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Correlation between genomic diversity and asiaticoside content in *Centella asiatica* (L.) Urban.

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Abstract. Asiaticoside has a tremendous medicinal value against leprosy. A comparative chemical analysis of ten ecotypes of *Centella asiatica* in India was carried out to estimate asiaticoside content in leaves. The higher amount of asiaticosides were obtained in ecotype II and X of subtemperate Himalaya having 0.114% and 0.105% respectively. Subsequently two newly reported B-chromosomes were noted in these races besides their normal karyotype 2n=18 chromosomes. The influence of genomic constituents on asiaticoside contents was statistically analysed. The asiaticoside contents differed significantly among the ecotypes examined, reflecting their genome variation. The possibility of utilizing the selected genotypes of subtemperate Himalaya for commercial exploitation was indicated.

Key words: Asiaticoside content; B-chromosomes; *Centella asiatica*; Ecological variation; Genomic diversity; Karyotype constitution.

Introduction

The plant *Centella asiatica* (L.) Urban. belonging to the family Umbelliferae is growing predominantly in southern hemisphere. It has of great medicinal value for its asiaticoside content in leaves. The asiaticoside, a glucoside, gave encouraging results against treatment of leprosy (Bailey, 1945; Boiteau *et al.*, 1949; Viala *et al.*, 1977). This active principle dissolve the waxy covering of *Bacillus leproe*, so that the causal organism becomes very fragile and may easily be destroyed (Bailey, 1945).

However, the previous cytological data have revealed the somatic chromosome number 2n=18 chromosomes forming regular nine bivalents (Sharma and Ghosh, 1954; Sharma and Bhattacharya, 1959, 1960; Bell and Constance, 1960; Liu *et al.*, 1961; Joshi and Raghuvanshi, 1970; Subramanian, 1986). Some conflicting report regarding chromosome number i.e., 2n=22 and 2n=33 have been reported by Sharma and Sharma (1957) and Mitsukuri and Kurahori (1959), respectively. No systematic attempt has, however, been made on the detailed cytological analysis of different ecotypic races of this species. Moreover, the asiaticoside contents have not yet been analysed in different races. The objectives of this study are to analyse the karyotype constituents and to find out if there is a correlation between genome and the amount of asiaticoside contents.

Materials and Methods

Plants of *C. asiatica* were collected from ten different ecotypic regions of India. The localities and the altitudes of the collection spots of the plants were given in Table 1. All these ecotypes were grown in the experimental plot of the Department of Botany, University of Calcutta.

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Table 1. *Karyotype constitution, asiaticoside content and habitats of ten ecotypic races of* ***C. asiatica***

Centella asiatica	Localities in India	Soil type	Altitude in metre	Somatic chromosome number (2n)	Karyotype formula	Total chromosome length in μm ± S.D.	Total chromosome volume in cu. μm ± S.D.	Total F%	Asiaticoside content in % of dry leaf wt. ± S.D.
Ecotype I	Konarak (Orissa)	Coastal saline	11.28	18	$B_2+C_4+D_4+E_8$	31.98±0.03	13.56±0.56	34.33	0.062±0.006
Ecotype II	Rudraprayag (Uttar Pradesh)	Rocky soil	609.60	18+2B	$C_2+D_{10}+E_6+2B$	16.56±0.07	1.71±0.11	41.67	0.114±0.004
Ecotype III	Sukhna (West Bengal)	Sandy loam soil with humus	360.00	18	$C_2+D_{14}+E_2$	26.86±0.18	16.92±0.72	43.63	0.075±0.004
Ecotype IV	Midnapore (West Bengal)	Laterite soil	8.53	18	$A_2+C_2+D_8+E_6$	38.66±0.01	23.68±0.82	39.27	0.082±0.004
Ecotype V	Darjeeling (West Bengal)	Rocky soil	2133.60	18	$C_4+D_4+E_{10}$	30.40±0.15	20.95±0.76	32.37	0.097±0.009
Ecotype VI	Kamakha Hill (Assam)	Sandy rocky soil with sand and gravel	249.90	18	$C_6+D_8+E_4$	31.46±0.03	12.52±0.61	36.11	0.088±0.013
Ecotype VII	Indian Botanical Garden (West Bengal)	Alluvial soil	4.57	18	$C_4+D_4+E_{10}$	34.54±0.33	25.08±0.54	34.34	0.006±0.002
Ecotype VIII	Sagar Island (West Bengal)	Coastal saline clay soil	1.34	18+1B	$B_2+C_4+D_{12}+1B$	26.36±0.17	10.42±0.82	43.06	0.060±0.006
Ecotype IX	Caluctta (West Bengal)	Sandy loam soil	6.09	18	$B_4+C_2+D_8+E_4$	32.00±0.05	22.12±0.71	37.50	0.017±0.005
Ecotype X	Shillong (Meghalaya)	Sandy rocky soil	1510.00	18+2B	$B_2+C_2+D_4+E_{10}+2B$	26.26±0.53	5.33±0.97	30.58	0.105±0.007

