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CYTOGENETIC DIVERSITY OF *ELSHOLTZIA CILIATA* BENTH. (LAMIACEAE) FROM KASHMIR HIMALAYA

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Our cytomorphological study of various populations of *Elsholtzia ciliata* (Lamiaceae) collected from high-altitude sites of Kashmir Himalaya revealed two euploid cytomorphotypes, diploid (n=8) and tetraploid (n=16), growing sympatrically but inhabiting two different habitats. This is the first report of tetraploid (4×) *E. ciliata* from the Indian subcontinent. We found the course of meiois to be normal in diploids, but tetraploid individuals showed chromosome and meiotic irregularities: cytomixis at early prophase I, stickiness at metaphase I, and chromosome bridges at anaphase I. In tetraploids, 23 of the 26 pollen mother cells observed at metaphase I showed 0–6 quadrivalents, suggesting that the tetraploid is a segmental allopolyploid. Microsporogenesis was also abnormal in tetraploids, showing the formation of triads. All these anomalies are conducive to lower reproductive potential (40.70%) in tetraploids than in diploids (90.50%). Significant morphological differences between the two cytotypes are presented.

Key words: Cytomorphotype, chromosome, diploid, tetraploid, *Elsholtzia ciliata*, quadrivalents, Kashmir Himalaya, chromosome stickiness.

INTRODUCTION

The genus *Elsholtzia* Willd. belongs to the Lamiaceae family (Elsholtzieae) and is distributed primarily in temperate regions of the Northern Hemisphere (Harley et al., 2004). The center of diversity of the genus is in East Asia, particularly China, Korea and Japan (Li and Hedge, 1994). *Flora of Pakistan* (Web) documents a total of 30 species of the genus in the world. *Elsholtzia ciliata* (= *E. cristata* Wild.), commonly known as Vietnamese Balm, is a small fragrant annual herb distributed in Himalaya from Kashmir to Arunachal Pradesh, reaching up to 3300 m a.s.l. (Blatter, 1928). It is a traditional medicine used as a carminative and astringent (Manandhar and Manandhar, 2002). The leaf juice is used as a diuretic and against coughs and colds (Rai and Lalramnghinglova, 2010). In obese mice, an ethanol extract of its dried aerial parts significantly decreased total serum cholesterol, triglycerides and leptones (Sung et al., 2011). A water extract inhibited mast-cell-mediated allergic inflammation (Kim et al., 2011). In view of the medicinal importance of this species we wanted to understand its meiotic behavior and cytomorphological variation, the microhabitat distribution patterns of the two sympatric cytomorphotypes, and their reproductive potential. The amounts of the active principle(s) in some medicinal plants significantly differ between intraspecific cytomorphotypes (Berkov, 2001), hence there is need to find and designate the best chemotypes. Little information is available on the distribution and interactions between polyploids and their diploid progenitors when they occur sympatrically (Thompson and Lumaret, 1992; Šafářová and Duchoslav, 2010; Šafářová et al., 2011). Understanding the distribution patterns of two (or potentially more) cytotypes within the sympatric zones of a species can shed light on the nature of interactions such as competition and mating between parental genotypes, and also into the genetic basis of their differences (Harrison and Rand, 1989).

MATERIALS AND METHODS

In botanical surveys of some high altitude sites of Kashmir Himalaya (32°20'–34°50' N; 73°55'–75°35' E) in the last two years, *Elsholtzia ciliata* was seen

