Chromosome relationships among 13 species and one variety of *Lachenalia* (Liliaceae) with the chromosome numbers of 2n=14 and 16 detected by FISH using 5S rDNA and 18S rDNA probes and DAPI staining

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**ABSTRACT.** The structural comparison of somatic chromosomes of 13 species and one variety of *Lachenalia* (Liliaceae) that contained the chromosome numbers either of 2n=14 or 2n=16 (the basic chromosome numbers may be x=7 or x=8) was performed by DAPI staining and fluorescence *in situ* hybridization (FISH) using 5S rDNA and 18S rDNA probes. The five taxa which had the chromosome number of 2n=16 (x=8) displayed similar characters and close relationships to each other. Eight species and one variety which had the chromosome number of 2n=14 (x=7) showed a variety of patterns. The cases were suggested as traces of additions to the 18S rDNA region, translocations of chromosomes and hereditary exchanges among the taxa with x=7 and a taxa with x=8. It was suggested that the chromosome diversity in *Lachenalia*, especially in the taxa of basic chromosome number of x=7, was resulting a speciation reflected by hereditary influences between the taxa which had other patterns of chromosome structures.

**KEYWORDS:** DAPI, 18S rDNA, FISH, 5S rDNA, *Lachenalia*, Liliaceae

*Lachenalia* Jacq. ex Murray is one of the perennial bulbous genera, which belongs to the Liliaceae or sometimes to the Hyacinthaceae. Approximately 110 species of *Lachenalia* are endemic to the western part of South Africa and Namibia. About 80 species may be found in the Cape Province of South Africa (Manning et al. 2002).

Based on morphological data, *Lachenalia* was firstly classified into five subgenera by Baker (1897). Subsequently, Crosby (1986) suggested to divide five groups and was followed by Duncan (1988) who segregated ten subgroups in the five groups. Manning et al. (2002) also suggested five groups. Then, Duncan et al. (2005) reported a phylogenetic tree on the basis of the cladistical analysis using 73 parameters transformed from morphological data. However, they reported that it was difficult to clarify relationships in the aforesaid classifications and their own results.

Chromosome numbers of 61 species and eight varieties of *Lachenalia* have been reported as 2n=10, 12, 14, 16, 17, 18, 21, 22, 23, 24, 26, 27, 28, 29, 30, 32, 40, 42, 44, 49 and 56 (e.g., Gouws 1965; Ornduff and Watters 1978; Nordenstam 1982; Johnson and Brandham 1997: Hamatani et al. 1998, 2004, 2007) and thus, 2n=14 and 2n=16 were abundant phenomena (Johnson and Brandham 1997).

Molecular systematics based on DNA sequences have been presented by some researchers (e.g. Spies 2004; Hamatani et al. 2008). Hamatani et al. (2008) studied the comparison of the internal transcribed spacer (ITS) region on the nuclear (nc) rDNA in 31 species, four varieties, and six cultivars. They found that the taxa with the basic chromosome numbers of x=7 or 8 had genetic homology and were different from the taxa placed with other than the basic chromosome numbers either of x=7 or of 8. They also suggested that the taxa with the basic chromosome number of x=8 were more closely related to taxa with the basic chromosome numbers either of x=7 or of x=8, however, the relationships among the taxa with the basic chromosome number of x=7 have not yet been studied.

Structural comparisons of somatic chromosomes in *Lachenalia* species by 4`,6-diamidino-2-phenylindole (DAPI) staining and fluorescence *in situ* hybridization (FISH) using 5S rDNA and 18S rDNA probes were here performed to clarify and justify the relationships among the problematic taxa of *Lachenalia* with respect to the basic chromosome numbers of x=7 or x=8.

**MATERIALS AND METHODS.**

**Plant materials.** Plants of 13 species and one variety of *Lachenalia* that were reported to have the basic chromosome numbers of x=7 or x=8 were used in this study and were tabulated in Table 1. They were cultivated in the Hiroshima Botanical Garden.

