

# Intraspecific evaluation of phenotypic variation of *Caryopteris incana* in western Kyushu, Japan

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## Abstract

*Caryopteris incana*, an endangered species distributed parts of western Kyushu in Japan, is composed of clusters of individuals that can be recognized as a population, and grow in a spatially isolated environment. The objective of this study was to evaluate the phenotypic variation of *C. incana* in western Kyushu and to clarify their relationships with natural environment and geographical structure, therefore, we investigated the growth survey using collected seeds under the same cultivation conditions. As a result, it was confirmed that each natural population showed various phenotypes in plant height, flower color, etc., and each population was classified into five groups by phenotype, showing the similar phenotype in each region. On the other hand, since different phenotypic groups were shown in the same region, it was inferred that each natural population acquired various phenotypes by differentiating independently in various natural environments. In addition, chloroplast DNA haplotypes differed between populations with the same phenotype, suggesting that they should be conserved as separate local populations to maintain genetic diversity. Similarly, because of the low seed-dispersal ability and the ecology as a lithophyte, even if haplotypes are common, when phenotypes differ between populations, they should be conserved as finely divided as possible. These results suggest that local populations and their ranges of high conservation priority should be set not only among regions but also within regions, and prompt actions should be taken.

## INTRODUCTION

*Caryopteris incana* (Thunb.) Miq. is a perennial plant or shrub natural to China, Taiwan, Korean Peninsula, and Japan.

Plant height may exceed 1 m from 30 cm, stand upright, and branch.

Opposite leaves egg-shaped have strongly aromatic with coarsely serrate, and their surface is a grey-green color [1].

Cymes extend from the axillae above the stem with blue-violet, fragrant flowers.

The inflorescence has a ring-like flower cluster on the stem and blooms from the lower part to the upper part.

It is a short-day plant that blooms from September to October, and the upper leaves die after 1 ~ 2 months of blooming.

In early winter, a few leaflets remain in a rosette and overwinter.

It is used for horticultural purposes, as well as for flower arrangement and garden planting.

In particular, many of cultivar group of *Caryopteris* × *clandonensis* A. Simmonds by mating *C. incana* (seed parent) with *C. mongholica* Bunge. (pollen parent) is used as a garden plant or cut flower mainly in Europe and the United States because it is a hardy horticultural plant [2].

However, cultivars of *C. incana* in circulation have only 3 color of flowers is blue-violet, white (*C. incana* f. *candida* C.K. Schneid.), pink (*C. incana* f. *rosea* Sugim.), few cultivars distinguished in the form except the flower color are produced.

Therefore, it is necessary to obtain phenotypic information in wild types as a breeding material for diversifying horticultural cultivars of *C. incana*, *C. ×clandonensis*.

Outside of these horticultural uses, *C. incana* contains several phenylpropanoid glycosides, including the proprietary incanoside (Gao and Han, 1997; Gao *et al.*, 1999, 2000; Li and Wang, 2004; Zhao *et al.*, 2009), which is used in China as folk medicine with medicinal properties against pertussis, menstrual irregularities, eczema, and rheumatic pain [1,3].

In addition, GC/MS analysis of volatile compounds showed that fumigants and essential oils from *C. incana* exerts potent insecticidal effects against the weevil, *Euscepes postfasciatus*, a stored grain pest in Poaceae [4].

In addition to the horticultural ornamental value, *C. incana* has the value as a functional plant.

*C. incana* is considered to be one of the continental plants that remain on the islands of western Kyushu and western mainland Japan (Ito, 1997), and its natural environment in Japan is limited to western Kyushu [5,6].

*C. incana*, which grows mainly in exposed rocky areas, shows lithophytic features, such as in soil and in crevices where organic matter accumulates. And is sometimes observed in the same assemblage as *Selaginella tamariscina* (P. Beauv.) Spr. [6].

Based on these natural environments, it is considered that each natural population of *C. incana* is distributed locally (not continuously), and that it exists in multiple local populations in the same region.

In addition, the number of indigenous populations has been declining due to destruction of natural environments, mainly due to land reclamation, road maintenance, trampling, etc., and was listed in the Red Data Book in 2000 as Endangered Class II (VU) [7].

An investigation of the habitat of *C. incana* in Japan was reported by Itow and Kawasato (1988) in 1988 [6].

