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Heterochromatic differentiation in barley chromosomes revealed by C- and N-banding techniques

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Summary. Heterochromatin distribution in barley chromosomes was investigated by analyzing the C- and N-banding patterns of four cultivars. Enzymatic maceration and air drying were employed for the preparation of the chromosome slides. Although the two banding patterns were generally similar to each other, a clear difference was observed between them at the centromeric sites on all chromosomes. Every centromeric site consisted of N-banding positive and C-banding negative (N^+C^-) heterochromatin in every cultivar examined. An intervarietal polymorphism of heterochromatin distribution was confirmed in each of the banding techniques. The appearance frequencies of some bands were different between the two banding techniques and among the cultivars. The heterochromatic differentiation observed is discussed with respect to cause.

Key words: *Hordeum vulgare* L. – C-banding – N-banding – Centromeric heterochromatin – Heterochromatic polymorphism

Introduction

Giemsa banding techniques have enabled the complete identification of individual chromosomes and chromosome arms in barley (*Hordeum vulgare* L.). This is due largely to the widely recognized fact that barley has a common basic banding pattern (Linde-Laursen 1975, 1978, 1981; Noda and Kasha 1978; Islam 1980; Singh and Tsuchiya 1981, 1982a, b; Fukui and Kakeda 1990). However, opinions are divided on whether or not this basic pattern is intrinsically modified by the technique adopted and/or the genotype used.

For example, Islam (1980) and Linde-Laursen (1981) stated that they observed centromeric bands that were produced by N-banding but not by C-banding. Linde-Laursen (1978, 1981) also pointed out that there are wide differences among various barley lines in the size of particular bands and the number of bands on the same chromosome. Conversely, Singh and Tsuchiya (1982a, b) reported that there is no centromeric band produced only by N-banding, and stated that intervarietal heterochromatin polymorphism is absent in barley. Eventually, based on the fact that more bands can be found at prometaphase to early metaphase than at midmetaphase, they drew the conclusion that the variation in banding pattern observed in barley might be ascribed only to the mitotic stage examined and the technical procedure used.

Such discrepancies have caused serious confusion and raised hard discussion among researchers conducting cytological studies on barley, suggesting that there should be a comprehensive reexamination of the banding pattern. In this study the chromosome banding patterns of four barley cultivars were investigated by using C- and N-banding techniques. We demonstrated that N-banding positive and C-banding negative (N^+C^-) heterochromatin is present at all centromeric sites. It was also proved that intervarietal banding pattern polymorphism results from the intrinsic native characteristics of chromosomes.

Materials and methods

Four two-rowed barley cultivars, 'New Golden', 'Shin Ebisu 16', 'Betzes' and 'Emir' were used. Either the C- or the N-banding pattern, or both, had been examined previously in these

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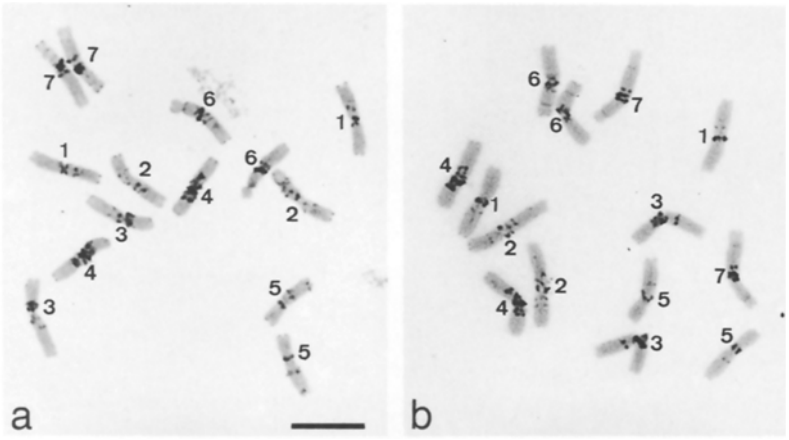

Fig. 1 a, b. Metaphase plates stained by C-banding (**a** 'Emir') and N-banding (**b** 'Shin Ebisu 16') treatments without earlier acetocarmine staining. *Bar* = 10 μm

cultivars (Linde-Laursen 1975, 1978, 1981; Noda and Kasha 1978; Islam 1980; Singh and Tsuchiya 1981, 1982a, b; Fukui and Kakeda 1990).