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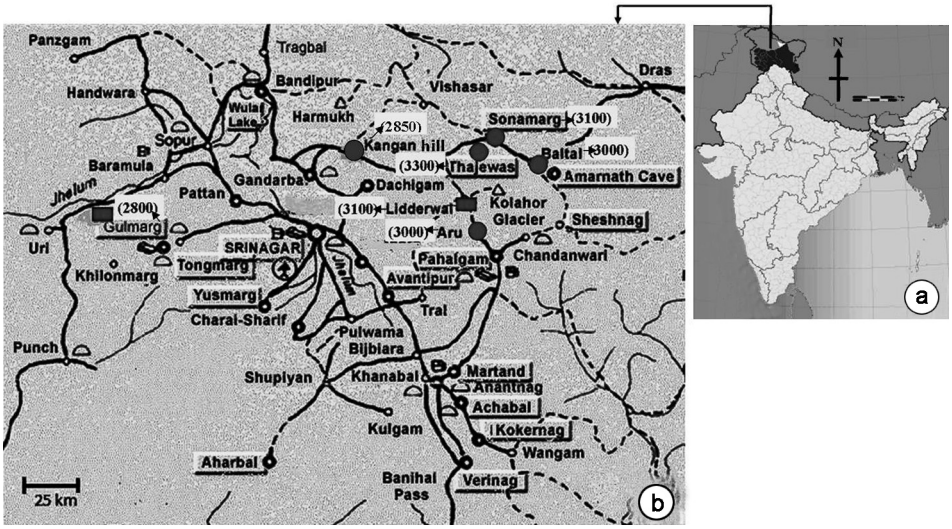

Fig. 1. (**a**) Map of India showing the location of Kashmir (dark shading), (**b**) Map of Kashmir showing the *Elsholtzia ciliata* collection sites marked with filled circles and rectangles (elevation in m a.s.l). Circles represent sites where both diploid and tetraploid individuals occur; rectangles represent sites where only diploid individuals were found.

growing as two morphotypes (Fig. 1). The young flower buds were collected from plants growing in their natural habitats and fixed in Carnoy's fixative (ethyl alcohol/chloroform/acetic acid 6:3:1, v/v/v). The meiotic studies were carried out on young flower buds using standard acetocarmine smear technique. Eight specimens each from two populations of the Thajwas area were taken for detailed meiotic and morphometric analyses. The difference in ploidy between the two morphotypes was confirmed by chromosome counts of at least one specimen from each site (Fig. 1b). One morphotype always came out with the same ploidy level, diploid (2×) or tetraploid (4×). Pollen fertility was estimated by stainability in 1% glycerol-acetocarmine. Well-stained and well-filled pollen grains were considered to be fertile, while unstained and shrunken pollen grains were regarded as sterile. For stomatal studies the leaves were immersed in 10% KOH for 10–15 min and the peels taken off for microscopic observations. Photomicrographs of pollen mother cells, pollen grains and stomata were taken (Nikon 80i Digital Imaging System). The plant specimens are deposited in the Herbarium of the Department of Botany, Punjabi University, Patiala (PUN).

RESULTS

The superficially obvious morphological differences between the two morphotypes of *Elsholtzia ciliata* prompted us to examine their cytomorphology in detail. Our analysis of the meiotic course in the two morphovariants revealed them to be cytological variants on the basis of ploidy level. One is diploid with a gametophytic chromosome number n=8 (Fig. 2a,b); the other is tetraploid, showing gametophytic chromosome number n=16 (Fig. 2c). Chromosome number n=8 has already been reported for this species (Gill, 1984); the present chromosome count of n=16 is given here for the first time from India and is already published as a chromosome number report (Malik et al., 2011).

Meiosis was found to be normal in the diploid cytotype but all tetraploid specimens showed some abnormalities during the male meiotic course. Cytomixis, the transfer of chromatin from one pollen mother cell (PMC) to another, was seen in 12% of the cells at early prophase I (Fig. 2c). Later, in metaphase I, ~42% of PMCs showed stickiness along the whole genome (Fig. 2h). At anaphase I, 5% of the PMCs showed chromosome bridge formation (Fig. 2i). Normal PMCs without sticky chromosomes at metaphase I in the tetraploid cytotype showed the proper frequency of quadrivalents (Tab. 2). The different configurations of bivalents and quadrivalents observed in 26 PMCs, along with their average frequency per PMC, are given in Table 2. The range of quadrivalents varied from 0 to 6, and all were of open chain type (Fig. 2e–g). These observations – cytomixis, stickiness, bridge formation and quadrivalents – have not been reported previously in this species. Table 1 presents the morphological characters along with stomatal and pollen features of the two cytomorphotypes, showing significant differences between them. Average plant height shows little variation, but average lam-