**Pre-treatment.** Root-tips of the plants listed in Table 1 were harvested, immersed and pretreated in 2mM hydroxyquinoline solution at 20°C for 2 h. Then, they were fixed in an ethanol acetic acid mixture (3:1) overnight at -20°C. Then, they were transferred into 70% ethanol and kept at -20°C for a few days. The root tips fixed were, then, transferred and hydrolyzed in an enzymatic mixture.
of 3% cellulase and 0.5% pectolyase at 37°C for 60 min. The treated root tips were then placed on glass slides and squashed with a drop of 45% acetic acid.

**Labeling with rDNA probes**  The fragments of 18S rDNA and 5S rDNA were obtained from the total genomic DNA of *Cucumis sativus* ‘Borszczagowski’ using polymerase chain reaction (PCR). They were amplified according to Sigin (1990) and Hizume (1993), respectively. For FISH experiments, the 18S rDNA probe was labeled using the BioNick labeling system (Invitrogen), while the 5S rDNA probes were labeled using PCR labeling method with 0.2mM dATP, 0.2mM dGTP, 0.2mM dCTP, 0.1mM dTTP, and 0.1mM tetramethylrhodamine-5-dUTP (Roche) as dNTP mixture.

**FISH**  FISH method was modified from Malinowski et al. (2004). Firstly, 100 µg/ml R-Nase were dropped over the pre-treated samples on the glass slides, and the samples were, then, covered by parafilm and kept at 37°C for 60 min in a humid container. Parafilm was removed and the samples on glass slides were immersed in 2x SSC (pH 7.0) for 10 min at room temperature (RT), then immersed in PBS for 10 min at RT. For the purpose of fixing the proteins on the chromosomes, samples on slides were immersed in 4% paraformaldehyde in PBS for 10 min at RT. For denaturing, samples on slides were immersed in 60% formamide in 2x SSC at 68°C for 60 sec. They were, then, immersed in 70% ethanol at -20°C for 10 min, 100% ethanol at -20°C for 10 min, and finally air dried.

Following drying, a hybridization mixture (5S rDNA and 18S rDNA probes in 2x SSC with 10% dextran sulphate sodium salt and 50% formamide) was dropped on the slides. The samples were, then, enclosed with cover glasses. These prepared samples were then heated at 73°C for 5 min, before being placed in a humid container and kept overnight at 37°C.

The next day after removing cover slips, the samples were immersed in 4x SSC at 42°C for 10 min, twice repeatedly, then they were immersed in 2x SSC with 0.2% Triton X at RT for 10 min. Subsequently, a blocking solution (2% bovine serum albumin (BSA) and 0.5% blocking reagent in 2x SSC) were dropped on the slides. The slides were then covered by paraffilms and kept at 37°C for 45 min in a humid chamber. Then, paraffilms were removed and 0.5% Alexa F488 in the blocking solution was dropped on the slides, slides were covered by paraffilms again and kept at 37°C for approximately 2 h in a humid container in dark condition.

Paraffilm was removed and the slides were then immersed in 2x SSC with 0.2% Triton X for 10 min at RT in dark conditions, this was repeated two times and followed by immersion in 2x SSC for 5 min (at RT), in distilled water for 5 min, in 30% ethanol for 5 min, in 70% ethanol for 5 min, in 100% ethanol for 5 min in the dark condition. Finally, the samples on the glass slides were dried in the dark, before covering with Vector Shield (Vector) supplemented with 0.2 µg/ml DAPI.

Observations of the prepared samples were carried out using fluorescence microscope FXA (Nikon) with UV-1A, B-2A, and G-2A filters. The obtained images were compounded and analyzed using Photoshop Elements 2.0 (Adobe).

**Results**  The FISH result in *Lachenalia* (Liliaceae) was reported here for the first time. The chromosome count of 2n=14 in *Lachenalia variegata* was here reported for the first time.

