.; |
| M. longifolia (sylvestra) | M. sapida Tausch.; |
| M. rotundifolia L.; | M. rotundifolia Linn.; |
| M. longifolia subsp. hypalaisiensis Briq.; | M. longifolia subsp. capensis (Thum.) Briq.; |
| M. longifolia subsp. polyadenia (Briquet) Briq.; | M. longifolia subsp. longifolia var. thyoides; |
| M. longifolia var. aquatica; | M. longifolia; |
| M. longifolia L. | |

are treated as sens. lat. under same synonym of *M. longifolia* (L.) L. to cover the large range of variations (Aswal and Mehrotra 1994). From the present study area variation in hairiness of stem; size, shape and hairiness of leaves; spikes and flowers were also reported by Aswal and Mehrotra (1994). In the present case intensive screening of populations of the species from Lahaul-Spiti area revealed four morphotypes (α, β, γ and δ) occupying different locations. It seems that the phenotype of the population is greatly influenced by altitudinal range and topography. The populations of the species collected from dry temperate (Key, Tiling, Mudh and Darcha) areas show high tomentum (DP and VP) and almost woolly outlook while those of moist temperate locations (Kukumsari, Zero-point, Kishori, Tosh and Kasol) were less hairy.

The variants in the taxa are distributed in the different ecological and environmental conditions. The flowering period among the different populations varies marginally with the difference of one month. It indicates that the variation might be due to ecological preferences as suggested in other flowering plants (Korner 1999; Parker et al. 2003; Andi et al. 2011).

Morphological characteristics are the consequences of the effect of various ecological factors on the genotype of the species. Stebbins (1950) suggested that intraspecific variations of morphological characters are dependent upon the environmental modifications, genetic recombination and mutations.

Cytological illustration

Presence of 2n = 24 in the present taxa agrees with the previous reports from India and outside (Table 2). Whereas the count of 2n = 18 in Darcha population (morphotype δ) also confirms the previous reports from India and from the other parts of the world (Table 2). However, the present study is the first report about the presence of B-chromosome in this species (n = 12 + 0-3B). Nearly all the described species of the genus are known cytologically (Table 3). The genus exhibits a wide range of chromosome numbers (2n = 10 to 144; highest in *M. piperita* L.; Luttov et al. 1966; Singh 1995) which indicate the polybasic nature of the genus with base numbers x = 5, 6, 9, 10, 12 and x = 13. The base number x = 9 and 12 are the most common in distribution (Table 3). Morton’s report (Morton 1973; Chambers and Hummer 1994) of 2n = 10 for *M. pulegium* from the Liège Botanical Garden makes the lowest base number x = 5 in the genus. The base numbers x = 7 and 8 are also suggested in the genus. But, true diploids (2n = 14, 16) based on these base numbers are not yet reported in nature. Singh (1995) suggested x = 12 as a secondary base number for the genus. While, Harley and Brightton (1977) suggested x = 12 as the ancestral (primary) base number for the *Mentha* L. This view was also supported by Bhat et al. (2002) and Lawrence (2007). However, literature confirms the polybasic nature of the genus with wide range of polyploids (100%) and variable reports based on different base numbers. Such a great variability in base number may be due to frequent interspecific hybridization, which makes it cytologically a complex genus.

In India nearly 13 *Mentha* species (including hybrids) are cytologically available. The species *M. longifolia* is already being reported with 2n = 18, 24, 27, 36 and 2n = 48 from different parts of the world (Table 2), it represent
the intraspecific chromosome variability at \( x = 9 \) and 12. Diploid nature of this species with \( x = 9 \) and \( x = 12 \) was supported by many workers (Schurhoff 1929; Morton 1956; Murray 1960; Harley and Brighton 1977; Lawrence 2007; Al-Rawashdeh 2011). Thus, present observation of \( 2n = 18 \) and \( 2n = 24 \) (or \( 2n = 24 + 1/2-3B \)) in \( M. \) longifolia are seems to be at diploid (2x) level which are based on \( x = 9 \) and \( x = 12 \).

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Mentha longifolia from cold desert regions

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