Seeds were germinated at 20 °C, and when the roots had elongated to about 1 cm in length, they were excised and pretreated with distilled water at 0 °C for 16 h. The root tips were fixed with freshly prepared Farmer's solution (methanol : acetic acid, 3 : 1) and stored in a freezer (–20 °C) for about 1 week. The fixed root tips were washed with distilled water and then macerated with an enzymatic mixture (4% Cellulase Onozuka RS, 1% Pectolyase Y-23, 75 m*M* KCl, 7.5 m*M* Na_2EDTA, pH 4.0; Fukui and Kakeda 1990) at 37 °C for 45–60 min. After rinsing, the root tips were each tapped carefully with the tip of a forceps on a glass slide with additon of a few drops of the fixative, then air-dried.

Some slides were stained with 0.5% acetocarmine solution for about 10 min and well-dispersed metaphase plates were photographed. These slides were then destained with 45% acetic acid for 5 min, washed with distilled water for 10 min and air-dried.

Both the acetocarmine-stained and the unstained slides were subjected to banding treatments after about 1 week of air drying at room temperature. For C-banding, the procedure reported by Giraldez et al. (1979) was used with the following modifications. The air-dried slides were incubated in 0.2 *N* HCl at 55 °C for 3 min, in 5% $Ba(OH)_2$ solution at 25 °C for 5 min, then in 2 × SSC solution at 55 °C for 20 min. Prior to $Ba(OH)_2$ treatment, the slides were air dried by a fan for 30 min–1 h. For N-banding, the air-dried slides were incubated in 1 *M* NaH_2PO_4 solution at 90 °C for 3 min according to Jewell (1981). In both the C-banding and N-banding procedures, the slides were rinsed with tap water or distilled water after each step of the treatment.

Treated slides were usually stained with 8% Wright solution (Merck) diluted with 1/30 *M* phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 6.8) for 1 h at room temperature, then briefly rinsed and air dried. The concentration of Wright solution and the duration of the staining were appropriately adjusted within the extents of 5%–10% and 30 min–2 h, respectively, according to the qualities of the slides and Wright solutions. Only well-banded metaphase plates were photographed.

In some slides combined treatments with C-banding and N-banding were given to the same preparation in the following way: Well C-banded (N-banded) metaphase plates were photographed, then subjected to N-banding (C-banding) treatment without destaining the C-bands (N-bands).

In addition to the above procedure, two others, one different in slide preparation technique and the other in band-staining technique were also attempted in order to examine whether band pattern varies with the technical procedures applied. One involved the squash method comprising root-tip prestaining with acetocarmine (0.5% for 30 min–1 h) instead of the method with enzymatic maceration and air drying, and the other used Giemsa solution (1%, diluted with 1/15 *M* phosphate buffer, pH 6.8) instead of Wright solution.

Results

Enzymatic maceration and air drying was a more effective method for obtaining numbers of quality metaphase plates on a glass slide than the squash method. The application of Wright solution improved the stain specificity of each band compared with the application of Giemsa solution, giving highly contrasted bands as shown in Fig. 1. However, these differences, which are a result of the techniques used, gave no significant difference in each of the C- and N-banding patterns within a cultivar.