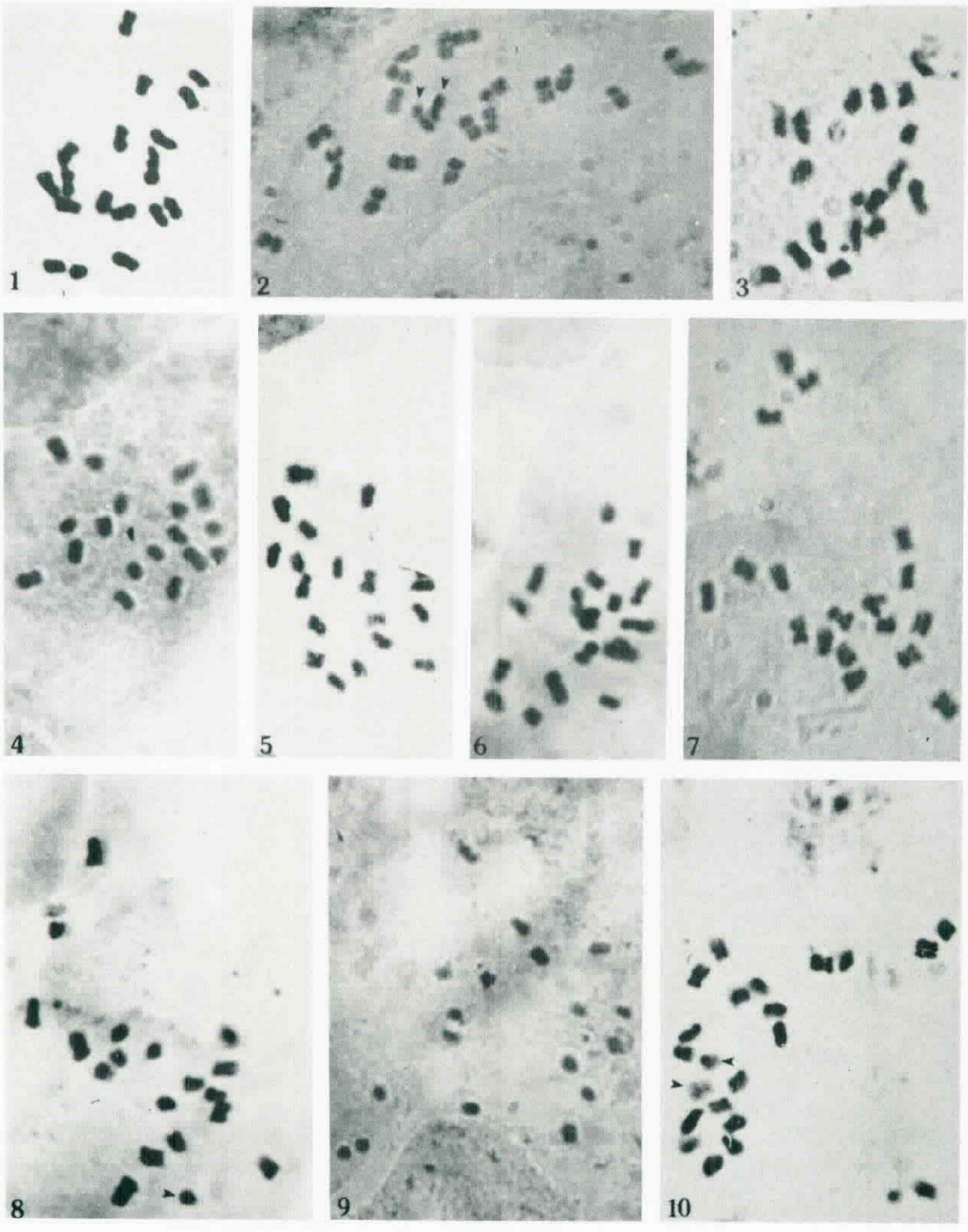
Cytological Techniques

Well scattered metaphase plates were obtained by pretreating the root tips with paradichlorobenzene aesculine mixture (2:1) for three hours at 12°C followed by overnight fixation in 1:3 acetic ethanol. Prior to staining cold hydrolysis of roots in 5N hydrochloric acid (HCl) at 16°C for 10 minutes was done. Staining was performed with 2% acetic-orcein solution for 3 hours at room temperature after 10 minutes of incubation at 60°C. Root tips were squashed in 45% acetic acid.

Total chromosome length of each ecotypes was calculated by adding the whole lengths of all the chromosomes, present in a complement. The breadth of individual chromosomes was measured from the karyogram plates and the chromosome volume was calculated applying the formula πr^2h where r and h are the radius and height of the chromosome respectively. Following the methods of Levan *et al.* (1964) total form percentage (TF%) was calculated in each ecotypes.

Chemical Analysis

For chemical analysis leaves of each ecotypes were oven dried at 60°C for 48 hours and crushed to make powder. Powdered leaves were hydrolysed with 30% V/V HCl for 4 hours on a waterbath at 100°C, neutralized with sodium hydroxide, filtered and washed thrice thoroughly with distilled water. Extraction of asiaticoside was done from finely dried leaf powders with pyridine (b.p. 115-116°C) continuously for 24 hours at 35-40°C in a Soxhlet apparatus. The extracts were