Bi-color photographs of somatic chromosomes stained by DAPI and FISH of 5S rDNA and 18S rDNA of each taxa studied are shown in Fig. 1. Idiograms based on the pictures are presented in Fig. 2 while the chromosome numbers (2n) and the numbers of 5S rDNA and 18S rDNA signals were shown in Table 1.

*Lachenalia carnosa, L. liliflora, L. namaquensis, L. splendida,* and *L. unicolor,* which had commonly the chromosome number of 2n=16, showed 14 clear DAPI bands. Each of the bands was observed near the centromeres, but was not present in the two smallest chromosomes (Fig. 2). However, six out of the other nine taxa which had chromosome number of 2n=14, such as *L. muiri, L. aloides var. aloides, L. aloides var. aurea, L. longibracteata, L. variegata,* and *L. viridiflora* showed 12 clear DAPI bands. The bands were observed near the centromeres of 12 chromosomes, but were not present in the two largest chromosomes. *Lachenalia mutabilis* showed 14 clear DAPI bands and each of the band was observed near the centromeres of each of the 14 chromosomes (2n=14). In contrast, *Lachenalia rubida* had the chromosome number of 2n = 14 and showed 14 clear DAPI bands each of which was observed on each of the 14 chromosomes, but the clear DAPI bands on the two largest chromosomes were placed in an intercalary positions of the long arm of the chromosomes while the clear DAPI bands on the other 12 chromosomes were located near the centromeres. However, *L. pusilla* of the other 2n=14 species showed no clear DAPI bands on any of the chromosomes.

**5S rDNA**  Four signals of 5S rDNA were observed on the chromosomes of *L. muiri,* while two signals were observed on the other 13 taxa. *Lachenalia carnosa, L. liliflora, L. namaquensis, L. splendida* and *L. unicolor* showed 5S rDNA-signals near the centromeres on the short arm of two small chromosomes with clear DAPI bands. *Lachenalia muiri* showed signals on the distal positions on the short arms of the largest two chromosomes and near the centromeres on the long arms of the two small chromosomes. *Lachenalia aloides var. aloides, L. aloides var. aurea, L. longibracteata, L. variegata,* and *L. viridiflora* showed signals on the distal positions on the short arms of the largest two chromosomes. *Lachenalia mutabilis* showed 5S rDNA signals near the centromeres.
on the long arms of the two large chromosomes. *Lachenalia rubida* showed signals on the intercalary positions of the long arms, near clear DAPI bands of the two large chromosomes. *Lachenalia pusilla* showed signals near the centromeres on two medium sized chromosomes. *Lachenalia mutabilis* had six signals of 18S rDNA on the chromosomes, while *L. viridiflora* had four signals on the chromosomes and the other 12 taxa had two signals.

18S rDNA  *Lachenalia mutabilis* had six signals of 18S rDNA on the chromosomes, while *L. viridiflora* had four signals on the chromosomes and the other 12 taxa had two signals.

*Lachenalia carnosa*, *L. liliflora*, *L. namaquensis*, *L. splendida* and *L. unicolor* showed 18S rDNA signals on satellites of the two largest chromosomes. *Lachenalia muirii* showed signals near the centromeres of the two small chromosomes which produced 5S rDNA signals. *Lachenalia aloides* var. *aloides*, *L. aloides* var. *aurea*, *L. longibracteata*, and *L. variegata* showed 18S rDNA signals on the intercalary positions of the short arm of the two large chromosomes, where also exhibited clear DAPI bands. *Lachenalia viridiflora* showed 18S rDNA signals on the intercalary positions of the short arm of another two large chromosomes, where also showed clear DAPI bands on their satellites. Molecular phylogenetics using *trnL-F* region by Spies (2004) and ITS region by Hamatani et al. (2008) suggested that the taxa with basic chromosome number of *x*=7 or 8 formed a clade distinct from taxa with another basic chromosome numbers. Moreover, the taxa with the basic chromosome number of *x*=8 formed smaller clade and showed more close relationship than the other taxa of *x*=7. The present result by molecular cytogenetics and DAPI-staining supported those molecular phylogenetical conclusions.