Since the distribution of *C. incana* had not been investigated since this survey, we conducted a habitat survey in western Kyushu, Japan [8,9].

We identified 72 populations on Tsushima, Nagasaki Prefecture, 16 populations on the mainland of Nagasaki, 16 populations on the Goto Islands, and 109 populations on Koshikijima Islands, Kagoshima Prefecture (Fig. 1).

Among these areas, we confirmed that the indigenous population tended to decrease in the mainland of Nagasaki.

In addition, DNA was extracted from the seedling of the seeds collected from each natural population, and the chloroplast DNA was sequenced.

As a result, interpopulation variation was identified in 6 regions of chloroplast DNA, and a total of 22 haplotypes were identified, including 6 types in Tsushima, 6 types in the Nagasaki mainland, 9 types in the Goto Islands, and 3 types in the Koshikijima Islands.

Phylogenetic analysis revealed multiple sequence variation between these haplotypes and populations distributed in the Tsushima, Nagasaki mainland, and the Goto Islands, the Koshikijima Islands, and parts

of Nagasaki mainland, and they were classified into two groups, suggesting early disjunction between these regions.

We also identified common haplotypes in natural populations in the northwestern part of Nagasaki mainland and the Koshikijima Islands.

Base on these results, we clarified the genetic structure and genetic diversity among wild populations of giant reed in western Kyushu based on chloroplast DNA.

On the other hand, the phenotypic diversity of the *C. incana* has not been studied.

As *C. incana* is characterized as a chasmophyte plant, it has a localized assemblage of individuals that can be recognized as a population and grows in a spatially isolated state.

Therefore, there is a possibility that the phenotype variation adapted to the natural environment occurs in each population.

The objective of this study was to investigate phenotypic variation in each natural population by growth survey and to evaluate its association with geographical structure.

These results suggest that the phenotypic diversity of the indigenous population in western Kyushu and comparison with the genetic structure in chloroplast DNA may clarify the relationship between phenotypic variation and genetic variation.

Information on these phenotypes may contribute to the utilization of plant resources such as breeding materials and the planning of conservation programs as endangered species.

## MATERIALS AND METHODS

### GROWTH SURVEY

For the growth survey, seeds of 20 populations selected from 72 populations Tsushima, and 37 populations in western Kyushu except Tsushima, were examined.

After sowing in April 2011, they were grown under natural sunlight in a greenhouse.

First, 50 grains of each population were sown on filter paper containing GA3 in a Petri dish, and the humidity was kept at 25.

After approximately 1 ~ 2 weeks, germinated plants were transferred to cell trays and maintained in shaded cultivation tunnels.

After confirming 4 ~ 6 leaves after about 1 month, they were put in No. 2.5 pot using a mixed culture soil of red soil: peat moss: perlite = 7:2:1.

Futhermore, after 8 ~ 12 leaves were confirmed, a maximum of 12 plants in each group were placed in No. 7 pot and managed until measurement.

In order to get close to the local environment, basal fertilizer was not used, Hyponex (N-P-K = 6 -10 -5, Hyponex Japan) which was diluted 2000 times in pots No. 2.5 and No. 7, was given once a month from 2 weeks to 1 month as additional fertilizer.

In August and October, one grain each of the promic tablets (N-P-K = 12-12-12, Hyponex Japan) was placed in each pot and given as fertilizer.

Measurements were started when flowering began from September to October.

Each measurement was performed using a digital caliper and a tape measure.

As measurement items, 36 items were set.

At first, flowering was defined the stage that the corolla of first flower completely opened and calculated the days from sowing to number of days to flowering.

Then, two flowers were randomly selected for each individual in each population, and seven floral morphology items were investigated.

Those items were length of corolla, stamen, pistil, calyx and peduncle, and width of corolla and peduncle.

The width of corolla was measured The width of corolla was measured from the upper part of the flower to the front of the lip in the longitudinal direction.

The stamens and pistils were measured by disassembling each flower.

Eight leaf morphologies were investigated on one leaf at the 10th stage-which had grown to mature enough in each individual.

The investigation items were length of leaf, petiole, and width of leaf, petiole, number of sawtooth, Depth of sawtooth lacinia, angle of leaf apex, angle between main stem and petiole.