screened for the presence of asiaticoside by thin layer chromatography (TLC) on silica gel G (type 60, Merck) coated glass plates. The samples were applied on the activated plates (2 h in 100°C oven) with the help of micropipettes, as very small spots of 2-5 mm in diameter on one end of the plate. Separation was carried out at room temperature (26-30°C) in diffused light. The plates were placed in a presaturated chambers filled with solvent chloroform: methanol in the proportion of 95:5 to a depth of about 0.5 cm and air-tighted with glass lid for ascending development. Caution was taken so that the whole breadth of the TLC plate dipped into the solvent to the same depth i.e. 0.5 cm but not to cover the spots at the start. The length of the run was 15 cm from the starting point. The plates were sprayed with Liebrman-Burchard reagent (Krebs *et al.*, 1969)



Figs. 1–10. Somatic metaphase plates of different ecotypes (I–X accordingly) of *Centella asiatica* (X2900). Arrows indicate the B-chromosomes in the karyotype.

and visualised by heating. Spectrophotometric analysis of asiaticoside was performed in Specord spectrophotometer after eluting the asiaticoside (Stahl, 1969) in pyridine from silica gel G at 4000 g by centrifugation. Quantitation was done from optical density at 783-785 nm, the absorption spectrum of asiaticoside, against standard curve of standard sample. For each ecotypic races the mean value of the asiaticoside was calculated from seven sets of experiments.