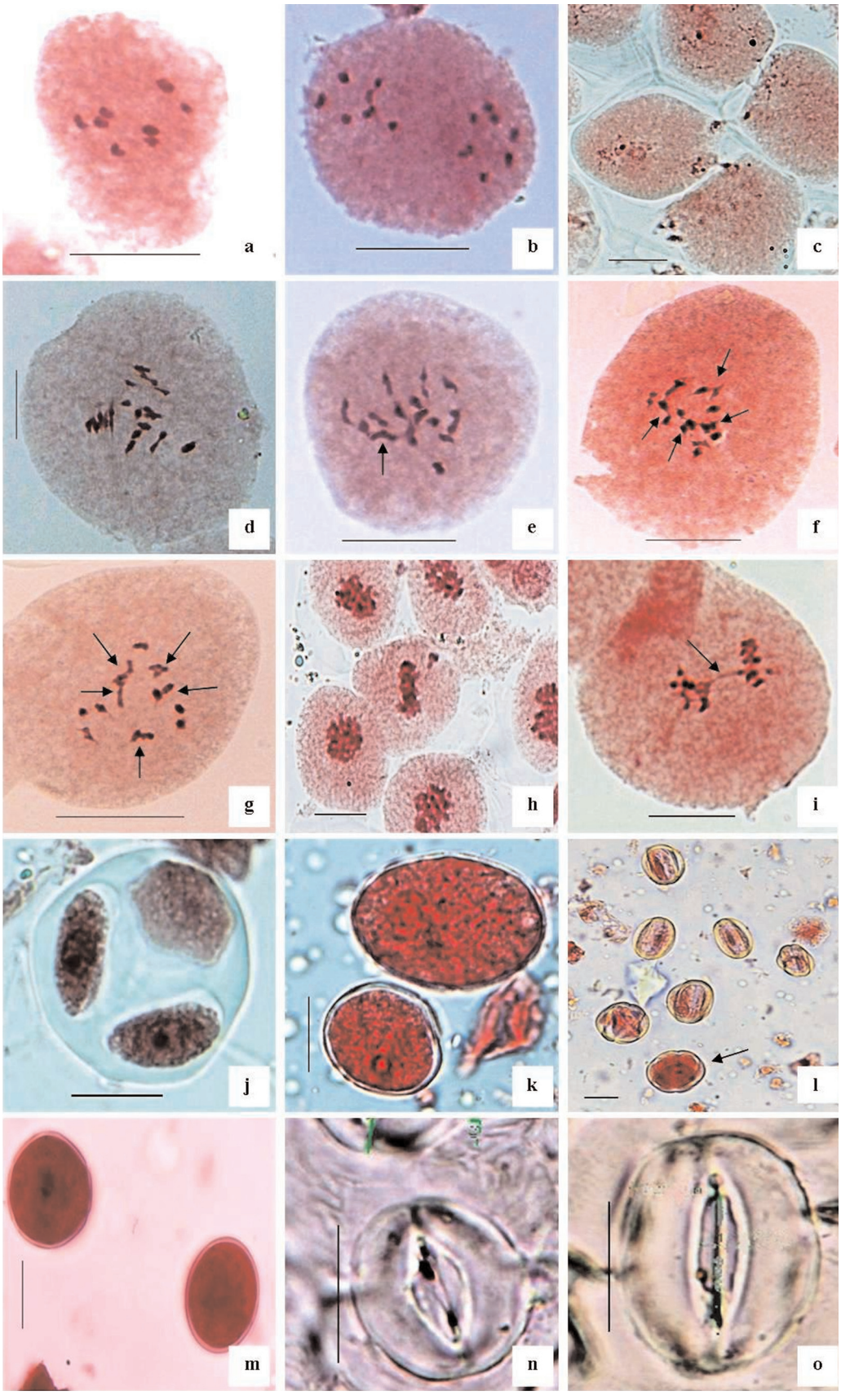
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TABLE 1. Comparison of morphological characters of diploid and tetraploid cytomorphotypes of *Elsholtzia cilia*