The depth of the sawtooth was measured as the distance between the tip of the fifth sawtooth and the front part of the sawtooth.

The angle of leaf apex was measured as the angle between the leaf apex and the fifth sawtooth, and the angle of main stem and petiole was measured as the angle on the adaxial side of the stem and petiole.

When all florets of the first inflorescence of each individual fully flowered, 9 items of plant shape at flowering were investigated.

The investigation items were the number of florets, the length and the width of the flower cluster, the length of intercluster, the distance from the ground to the first flower cluster, the first flowering nodal position, the height of plant at flowering, the width of plant and the length of internode.

The number of florets, the length and the width of the flower cluster were measured using the first flower cluster of the main stem.

The distance from the first flower cluster to the second flower cluster was measured for intercluster length, and the distance from the first flower cluster to the lower node was measured for internode length.

The number of days from flowering was calculated as the flowering period when all flowers of each plant were completely closed, and 9 items of plant form at the time of closing flowers were investigated.

The investigation items were the number of flower clusters on the main stem, the height of plant at setting, the number of flowering branches, and the width of main stem.

Three items of flower color and three items of leaf color were investigated using a spectrophotometer CM-700 d (Konica MINOLTA Inc.).

The investigation items were flower and leaf color value of  $L^*$ ,  $a^*$ ,  $b^*$  using Hunter Lab values.

Leaf color was measured during leaf morphometry, and flower color was measured on the lip of fresh flowers from immediately after to 1 day after anthesis.

Seven items related to flower shape, such as corolla length, and 13 items related to plant shape, such as plant height and plant width, as well as days to flowering and flowering period, were investigated for 10 individuals in each population.

Eight items related to leaves, such as leaf height, and 6 items related to colors, such as flower color, were measured for 5 individuals in each group.

Nine additional secondary variables were added to these items, and a total of 45 items were compared in each group.

## STATISTICAL ANALYSIS

IBM SPSS Statistic (International Business Machines Corp.) was used for statistical analysis, and principal component analysis was performed between each indigenous population.

To compare the phenotypes of each population, principal component analysis with varimax rotation was performed.

Dendrograms were generated by Ward's cluster analysis for phenotypic grouping.

Furthermore, one-way ANOVA was performed to determine significant differences in variables between regions.

The tests were performed using Tukey B or Hochberg's GT2 if the sample size was significantly different, and Dunnett T3 if the isodispersibility was not assumed.

Pearson's product-moment correlation coefficients were also obtained to measure correlations between variables and field data.

## RESULTS

### DAYS TO FLOWERING AND FLOWERING PERIOD

The mean days to flowering was 168.62  $\pm$  0.50 days for all individuals of the 57 western Kyushu populations.

The mean days to flowering in each region were 165.12  $\pm$  0.87 days in the Tsushima populations, 166.78  $\pm$  0.76 days in the Nagasaki mainland populations, 171.73  $\pm$  0.87 days in the Goto Islands populations, and 180.28  $\pm$  1.75 days in the Koshikijima Islands populations.

The shortest population (C 36: population number [8,9]) of days to flowering indicated 152.00  $\pm$  4.00 days and the longest population (C 7) indicated 193.83  $\pm$  6.70 days.

Both of these populations were the Tsushima populations, but the mean value in all Tsushima populations was 165.12 days, suggesting that the C7 population represents an outlier among the Tsushima populations studied.

The mean flowering period of all individuals of 57 populations in western Kyushu indicated 82.5  $\pm$  2.7 days.

The mean flowering period in each region were 77.19  $\pm$  2.32 days in the Tsushima populations, 88.87  $\pm$  2.65 days in the Nagasaki mainland populations, 83.50  $\pm$  3.48 days in the Goto Islands populations, and 76.23  $\pm$  4.67 days in the Koshikijima Islands populations.

### PRINCIPAL COMPONENT ANALYSIS

The cumulative percentages of PC1, PC2, PC3, and PC4 in all morphological variations were 42.9%. (PC1 = 15.0%, PC2 = 10.5%, PC3 = 9.1%, PC4 = 8.3%; Table 1)

PC1 showed positive and large values above 0.7 for internode length, intercluster length, distance from the ground to the first inflorescence, plant height at flowering, plant height at fruiting/number of inflorescences, plant height at fruiting and spike length/(number of inflorescences -1).