Statistical Analysis

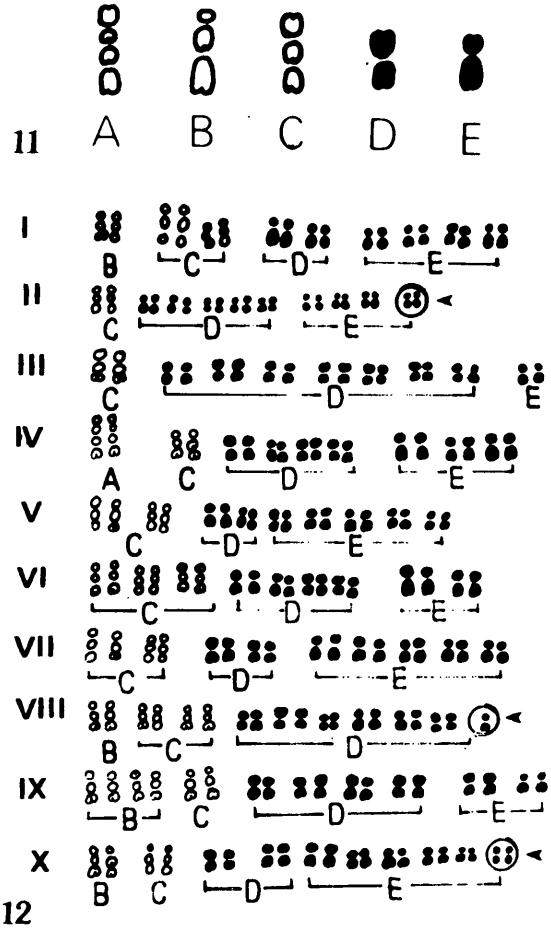
For the statistical analysis the mean numbers in each set of asiaticoside content in leaves were used. ANOVA test (Sokal and Rohlf, 1973) followed by Duncan's multiple range test (Harter, 1960) have been performed for finding out the significant differences, if any, between different ecotypic races of *C. asiatica*. Correlation coefficient (r) value was also analysed to find out the relationships between altitudinal differences and asiaticoside contents as well as chromosome volumes and asiaticoside contents.

Results and Discussion

Karyotype Analysis

The general karyotype analysis of ten ecotypes of *Centella asiatica* showed some numerical and structural alteration of chromosomes in their genome (Figs. 1-10, Table 1). Normally the plants growing in the tropical plains showed 2n=18 chromosomes whereas the plants growing in higher altitudinal area (1510.0 m - 2135.6 m) as well as in subtemperate zones (Figs. 2, 10) and halophytic zones of plains showed B-chromosomes (Fig. 8) in addition to their normal chromosomes. The presence of B-chromosomes as well as variation in number of chromosomes may be due to the adaptation of the plants to different environments. Eventually, the macroenvironmental adaptation among the ecotypes might be occurred by the changes of chiasma frequency as well as recombination of genetic make up of an organism (Barker, 1960; John and Hewit, 1965; Barlow and Vosa, 1970; Yrjo, 1972; Jones and Rees, 1968; Hamal and Koul, 1986).

The different ecotypes showed similarity in gross chromosome structure as revealed by the graded karyotype. Out of total five types of chromosomes (Fig. 11) i.e., A, B, C, D and E, the chromosomes with secondary constrictions (A, B, C types) were varied from 2-6 in



Figs. 11-12. Fig. 11. Type chromosomes of *C. asiatica*. Fig. 12. Comparative karyograms of different ecotypes of *Centella asiatica*.