Cyto-type	Locality/ Acc. No. (PUN)	Average plant height (cm)	Average lamina size (cm)	Average petiole length (cm)	Leaf shape	Flower color	*Pubescense	Stomatal size (µm)	Pollen fertility (%)	+Pollen size (µm)
Diploid	Thajwas/ 55174	22	3.8 × 2.0	1.5	Ovate	Light purple	Very sparse	16.35±1.18 × 13.66 ±1.09	91.00	21.84±1.94 × 18.55±1.39
Tetra-ploid	Thajwas/ 55193	25	3.2 × 1.3	2.2	Ovate lanceolate	White	Much pubescent	18.72±0.80 × 15.79±0.69	40.70	17.56±0.95 × 15.88±0.082

* – pubescence present on abaxial surface of leaves and mainly young portions of stem; ± – standard deviation; + – pollen size of fertile pollen grains; PUN is the code of the Herbarium of the Department of Botany, Punjabi University, Patiala.

TABLE 2. Chromosome configurations observed at M-I in tetraploid *Elsholtzia ciliata*

	Configuration		PMCs observed	
	II	IV	Number	%
	16	0	3	11.54
	14	1	4	15.38
	12	2	6	23.08
	8	4	5	19.23
	6	5	4	15.38
	4	6	4	15.38
Total	256	80	26	
Average frequency/ PMC	9.85	3.07		
% of chromosomes involved	61.53	38.46		

ina size is larger in the diploid than in the tetraploid. The flowers are light purple in the diploid and white in the tetraploid. The diploid cytomorphotype shows very sparse or no pubescence, while the leaves as well as aerial parts are obviously pubescent in the tetraploid cytomorphotype (Fig. 3d). The stomata in both the diploid (Fig. 2n) and tetraploid (Fig. 2o) cytomorphotypes are of diacytic type. The diploid cytomorphotype has comparatively larger lamina (Fig. 3a, 3c) and larger pollen (Fig. 2k), but the tetraploid slightly exceeds the diploid in plant height, petiole length (Fig. 3b) and stomatal size (Fig. 2o).

Interestingly, though both cytomorphotypes occur sympatrically, they inhabit different microhabitats. The diploid flourishes well on slopes, whereas the tetraploid thrives well on exposed plane surfaces as well as slopes.

DISCUSSION

The genus *Elsholtzia* shows a base number of x=8 (Darlington and Wylie, 1955). Nineteen species of *Elsholtzia* ranging from 2n=16 to 2n=58 are known from the literature. To our knowledge no species of the genus from Indian subcontinent has been reported to have base number x=9. Previous chromosome reports of *Elsholtzia ciliata* from India (Gill, 1984), Poland (Pogan, et al., 1983) and Russia (Probatova and Sokolovskaya 1990) give 2n=2x=16. The only tetraploid (4×) report (2n=32) for the species is also published from Russia (Nishikawa, 1985), and it too is based on x=8. However, 2n=18 has also been reported from China (Zhang et al., 1993) and Russia (Uhrikova and Majovsky, 1983). The authenticity of chromosome numbers on base number x=9 needs to be validated. Meiotic studies in natural populations of *Elsholtzia ciliata* confirmed the existence of two euploid cytotypes – diploid and tetraploid. Analysis of meiosis in tetraploid specimens showed chromatin transfer through narrow cytoplasmic channels between proximate meiocytes at early prophase I. This phenomenon was first recorded by Kornickle (1901) in *Crocus sativus*. Such a phenomenon has a profound impact on the meiotic process, meiotic end-products, and the overall reproductive potential of the species. The literature suggests many factors responsible for cytomixis, including temperature (Narain, 1976), stress factors coupled with genetic control (Ghanima and Talaat, 2003), and direct genetic control (Bellucci et al., 2003, Haroun et al., 2004). It has also been attributed to fortuitous causes such as sublethal artifacts produced by fixation, mechanical injury or