These results suggest that PC1 is an index of plant height.

PC2 showed large positive or negative values above 0.7 for flower color value of L\*, a\*, b\*.

PC3 showed positive and large values (more than 0.7) for days to flowering, first flowering nodal position and corolla length.

PC4 showed large positive values above 0.7 for leaf color color value of L\*, a\*, b\*..

The scatter plots of the scores of the principal components PC1 and PC3 scores for plant shape, days to flowering, and PC2 and PC4 scores for color showed differed among the regions (Fig. 2A, 2B).

In Fig. 1A, the Tsushima populations tended to show positive values for PC1 and negative values for PC3, indicating high plant height and early flowering.

On the other hand, the Nagasaki mainland populations tended to be negative values for PC2, indicating a low plant height characteristic.

The Goto Islands populations showed no tendency for PC1 and PC3.

The Koshikijima Islands populations tended to show a positive value for PC3, indicating late flowering.

Subsequently, Figure 1B showed that the Tsushima populations tended to show positive values for PC2 and negative values for PC4, indicating a characteristic of deep flower and leaf colors.

The populations in Nagasaki mainland and Goto Islands had the characteristics which the color of flowers was light because the value of PC2 tended to show negative in those populations.

The population of the Nagasaki mainland populations and the Goto Islands populations showed a tendency toward negative values of PC2, indicating a characteristic of light flower color.

The Koshikijima Islands population showed characteristics intermediate to those of other regions.

### **CLUSTER ANALYSIS GROUP CLASSIFICATION**

Dendrograms by cluster analysis on a population mean of 45 variables were classified into 5 groups, from the 57 western Kyushu natural populations A to E.

The geographical distribution of the classified groups and each indigenous population was shown in Fig. 3.

Populations classified into groups A and B were distributed in Tsushima.

Except for two populations in group A, populations classified into groups C and D were distributed in Tsushima.

Groups classified as Group E were distributed in the Koshikijima Islands.

### **LOCAL PHENOTYPES**

By the principal component analysis, we clarified populations distributed each area show the characteristics which different phenotypes each area.

On the other hand, we conducted one-way ANOVA to understand the phenotypes in each group because they were classified into different phenotypic groups within the region by cluster analysis.

The mean values of some variable between groups and the results of one-way ANOVA are shown in Table 2.

There were significant differences among the groups in 43 variables, except for stamen length and petiole length.

In group A, the plant height was lower and the flower color was darker.

In group B, flowering period was shorter, plant height was higher, and leaf and flower colors tended to be darker.

In Group C, the flowering period was long, the plant height was low, and the number of florets per cluster was small, but the clusters were dense and the flower color tended to be light.

In group D, flowering was slightly delayed and the number of inflorescences was small, while the number of lateral shoots was large and the flower color was light.

In Group E, flowering was delayed, the inflorescence and flower cluster was large, the leaves tended to be broad but less serrated.

As a result of correlation analysis, The number of florets showed a positive correlation with the longitudinal diameter of the inflorescence and the transverse diameter of the inflorescence (0.72, 0.61), indicating that the inflorescence size increased in proportion to the number of flowers.

The days to flowering were not correlated with the number of inflorescences and the distance from the length/threshold of the inflorescence to the first inflorescence, but correlated positively with the first flowering nodal position (0.68).

As there were significantly different variables among the regions, we performed correlation analysis among the populations within each region, but no correlation coefficient of 0.5 or more was shown.

## DISCUSSION

### PHENOTYPES SPECIFIC TO EACH GROUP AND REGION

Growth survey revealed that the natural population of *C. incana* throughout western Kyushu had diverse phenotypes.

On the other hand, since no correlation was shown between these phenotypes and these environments in Tsushima, suggesting that they acquired various phenotypes by differentiating independently in each environment.

Cluster analysis of phenotypes revealed that each group was largely classified by region.

Groups A and B distributed in Tsushima showed a common tendency for earlier flowering, more serrated leaves and darker flowers than those in other areas.

On the other hand, there was no difference in the number of inflorescences between the two groups, but the plant height tended to be different between the two groups.

In particular, Group B was the most tallest of all the groups and was not be distributed outside Tsushima, suggesting a unique phenotypic group in Tsushima.