Figure 2 shows two acetocarmine-stained metaphase plates of 'New Golden' (a, b) and the same plates subsequently stained by C-banding (c) and N-banding (d) treatments. In Figure 3 the individual chromosomes that appear in each plate of Fig. 2 have been arranged in order of chromosome number. Figure 3 clearly shows a difference between C- and N-banding patterns: N-bands, but no C-bands, appeared at the centromeric sites of all chromosomes. Confirmation of this was (1) the appearance at the centromeric sites of diamond-shaped structures, such as those reported by Singh and Tsuchiya (1982a, b), which were stained only by the N-banding treatment (e.g., chromosomes 2–4 in Fig. 3b), and (2) the occasional appearance at these sites of only negatively stained pairs with dots or diamond-shaped structures under the microscope with the C-banding treatment.

The existence of such N-banding specific bands was strongly supported by Fig. 4, which illustrates the result of combined treatments with C-banding and N-banding

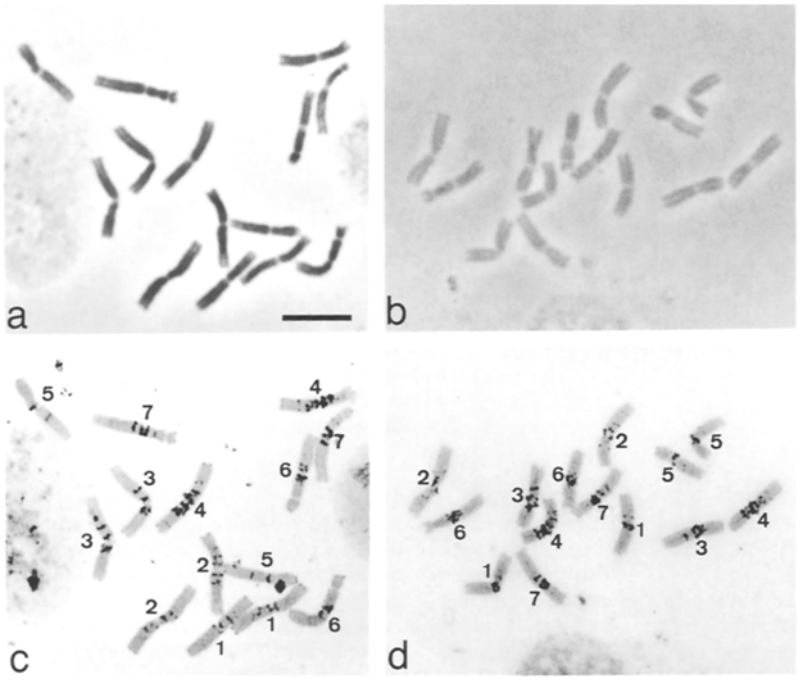

Fig. 2a–d. Acetocarmine-stained metaphase plates of 'New Golden' (**a**, **b**), and the same plates subsequently stained by C-banding (**c**) and N-banding (**d**) treatments. *Bar* = 10 μm

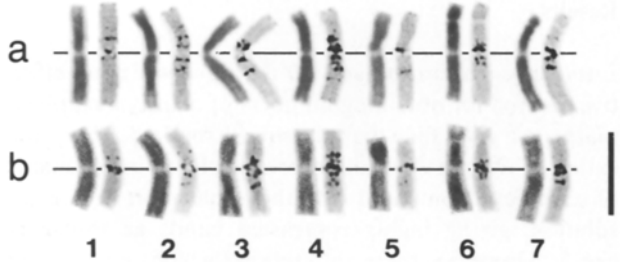

Fig. 3a, b. Acetocarmine-stained chromosomes (**a, b** *left-hand side*) and the same chromosomes subsequently stained by C-banding (**a**) and N-banding (**b**) treatments (*right-hand side*). Individual chromosomes were taken from the plates of Fig. 2. The *horizontal lines* indicate the position of the centromere. Assignment of the long and the short arm of chromosome 1 follows Singh and Tsuchiya (1982b). *Bar* = 10 μm

in 'Shin Ebisu 16'. New bands or more darkly stained regions appeared at the centromeric sites (Fig. 4b, arrows) when the N-banding treatment was subsequently applied to C-banded chromosome plates. Conversely, the centromeric N-bands disappeared or remained as only traces when a N-banded chromosome slide was subjected to the C-banding treatment.