number. The striking feature was the presence of supernumerary constricted (A types) i.e. chromosomes having constriction other than primary and secondary constrictions in ecotype IV. Whereas B types were present in Ecotypes I, VIII, IX and X (Fig. 12). However, the increase or decrease in number of chromosomes with secondary constrictions might have been due to duplication of chromosomes or translocation between the chromosomes with or without secondary constrictions at a very early stage of evolution. Again, the variation in total chromosome length and volume were also observed (Table 1). Consequently, the appar-

ent increase or decrease in length, attributed to the variation in size has affected the overall chromosome complement as evidenced by total F% (TF%) values as well. The lowest total chromosome length was noted in ecotype II which showed extra two B-chromosomes. Reduction in the size of autosomes were recorded with the presence of B-chromosomes. The reduction of total chromosome volumes have been noted in ecotype II (1.71 cu. μm) and ecotype X (5.33 cu. μm) also. In *Petunia hybrida* the size of A-chromosomes gets reduced with the incorporation of B's (Gohil and Kaul, 1980). Reduction in the size of A-chromosomes might be justified on the basis of reduction caused in the quantity of nuclear proteins and RNA in B plus plants (Kirk and Jones, 1970). Correlation coefficient value (r) = (-) 0.65 was analyzed between different genomic chromosome volume and asiaticoside content, which confirms the high negative correlation between them. Such reduction of chromosome volume with the increase of asiaticoside may be due to the presence of B-chromosomes with autosomes, which coincide with previous views. Again high amount of asiaticoside content was noted in B plus karyotype. So, B-chromosomes might influenced the genomic length as well as volume on one hand and asiaticoside content on the other hand. Evidently, the variation of the total chromosome length and volume projected the differential spiralization and differences of histone protein components of genome in addition to DNA (Sharma and Mukhopadhyay, 1984; Das and Mallick, 1989a,b; Kuo, 1989). In addition to point mutation, segmental duplication and structural alteration of chromosomes have had played an important role in evolution as well as cytotypic genome variation.

Alkaloid Content in Relation to Genomic Variation

Qualitative analysis of asiaticoside in the dry leaves of *C. asiatica* showed the presence of this chemical in different level in all the studied ecotypic races. The range of asiaticoside contents were 0.006% to 0.114% in ecotype VII and ecotype II respectively. The plants grown in subtemperate Himalayas viz., ecotypes II, X and V showed higher amount of asiaticoside con-

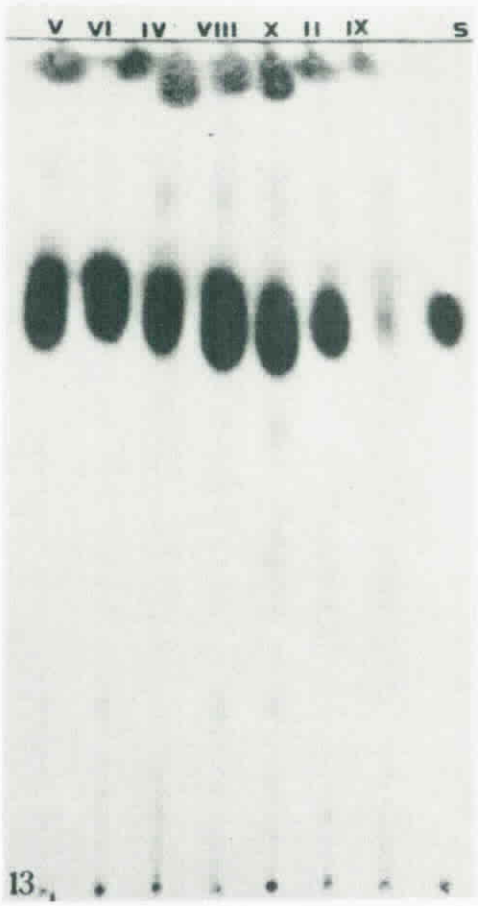


Fig. 13. Chromatogram showing separation of asiaticoside in the leaf extracts of different ecotypes of *C. asiatica* × 0.55 (approx.). S = Authentic sample of asiaticoside.