Nine taxa with the chromosome number of *2n*=14 (*x*=7) varied patterns in positions of DAPI bands and the 5S rDNA and 18S rDNA signals: Six taxa, such as *L. muirii*, *L. aloides* var. *aloides*, *L. aloides* var. *aurea*, *L. longibracteata*, *L. variegata*, and *L. viridiflora* did not show clear DAPI bands on the largest two chromosomes but showed clear DAPI bands on the other 12 chromosomes. They showed

### DISCUSSION

Figure 2 shows the idiograms of *Lachenalia* taxa studied which indicated the positions of DAPI bands and the 5S rDNA and 18S rDNA signals.

The five taxa which had commonly the chromosome number of *2n*=16 (*x*=8) and similar karyotype characters to each other were closely related to each other. Each of the taxa showed no clear DAPI band in their two smallest chromosomes, but clear DAPI bands in the other 14 chromosomes, and furthermore, showed similar clear DAPI bands on equivalent chromosomes, two small chromosomes with a 5S rDNA signal near their centromeres and the two largest chromosomes with a 18S rDNA signal on their satellites. Molecular phylogenetics using *trnL-F* region by Spies (2004) and ITS region by Hamatani et al. (2008) suggested that the taxa with basic chromosome number of *x*=7 or 8 formed a clade distinct from taxa with another basic chromosome numbers. Moreover, the taxa with the basic chromosome number of *x*=8 formed smaller clade and showed more close relationship than the other taxa of *x*=7. The present result by molecular cytogenetics and DAPI-staining supported those molecular phylogenetical conclusions.

<table>
<thead>
<tr>
<th>Species and variety</th>
<th>Chromosome number (2n)</th>
<th>5S rDNA signals</th>
<th>18S rDNA signals</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. aloides</em> (L.f.) var. <em>aloides</em></td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. aloides</em> var. <em>aurea</em> (L.f.) Engl.</td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. carnosa</em> Bak.</td>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. liliflora</em> Jacq.</td>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. longibracteata</em> Phillips</td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. mutabilis</em> Sweet.</td>
<td>14</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td><em>L. muirii</em> W.F.Barker</td>
<td>14</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><em>L. namaquensis</em> Schltr. ex W.F.Barker</td>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. pusilla</em> Jacq.</td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. rubida</em> Jacq.</td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. splendida</em> Diels</td>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. unicolor</em> Jacq.</td>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. variegata</em> W.F.Barker</td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>L. viridiflora</em> W.F.Barker</td>
<td>14</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1. Chromosome numbers and numbers of FISH signals of 13 species and one variety of *Lachenalia* studied
Fig. 1. FISH signals of 5S rDNA and 18S rDNA sites of the metaphase on somatic chromosomes in 13 species and one variety of *Lachenalia* studied. 5S rDNA probed were labeled by tetramethylrhodamine and 18S rDNA probes were labeled by biotin. Biotin-labeled signals were detected with Alexa F488. Blue areas were stained by DAPI, red signals were hybridized with 5S rDNA and yellow signals were hybridized with 18S rDNA.

5S rDNA signals on the distal position of the short arm on the two largest chromosomes that did not show clear DAPI bands. In addition, five out of the six taxa, except for L. muirii, displayed 18S rDNA signals on the two large chromosomes that had clear DAPI bands. These 18S rDNA signals was located near the secondary constrictions of the short arm referred from the appearances of L. longibracteata and L. variegata. These similarities suggested a close relationship among the five taxa. Additionally, L. viridiflora had more signals of 18S rDNA than the other four taxa such as L. aloides var. aloides, L. aloides var. aurea, L. longibracteata, and L. variegata.