Figure 5, which shows C-banded (a) and N-banded (b) chromosomes of the four cultivars, also confirms that the N-banding positive and C-banding negative (N^+C^-) manner of the centromeric bands was common to all the cultivars. Thus, it may safely be concluded that all the centromeric sites of barley chromosomes consist of N^+C^- heterochromatin.

A distinct intervarietal polymorphism in banding pattern is shown in Fig. 5. The interstitial small band located on the middle distal part of the long arm of chromosome 4 (4L) appeared specifically in 'New Golden' and 'Betzes', and the subterminal band on 5L appeared uniquely in 'Emir' (refer also to Fig. 7). The interstitial band on the middle part of 3L was larger and denser in 'New Golden' and 'Shin Ebisu 16' than in 'Betzes' and 'Emir', while that on the middle part of 5L was smaller and lighter in the former two than in the latter two (refer also to Fig. 7). This mode of polymorphism was common to both C- and N-banding patterns.

Figure 6 shows C-band (a) and N-band (b) patterns of 'New Golden' at four mitotic stages from early metaphase to midmetaphase. The N-banding specific centromeric band kept the N^+C^- property throughout the four mitotic stages examined. Among the bands which showed intervarietal polymorphism (Fig. 5), the interstitial band on the middle distal part of 4L appeared to be stable, and the interstitial bands on the middle parts of 3L and 5L maintained both their sizes and their densities. The situation was almost the same in the three other cultivars. These results clearly indicate that differences in mitotic stages do not result in any significant change in banding pattern.

Figure 7 summarizes the banding pattern variations in C-banded (a) and N-banded (b) chromosomes of the four cultivars. In this idiogram each band was differentially shaded according to its appearance frequency (see figure legend). The idiogram was based on observations made on metaphase plates which had undergone direct

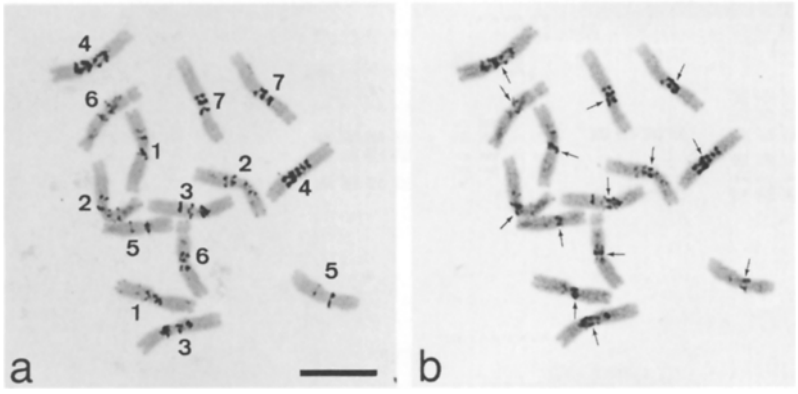

Fig. 4 a, b. Metaphase plate ('Shin Ebisu 16') stained by C-banding treatment (**a**), and the same plate subsequently stained by N-banding treatment (**b**). *Arrows* indicate the centromeric bands that appeared after N-banding treatment. *Bar* = 10 μm