Group C and group D distributed in the Nagasaki mainland and the Goto Islands showed a common tendency, which was that the leaf and flower colors were brighter and the lateral shoots spread in the horizontal direction.

However, there were significant differences in flowering period and stem diameter between the two groups.

The number of florets per cluster was inversely proportional to the number of florets, and there were significant differences in plant height and spike length.

Therefore, the C group showed the most shotrest trait with many flower clusters in long spikes, while the D group showed the trait with few flower clusters with many florets in short spikes.

Group E distributed in the Koshikijima Islands had slower flowering, larger flower buds, larger flower clusters, wider leaves with less serrated edges, and smaller plant width than those in other areas.

These phenotypes are unique to the Koshikijima Islands, which have not been observed in other regions.

Groups B and D were common in each region in that they were taller than the other group.

Group B, however, showed a tendency to elongate in the vertical direction because of the small number of lateral branches and the long spike length relative to the distance from the ground to the first inflorescence, whereas group D showed a tendency to elongate in the horizontal direction because of the opposite characteristics.

This Difference in AN flower colMmor was common among the groups in each region, suggesting that it is a characteristic of each region.

### COMPARISON OF PHENOTYPES AND CHLOROPLAST DNA HAPLOTYPES

We compared the phenotypically classified groups based on the results of growth surveys with the previously reported phylogenetic analysis of chloroplast DNA haplotypes [8].

First, in Tsushima, six types of haplotypes were distributed and classified into three groups according to sequence variation.

Haplotypes revealed that H1 (Haplotype group 1), H2, and H3-H6 are widely distributed in southern, eastern, and northern southern and northern, respectively.

On the other hand, the phenotypes were classified into 2 groups by growth survey in 20 populations on Tsushima out of 57 populations in western Kyushu, and there was no tendency in the geographical distribution of each group.

Therefore, since the association between haplotypes and phenotypic groups in Tsushima was low and there were different phenotypic groups among populations with common haplotypes, it was considered that each natural population acquired various phenotypes by adapting to various natural environments after colonizing in the southern and expanding its distribution toward the northern.

Subsequently, nine haplotypes were distributed in the Goto Islands, which revealed a relatively high level of genetic diversity [9].

Populations with H 12, H 16, and H 21 showed the phenotype of group C, and groups of common phenotypes within populations showing common haplotypes.

However, these haplotypes belonged to different subgroups within the group of the Goto Islands in the phylogenetic tree and haplotype network, suggesting a low correlation between the timing of distribution formation and the phenotype.

These results suggest that in the Goto Islands, as in the Tsushima, various phenotypes have been acquired through the progress of adaptive differentiation to each natural environment after isolation to each population.

On the other hand, the above populations showing common haplotypes were classified into a group of common phenotypes, suggesting that similar environmental factors might have caused differentiation into similar phenotypes in these populations, and that populations showing the same haplotypes that had been connected or had seed exchange until recently might have been divided, showing the present geographical distribution.

In Nagasaki mainland, 6 haplotypes are distributed and classified into 3 groups based on sequence variation, with H7 to H 10 found in the southern part of Hiradojima Island in the northwest and around Nagasaki City in the southern part, H 13 in the northern part of Hiradojima Island, Shiradake (C 82) and Matoyama-oshima (C 109), and H 22 in the central part of Nagasaki mainland (C 83).

The population in the central part of Nagasaki mainland (C83) with H22 from the Goto Islands, showed the phenotype of group D common to the population with H22 distributed in the Nakadori Islands of the Goto Islands.

On the other hand, populations with H 13, which was considered to be derived from Kyushu mainland and shared with the Koshikijima Islands, and populations showing H7, which was considered to be an ancestral haplotype that settled in this region after being separated from the Goto Islands, tended to show the phenotype of group C.

These results suggest that geographical variations in the Nagasaki mainland were caused by differences in natural environments between the southern and northwestern parts of the island, rather than differences in the timing of distribution formation with haplotypes, resulting in different phenotypes in plant height and earliness of flowering.

In the Koshikijima Islands, 3 haplotypes were distributed, and it is considered that ancestral populations with H 13, which is common to the northwest of the Nagasaki mainland, expanded its distribution to Shimokoshikijima after moving from the Kyushu mainland to Kamikoshikijima.