Fig. 2. Microsporogenesis and pollen of *Elsholtzia ciliata*. Diploid cytotype (**a, b, k, n**). (**a**) PMC with 8 bivalents at Metaphase I, (**b**) PMC with 8+8 distribution of chromosomes at Anaphase I, (**k**) Fertile pollen grains with significant size variation, (**n**) Stomata. Tetraploid cytotype (**c–j, l, m, o**). (**c**) PMCs at early prophase I showing cytomixis, (**d**) PMC with 16 II at M-I, (**e**) PMC with 1 IV+14 II at M-I, (**f**) PMC with 4 IV+8 II at M-I, (**g**) PMC with 5 IV+ 6 II at M-I, (**h**) PMCs showing chromosome stickiness at M-I, (**i**) Chromosome bridge at A-I (arrow), (**j**) Triad, (**l**) One fertile pollen grain (arrow) among sterile pollen grains, (**m**) Fertile pollen grains with no significant size variation, (**o**) Stomata. PMC – pollen mother cell; M-I – metaphase I; A-I – anaphase I; II – bivalent; IV – quadrivalent. Bar = 10 µm.

Fig. 3. Herbarium specimens. (**a**) Diploid cytotype, (**b**) Tetraploid cytotype, (**c**) Ovate leaf of diploid individual with no hairs, (**d**) Ovate-lanceolate leaf of tetraploid plant with hairs.

pathological anomalies (Takats, 1959; Gottschalk, 1970; Morisset, 1978). According to Levan (1941), Zheng et al. (1987), Ghanima and Talaat (2003) and Kim et al. (2009), cytomixis plays a major role in chromosomal diversity and speciation of taxa. Another unusual behavior is chromosome stickiness. It is characterized by intense clustering of chromosomes during any phase of the cell cycle (Rao et al., 1990). In the tetraploid cytomorphotype we observed stickiness along the whole genome in 42% of the PMCs at metaphase I. Chromosome stickiness has been reported in several plant species (Mendes-Banato et al., 2001). Beadle (1932) reported chromosome stickiness in maize for the first time and attributed the irregularity to a recessive mutant gene called *sticky (st)*. It has been reported in different *Brachiaria* species (Mendes-Bonato et al., 2007; Pagliarini et al., 2008; Risso-Pascotto et al., 2009) with suggestions that chromosome stickiness may be under genetic control: controlled by a single pair of genes, two pairs of genes, or by the interaction of several genes which may be recessive or dominant. Stickiness might also be caused by environmental factors such as X-rays, temperature and soil elements (Mendes-Bonato et al., 2001).

Meiosis in the tetraploid showed the presence of 0–6 quadrivalents per PMC at metaphase I, suggesting that it is a segmental allopolyploid. All the quadrivalents observed were of open chain type. This open chain quadrivalent behavior might be due to short-sized interchange segments (Biswas and Biswas, 2006). Out of 26 metaphase I PMCs, 23 showed the presence of quadrivalents. The occurrence of such a proper frequency of PMCs with quadrivalents indicates that the tetraploid cytotype may have descended from at least one parent from a different species of the same genus. Chromosome bridge formation was also observed in the tetraploid cytotype at anaphase I in 2% of the PMCs. Anaphase I bridge formation in only tetraploid individuals might be explained by paracentric inversion. According to Saylor and Smith (1966), the formation of bridges can be due to failure of chiasmata in a bivalent approaching terminalization, followed by stretching of the chromosomes between the poles. However, no micronuclei were found in the tetrads. The occurrence of quadrivalents along with chromosomal irregularities during meiosis is the main cause of reduced pollen fertility of the tetraploid specimens (40.70%); the diploids, showing a normal meiotic course, had much higher pollen fertility (90.5%).