Lachenalia muirii showed similar characteristics to the five taxa mentioned above. These six taxa showed commonly 5S rDNA signals on the distal positions of the short arm of the two largest chromosomes that did not indicate clearly DAPI bands. However, L. muirii showed different characteristics from the other five taxa mentioned above. Lachenalia muirii showed a 5S rDNA signal near the centromere on the long arm of two small chromosomes and a 18S rDNA signal near the centromere of the same two chromosomes, while L. aloides var. aloides, L. aloides var. aurea, L. longibracteata, L. variegata, and L. viridiflora did not. Moreover, the molecular phylogenetic analysis using ITS sequences (Hamatani et al. 2008) suggested that L. muirii was more closely related to the taxa of x=8 than those of x=7. It was seemed that L. muirii is related to the taxa of x=7 as well as those of x=8. Lachenalia muirii was similar in karyotype and sites of clear DAPI bands to the taxa of x=7 such as L. aloides var. aloides. The two signals of 5S rDNA on the two chromosomes had similar characters to the signals of 5S rDNA in the taxa of x=8 (Fig. 3). These two 5S rDNA signals of L. muirii could be one of the evidences of the relationship between

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**Fig. 2.** The idiograms of somatic chromosomes at metaphase with DAPI bands and signals of 5S rDNA and 18S rDNA in the 13 species and one variety of Lachenalia studied.
L. muirii placed in the taxa of x=7 and the taxa of x=8.

The L. mutabilis karyotype displayed 18S rDNA signal in an intercalary position of the short arm of the two large chromosomes (Nos. 3 and 4 in Fig. 2). This was the common character shared with the five taxa such as L. aloides var. aloides, L. aloides var. aurea, L. longibracteata, L. variegata, and L. viridiflora. In contrast, L. mutabilis showed no chromosome which had 18S rDNA signal other than the chromosomes Nos. 1 and 2 and no chromosome which corresponded to the chromosomes Nos. 3 or 4.

Lachenalia pusilla might be a heterogeneous species among the taxa of the 2n=14 (x=7) group studied because of its unclear DAPI bands and its different 5S rDNA and 18S rDNA positions.

Though reports of karyomorphological comparisons (Hamatani et al. 1998, 2004 and 2007) and reports of trnL-F region comparisons (Spies 2004) and of ITS region comparisons (Hamatani et al. 2008) suggested that the taxa of x=8 studied were closely related to each other and the taxa of x=7 studied were divided into groups and were not closely related each other in compared those of x=8. In the present study, the A-T rich regions due to DAPI bands and the positions of 5S rDNA and 18S rDNA signals confirmed that there were several morphological alterations of chromosomes in the taxa of x=7. Kleynhans et al. (2009) revealed that cross-hybridization among the Lachenalia species with same basic chromosome numbers occurred easily. The diversity in the taxa of x=7 studied descended from the transformations influencing hereditarily between their ancestral species which underwent morphological alterations on their chromosomes.

The present results suggested that the taxa of x=7 might be generated from some ancestral races, while the taxa of x=8 might be generated from an ancestral races and then, performed continuously further speciation. The molecular phylogenetic analysis of ITS regions of Lachenalia made it possible to speculate that the taxa of x=7 and x=8 might have the common ancestral race (Hamatani et al. 2008). More than two 18S rDNA signals observed in L. viridiflora and more than four 18S rDNA signals observed in L. mutabilis might lead gene-flows among the populations with intermixed species and recombinant chromosomes, since the two chromosomes which contained 5S rDNA and 18S rDNA signals in L. muirii might be influenced by DAPI bands and rDNA signals.

![Speculated progress of speciation of Lachenalia muirii.](image)
from both taxa of x=7 and x=8. According to Hamatani et al. (2008), L. muirii and L. pusilla might be closely related with the taxa of x=8 even the two species had basic chromosome number of x=7. It was difficult to elaborate the relationships between L. pusilla and other taxa, since L. pusilla was not similar in molecular cytogenetics to those of the other taxa. Lachenalia pusilla had unique morphological characters among the species of the genus. This species was segregated into an independent group by Baker (1897) and Crosby (1986). Many more study is needed to characterize L. pusilla.

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LITERATURE CITED