Table 2. *Anova test showing significant differences in the asiaticoside contents among the ecotypes of* ***C. asiatica***

Source	Degrees of freedom	Sum of Squares	Mean sum of Squares	'F' ratio
Between ecotypes	9	0.083	0.00922	184.40**
Within ecotypes	60	0.003	0.00005	

**Significant both at 5% and 1% levels.

Table 3. *Non-significant blocks of asiaticoside content (%) in* ***Centella asiatica***

Ecotype VII	Ecotype IX	Ecotype VIII	Ecotype I	Ecotype III
2n=18	2n=18	2n=18+1B	2n=18	2n=18
0.006	0.017	0.060	0.062	0.075
Ecotype IV	Ecotype VI	Ecotype V	Ecotype X	Ecotype II
2n=18	2n=18	2n=18	2n=18+2B	2n=18+2B
0.082	0.088	0.097	0.105	0.114

tents i.e., 0.114%, 0.105% and 0.97% accordingly, but the plants grown in sandy loomy plains of India showed minimum amount of asiaticoside content (Table 1, Fig. 13). Moreover, ecotype II and ecotype X showed a pair of B's in their genotypes (Figs. 2, 10). The analysis of variance test revealed that in general, there was a highly significant difference in the alkaloid content between ecotypes (Table 2). However, a number of non-significant blocks of ecotypes were recorded from Duncan's multiple range test (Harter, 1960). The clear correlation between altitudinal variation and asiaticoside content was observed (Tables 1, 3). The correlation coefficient value (r) = 0.60 was obtained. This positive correlation indicate the significant correlation between asiaticoside content and altitudinal variation among the ecotypes. It was evident that in karyotype when a pair of B-chromosomes were present along with C, D and E type of chromosomes the alkaloid contents were also increased as showed in ecotypes II and X only. In other combination, the plant did not produce high amount of asiaticoside which was observed in ecotype VIII where single B-chromosome was present along with C and D types of chromosomes. Possibly the asiaticoside content may be influenced by the interactions between B-chromosomes and genomic complements of the plant. It was recorded that the presence of 4-6 secondary constricted chromosomes having medium and submedium or submedium and subterminal or both submedium constrictions gave moderate amount of asiaticoside. B-chromosomes in addition with the genetic make up caused higher amount of asiaticoside. Perhaps, the extra-chromosomal inheritance in combination with environmental factors might play some role on asiaticoside content of plants. The genetic variability of rice also significantly differed by their single seed protein pattern (Sarkar and Bose, 1984). Again, the rate of meristem formation is influenced by genomic diversity in musa (Banerjee and Sharma, 1988). Thus, the variations might be in repetitive or unique sequences which regulates the transcriptional production through enzyme action.

The differences of such karyotype vis-a-vis genotypic differences with the differences in asiaticoside content demand special interest. The genotypic control of the alkaloid content appears to be quite evident. The ecotypes II and X too have been collected from subtemperate zones of Himalaya where the asiaticoside contents were high. The present investigation, therefore, clearly indicated the possibility of utilizing the genotypes of *C. asiatica* ecotypes II and X growing in the Himalaya for commercial exploration as the asiaticoside is a preventive drug for leprosy as well.

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雷公根(*Centella asiatica*)基因組歧異性與 asiaticoside 成份之相關

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Asiaticoside對治療痲瘋有極高的價值。本文從 10 個印度生態系之雷公根(*Centella asiatica*)以比較化學分析之方法來估算葉部組織之 asiaticoside 含量。在亞溫帶喜馬拉雅山生態系 II 和 X 發現較高之 asiaticoside 含量，其分別含有 0.114%及 0.105%。此外，新近被報告在正常染色體核型(2 n=18)外有之兩B染色體亦在這些生理小種內發現。利用統計方法進行基因組組成對 asiaticoside 含量影響之分析，顯示不同生態原其 asiaticoside 含量明顯地與其基因組之不同有關。本文並討論利用亞溫帶喜馬拉雅山所選拔出之基因型以充當商業用途之可能性。