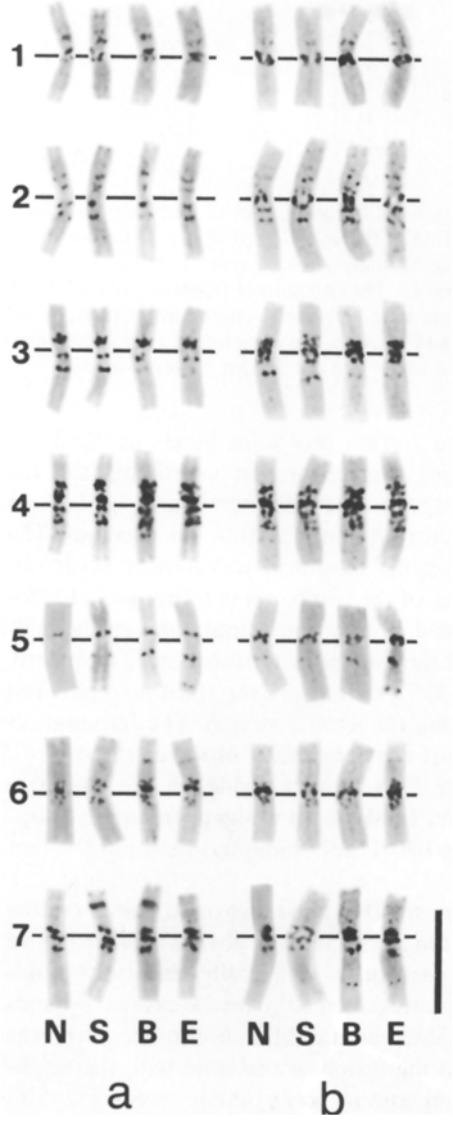

Fig. 5 a, b. C-banded (**a**) and N-banded (**b**) chromosomes of four cultivars 'New Golden' (*N*), 'Shin Ebisu 16' (*S*), 'Betzes' (*B*) and 'Emir' (*E*). *Bar* = 10 μm

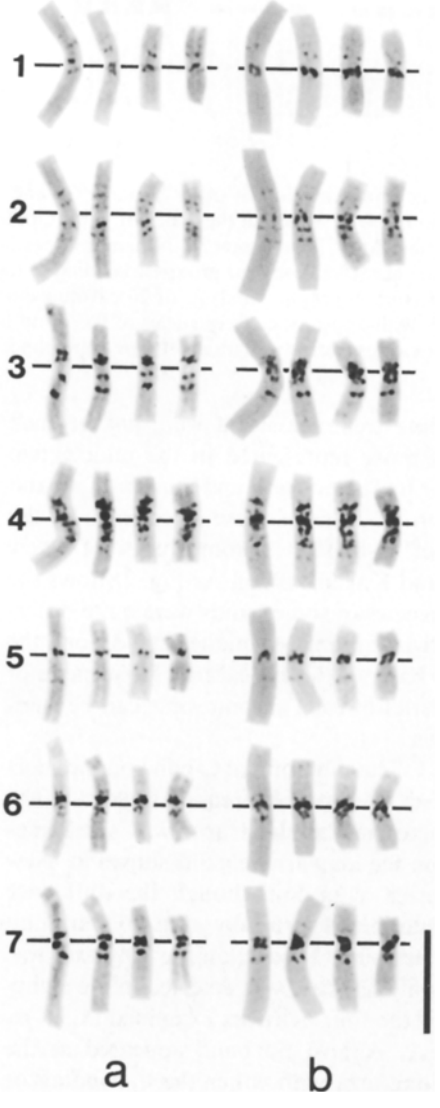

Fig. 6 a, b. C-banded (**a**) and N-banded (**b**) chromosomes of 'New Golden' at different mitotic stages from early metaphase (*left*) to mid metaphase (*right*). Chromosomes in vertical arrangement are those from a single cell. *Bar* = 10 μm