Morphologically the two cytotypes differ significantly and hence can be called cytomorphotypes. Previous studies have reported that tetraploids are usually taller than their diploid counterparts (Berdhal and Ries, 1997; Muntzing, 2010). We found that on average the tetraploid is 3 cm higher than the diploid. Leaf morphology varies quantitatively as well as qualitatively between the two cytotypes. Petioles are longer in the tetraploid, apparently at the cost of lamina size; they are smaller than in diploids. Many studies have demonstrated that a decrease in a quantitative character like leaf size can be accompanied by an increase in other quantitative characters like leaf number per branch or petiole length (Powell, 1992). The intraspecific variability of chromatin/chromosome behavior, meiotic behavior and qualitative and quantitative morphological differences (Tab. 1) in the two cytomorphotypes reflects genetic diversity within the species. There is much research on the genetic control of such characters as variation in leaf shape (Tsukaya, 2005) and pubescence within species (Agren and Schemske, 1992). The differences we found in quantitative traits including average plant height, lamina size, petiole length and pollen size between the two cytomorphotypes are in accordance with earlier research by Srivastava and Srivastava (2002), Zlesak (2009) and Omidbaigi et al. (2010). Tetraploid cytotypes usually are found to produce larger pollen grains; the smaller pollen in our study may be due to gene interaction between two different genetic components. In Table 1 the standard deviation for pollen size is higher for diploids, indicating a very significant size difference between the smallest and largest pollen grains (Fig. 2k). The stomata of diploids also show such a large size range. In tetraploids those size ranges are much smaller, as reflected in the standard deviations (Fig. 2m). The lower variability of quantitative characters like pollen size and stomatal size within the tetraploid cytomorphotype can be explained by Stebbin's (1956) buffering effect of polyploidization: each gene affecting a quantitative character makes a smaller contribution to variation at the tetraploid level than at the diploid level.

Generally the ecological amplitude of polyploids is thought to be broader than that of their ancestors, as they combine features of the parental genomes (Brochmann et al., 2004). We found tetraploids growing on both sloped and plane surfaces, unlike diploids which flourished only on slopes where moisture cannot accumulate. Polyploids usually have different geographical ranges than their diploid progenitors (Lewis, 1980). Our study helps explain the habitat niche difference between the two cytomorphotypes.

Polyploidy is recognized as a significant factor and as a major driver of the evolution of many eukaryotes. Recent genomic investigations indicate that most if not all angiosperm species have undergone at least one genome-wide multiplication event in their evolutionary history (Bowers et al., 2003, Blanc and Wolfe 2004, Mayrose et al., 2010). Here it seems that another *Elsholtzia* species growing on the same spot or nearby, *E. densa*, may have played a role as one of the probable progenitors of the tetraploid *E. ciliata*. Different cytotypes may coexist in one population, resulting in cytotype mixing (Baack, 2004; Safářová and Duchoslav, 2010; Šafářová et al., 2011). The coexistence may be temporary, with one cytotype dominating, or it may be permanent, due to reproductive isolation, potentially leading to speciation (Husband and Schemske, 1998; Baack, 2005). Will the tetraploid cytomorphotype of *E. ciliata* be able to dominate in the future? We have already met such examples (Felber, 1991; Treier et al., 2009).

Kashmir Himalaya is a hub of very important medicinal and aromatic plants, most of which are unexplored in all or many aspects. They need special attention. Studies like ours can be useful in germplasm evaluation, chromosomal database cataloguing, understanding the nature of intraspecifically variable chromosome/meiotic behavior, and chromosomal evolution in important Himalayan plant species.

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