In terms of phenotypes, the population in Kamikoshiki Island (C106) showed the phenotype of group C distributed in the Goto Islands and the Nagasaki mainland, and the other 4 populations showed the phenotype of group E that were greatly different from those in other regions in terms of floral organs and flower cluster sizes.

Since the former population is located along the road where slope machining work was carried out, it was considered that the population was formed by the planting brought in from Nagasaki mainland or the Goto Islands.

## LOCAL POPULATIONS WITH HIGH CONSERVATION PRIORITY

Based on the phenotypes and haplotypes of *C. incana* revealed in previous study and the current status of its environment, we discuss the endangered local populations and their range listed in the Ministry of the Environment's Red List Category, which is considered to be of high conservation priority [7].

First, populations in Tsushima were classified into two groups when compared with the whole of western Kyushu and five groups when compared with the inside of Tsushima based on the phenotype, and there were no relation with their geographical distributions.

On the other hand, the haplotypes were classified into three groups, and there were trends in their geographical distribution, but no association with phenotypic groups.

Therefore, it is difficult to define a large range of local populations taking into account both phenotypic and haplotype characteristics.

As for the present conditions, the disappearance of the natural population and the decrease in the population were confirmed for 7 years from 2007 to 2014, but the large population with numbers of individuals tended to be many in Tsushima than in other areas [8].

These results suggest that the risk of extinction in Tsushima is lower than that in other areas, and that there were no phenotypic or haplotype groups composed only of endangered populations, suggesting that conservation is not very urgent.

Subsequently, in Nagasaki mainland, the natural populations distributed in Hirado-jima and surrounding areas in the northwest showed a common phenotypic group, but different haplotypes were found between the northern and southern populations of Hirado-jima.

Among the populations reported by Ito and Kawasato [6], many of those assumed to have disappeared were distributed in the Nagasaki mainland, confirming that they were recently affected by human activities.

On the other hand, number of individuals in the natural populations which distributed in the northwest tended to be larger than that of the southern population, and they were found in exposed rocky areas at high altitudes, which were considered to be less affected by human activities.

Therefore, the conservation areas in Nagasaki mainland were classified into three groups, the group with H7 in the southern part of Hirado-jima, the group with H 13 in the northern part of Hirado-jima and its surroundings, the population in the central coastal area (C83), and the group with H 8 ~ H10 in the southern part.

In particular, number of individuals the southern group tended to be low, suggesting a high conservation priority.

In the Goto Islands, 4 populations out of 5 populations distributed in southern Fukue Island and its north-eastern part, Kuga Island, were considered to be populations with high conservation priority, because number of individuals in their populations was less than 20, and especially, C 88 and C 102 distributed in the western

and southern parts of Fukue Island showed haplotypes close to the ancestral population established early in the Goto Islands and were classified into different phenotypic groups.

No phenotypic or haplotype groups composed only of small populations with less than 20 individuals were found in the central part, Narushima and its northeast part, Nakatsushima Island, suggesting that conservation is not very urgent.

In the Koshikijima Islands, there are considered to be a high conservation priority area because they show characteristic phenotypes among the Japanese natural population.

In particular, populations distributed in Kamikoshikijima Island and Nakakoshikijima Island (C 103 to C 105) are considered to be conservation priority populations because of their small population size.

Haplotypes were also different between natural populations in Nagasaki mainland and the Goto Islands, which showed the same phenotypic group, suggesting that they should be conserved as separate local populations to maintain genetic diversity in all natural populations in Japan.

In addition, the low seed-dispersing ability of *C. incana* and its ecology as a lithophyte suggest that even within regions with the same haplotype, if phenotypic groups differ among populations, they should be preserved as finely divided as possible.

The delay in response of plant species to anthropogenic environmental changes suggests that the potential extinction crisis is more advanced than the present natural state [9,10]

These results of this study suggest that local populations and their ranges of high conservation priority should be set not only among regions but also within regions, and prompt actions should be taken.

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Table 1. Eigenvalue, contribution ratio, and principal component load of each principal component in the growth survey of Label

X1  
X2  
X3  
X4  
X5  
X6  
X7  
X8  
X9  
X10  
X11  
X12  
X13  
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X39  
X40  
X41  
X42  
X43  
X44

Table 2. Results of analysis of variance of some variable among 5 groups in growth survey of 57 native populations in western