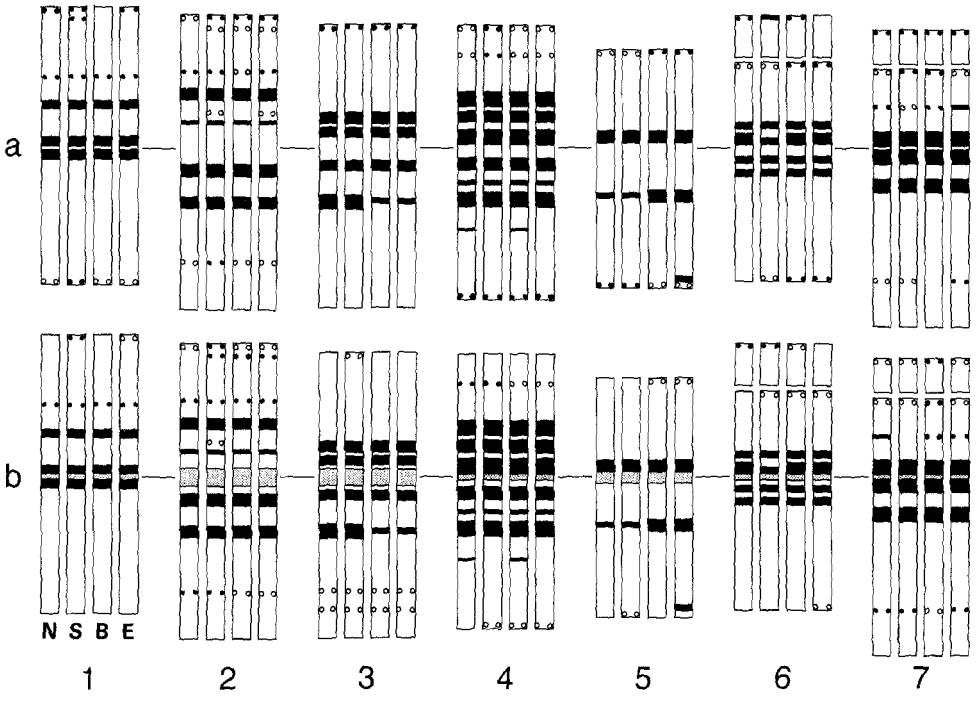

Fig. 7a, b. Idiogramic representation of the C-banding (**a**) and the N-banding (**b**) patterns of chromosomes in four cultivars. Each set of four chromosomes is arranged in the order of 'New Golden' (*N*), 'Shin Ebisu 16' (*S*), 'Betzes' (*B*) and 'Emir' (*E*) from left to right (See the example below Chromosome 1). N-banding specific bands located at the centromeric sites are represented by the *dotted parts*. Other bands are classified into four groups according to their frequency of appearance. The appearance frequency of each band was obtained by microphotographic analysis of 20 chromosomes from ten quality metaphase plates in each banding technique for each cultivar. Bands with appearance frequencies of 95% and higher, 45%–94%, and 11%–44% are depicted as *solid bands*, *solid circles* and *open circles*, respectively. Bands with an appearance frequency of 10% and under are not shown in the idiogram

staining by banding treatments and were just at such mitotic stages as those represented in the middle two lanes of Fig. 6a or b. The position and the size of a band were mostly set on the basis of those determined by the image analysis of N-banded chromosomes of 'New Golden' (Fukui and Kakeda 1990). As Fig. 7 shows the appearance frequencies of some bands were quite different between the two banding techniques and among the cultivars. On the basis of the appearance frequencies of bands, characteristics of each chromosome can be summarized as follows.

Chromosome 1. The subterminal C-band on the short arm of 'Shin Ebisu 16' appeared frequently (85%). The centromeric C-band on the short arm was sometimes larger than that on the long arm, e.g., as shown in 'New Golden' and 'Betzes' (Fig. 5a), though the difference could not be determined precisely due to variation among cells. The terminal N-band on the long arm was rarely observed; only one case was detected in the eighty chromosome ls of the four cultivars examined (1.3%).

Chromosome 2. Almost no band appeared at the terminal of the long arm with either the C-banding or N-banding technique (1.9%). The appearance frequency of the subterminal band on the short arm was higher in N-banding (44% averaged over the four cultivars) than in C-banding (15% on average).

Chromosome 3. Two very faint bands in the distal regions on the long arm appeared in N-banding (22% for both bands averaged over the four cultivars), while these scarcely appeared in C-banding (6% on average). The terminal band on the long arm was almost never observed with either of the two banding techniques (1.9%).

Chromosome 4. A faint interstitial band on the middle distal part of the short arm appeared more frequently in N-banding (43% averaged over the four cultivars) than in C-banding (26% on average). The terminal N-band on the short arm was rarely observed (5%).

Chromosome 5. A terminal band on the long arm appeared in 'Shin Ebisu 16' at a higher frequency (80% in C-banding and 40% in N-banding) than in the other cultivars.

Chromosome 6. The most proximal band on the short arm of 'Shin Ebisu 16' was always smaller in size than its counterparts in the other cultivars with C-banding (Fig. 5a). This also seemed to be the case in N-banding, though the intervarietal difference in band size was indistinct due to the fusion of the band with the neighboring N-banding specific centromeric band (Fig. 5b). The band adjacent to the secondary constriction on the short arm was frequently more prominent in C-banding than in N-banding, though its size was so unstable among cells that its intervarietal difference could not be

determined. The terminal band on the satellite seldom appeared in 'Emir' with both the banding techniques (2.5%, only one case with C-banding).

Chromosome 7. The situation regarding the band adjacent to the secondary constriction on the short arm was almost the same as that of chromosome 6. In some cells, however, the C-band was more prominent than that of chromosome 6, as exemplified in 'Shin Ebisu 16' and 'Betzes' in Fig. 5a. The interstitial bands on the middle part of the short arm of 'Shin Ebisu 16' and the distal part of the long arm of 'Betzes' appeared at lower frequencies than those of the other cultivars with both banding techniques. The N-banding specific band at the centromeric site seemed larger in 'Shin Ebisu 16' than in the other cultivars. No terminal band was observed on the long arm with either of the two banding techniques.

Discussion

In the present study, N-banding positive and C-banding negative (N^+C^-) bands were confirmed at all the centromeric sites of barley chromosomes (Figs. 3–6), and in each chromosome the locations of the N^+C^- band and N^+C^+ band(s) in the proximal region were clearly demonstrated as represented in Fig. 7. The intervarietal banding pattern polymorphism observed in this study is almost identical with that obtained from the C-banding of 'Shin Ebisu 16', 'Betzes' and 'Emir' (Linde-Laursen 1978) and that from the N-banding of 'Betzes' and 'Shin Ebisu 16' (Islam 1980).

Singh and Tsuchiya (1982a, b) reported that banding pattern variations are due to the technical procedures used and the mitotic stages examined, and concluded that no heterochromatic differentiation exists in barley chromosomes. In our study, however, variations in the banding pattern appeared to be stable throughout the four mitotic stages examined (Fig. 6) regardless of the preparation method (enzymatic maceration or squashing) or the staining solution (Wright or Giemsa). It can therefore be concluded that the banding pattern variations originate in the heterochromatic differentiation existing in barley chromosomes.

The banding patterns observed in the present study differ in several ways from those reported in a number of previous reports (Linde-Laursen 1978, 1981; Noda and Kasha 1978; Islam 1980; Singh and Tsuchiya 1982b; Fukui and Kakeda 1990). Firstly the idiograms of Islam (1980) and Linde-Laursen (1981) displayed no centromeric N-bands on some chromosome arms. This would be due to the fusion of the N^+C^- band with the adjacent N^+C^+ band(s), as suggested by Linde-Laursen (1981). Secondly, Linde-Laursen (1978) observed in 'Shin Ebisu 16', 'Betzes' and 'Emir' that there were intervarietal differences in the size of the C-band at the distal part on 2L, the parts adjacent to the secondary constrictions on 6S and 7S, the most proximal part on 7S, and the proximal interstitial part on 7L. However, these differences could not unequivocally be determined in our study. Thirdly, Linde-Laursen (1981) observed N-banding negative and C-banding positive (N^-C^+) bands at the terminals of all the chromosome arms, the distal part on 2L, and the parts adjacent to the secondary constrictions on 6S and 7S. In this study, however, except for the terminal bands on 2L, 3L and 7L (Fig. 7), these "N^-C^+" bands were all categorized to be N^+C^+ bands, even though they were often less conspicuous with N-banding than with C-banding. The terminal bands on 2L, 3L and 7L were rarely observed with either the C-banding or N-banding technique. Fourthly, compared with the previous N-band pattern of 'New Golden' (Fukui and Kakeda 1990), the present N-band pattern (Fig. 7b) showed an increased number of bands (e.g., at the interstitial sites of 2S, 3L, 4S, 7S, 7L, etc.) and the stable appearance of some bands (e.g., the interstitial bands of 1S, 2L, 6L, etc.). This would be due mainly to staining with Wright solution instead of Giemsa solution. In the present N-banding procedure, the temperature of the treatment with 1 *M* NaH_2PO_4 was reduced from 94 °C to 90 °C to minimize any disruption of chromosome shape. This modification may also have influenced the above-described differences between the two N-band patterns, though it did not produce such a drastic change of banding pattern as Jewell (1981) reported.

The heterogeneity between C-banded and N-banded heterochromatin shown in this study is of special interest because N^+C^- heterochromatin is probably a novel kind of heterochromatin in *Triticeae* species. Except for the special heterochromatin found at the nucleolar regions of two wheat chromosomes, which changes from $N^-(C^-)$ to $N^+(C^-)$ when the temperature of the NaH_2PO_4 treatment is reduced (Jewell 1981), only two kinds of heterochromatin, N^+C^+ and N^-C^+ heterochromatin, have so far been reported in wheat (Gill 1987), rye (Schlegel and Gill 1984) and *Elymus* (Morris and Gill 1987). With respect to the relationship between the two kinds of heterochromatin, Gill (1987) suggested that C-banding visualizes all classes of heterochromatin while N-banding visualizes only some specialized type of heterochromatin containing polypyrimidine sequences. This explanation is most likely also applicable to the N^+C^+ heterochromatin in barley chromosomes (Dennis et al. 1980), but is obviously not applicable to the formation of N^+C^- heterochromatin.

According to Burkholder (1988), one of major effects of the C-banding treatment is the selective extraction of DNA and proteins from the negatively banded chromosomal regions. Thus, in the C-banding of barley chromosomes, large portions of DNA and proteins could be extracted from the centromeric sites. In our study, how-

ever, N-banding produced new centromeric bands even after the C-banding treatment (Fig. 4). This suggests that at the centromeric sites some chromosomal material still remains after C-banding treatment and its structure is altered so as to be stainable by the subsequent N-banding treatment. Thus, the differential alteration of chromosome structure, rather than the differential extraction of chromatin, to N- and C-banding treatments may cause the formation of N^+C^- heterochromatin. The chromosomal material specifically stained by the N-banding treatment may be a phosphoprotein (Buys and Osinga 1982).

As to the intervarietal heterochromatin polymorphism, several causes are possible. For example, the 'Emir'-specific subterminal band on 5L is considered to have been introduced through a variety of *H. distichum* var. *laevigatum* (Linde-Laursen et al. 1982). The site-specific amplification of repeated DNA sequences, unequal crossing-over, the structural change of a chromosome, etc. may also be involved in the factors causing heterochromatin polymorphism. Further cytological and molecular approaches will be needed to elucidate the causes.

The information on heterochromatic differentiation obtained in this study is indispensable for further cytological studies in barley, e.g., for the analysis of chromosomal structural changes. As has been reported, the application of the C-banding technique is indeed useful for localizing the breakpoints of translocation chromosomes (Linde-Laursen 1988; Konishi and Linde-Laursen 1988), but it is difficult to determine whether the breakpoint is located just on the centromere or around the centromere by C-banding only. In such a case, the N-banding technique should enable the determination of the breakpoints more precisely because the N^+C^- centromeric bands form additional landmarks. A knowledge of intervarietal polymorphism of heterochromatin would also be important in further studies on chromosomes. In the analysis of translocation chromosomes of various origins, for example, the mis identification of breakpoints would be unavoidable without the premise that heterochromatic polymorphism could occur.

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