Molecular phylogenetic relationships among *Lachenalia*, *Massonia* and *Polyxena* (Liliaceae) on the basis of the internal transcribed spacer (ITS) region

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ABSTRACT. A molecular phylogenetic analysis based on internal transcribed spacer (ITS) sequences in 31 species, four varieties and four cultivars of *Lachenalia*, two species of *Massonia* and three species of *Polyxena* was made. These three genera have been considered as closely related with each other. The ITS sequence data confirmed that *Massonia* was clearly different from *Lachenalia* and *Polyxena*. *Polyxena* constructed one clade distinguished from *Lachenalia*. The species of *Lachenalia* used showed a relationship between ITS sequence data and karyotypes, excepting the species with the basic chromosome numbers $x=7$ and $x=8$ did not show any correlation with those with the other basic chromosome numbers. Similarities of their arrays of ITS sequence data among species with $x=8$ were greater than those among the species with $x=7$. *Lachenalia pusilla* and *L. muirii* which had $x=7$ formed a clade with the species with $x=8$. Differences in the species with the basic chromosome numbers of $X=7$ and 8 showed less differences in correlation between ITS sequence data and karyotypes than those with other basic chromosome numbers. It was suggested that the species of *Lachenalia* with the basic chromosome numbers of $x=7$ and 8 might be originated from a common ancestor, while the other species of the genus with basic chromosome numbers other than $x=7$ and 8 might be originated from other ancestor-else.

KEYWORDS: *Lachenalia*, *Massonia*, *Polyxena*, Phylogenetic analysis, ITS, Karyotype

Lachenalia is an important genus in the Liliaceae, sometimes placed in the Hyacinthaceae. The genus is endemic to the western part of southern Africa and consists of approximately 115 species (Duncan 1998).

Since the genus has subtle, morphological differences in rather small area, it has been classified into numerous species. However, it has been difficult to study phylogenetic relationships, speciation and other species biology. The previous cladistic analyses by Duncan (1988) and Duncan *et al.* (2005) made in 73 characters included flowers, bulbs and seeds in the members of the genus indicated that all those morphological data could be inadequate to clarify and justify the species relationships. They suggested that molecular comparisons in *Lachenalia*.

Molecular data in *atpB* and *trnL-F* chloroplast DNA sequence data were studied in six species in the tribe Massonieae, the family Hyacinthaceae by Pfosser *et al.* (2003) suggesting *Massonia* and *Polyxena* were closely related to *Lachenalia*.

Chromosome numbers of 61 species and eight varieties in *Lachenalia* documented showed aneuploid chromosome numbers of $2n=10, 12, 14, 16, 17, 18, 21, 22, 23, 24, 26, 27, 28, 29, 30, 32, 40, 42, 44$, and 56 (e.g., Gouws 1965, Ornduff and Watters 1978, Nordenstam 1982, Johnson and Brandham 1997, Hamatani *et al.* 1998, 2004, 2007).

Karyotypes of 35 species and four varieties of the genus were studied and classified into nine groups (Hamatani *et al.* 1998, 2004, 2007), however, they were not consistent with those of the cladistic analysis of Duncan *et al.* (2005). More deep studies in their karyotypes deposited problematic with patterns such as intermediate karyotypes among certain groups, triploid-like and natural hybrid-like karyotypes, non-uniformity of intraspecific karyotypes. It seemed that morphological and karyomorphological data were not sufficient to construct phylogenetic relationships of the species in *Lachenalia*.

The present phylogenetic study was conducted in the internal transcribed spacer (ITS) region in nuclear DNA sequences in 31 species, four varieties and four cultivars in *Lachenalia*, two species of *Massonia* and three species of *Polyxena*.

MATERIALS AND METHODS

Plant materials The plants used in this study were tabulated in Table 1. They were cultivated in the Hiroshima Botanical Garden and Kyoto Botanical Garden. *Ornithogalum*, the Scilloideae, the Liliaceae was used as the outgroup in this study.

DNA isolation, PCR and Sequencing Total DNA of each species studied was extracted from fresh leaves using the

Table 1. The materials used in this DNA analysis and intra-individual variation in ITS region in *Lachenalia*, *Massonia*, and *Polyxena* species

Species and variety	Voucher specimen	ITS length (bp)			Database accession	Chromosome informations	
		ITS1	5.8s rRNA	ITS2		Chromosome number (2n)	Reference
<i>Lachenalia algoensis</i> Schönl.	HIRO 5756-LPCGS	248	154	248	AB304971	14	Hamatani <i>et al.</i> 2007
<i>Lachenalia aloides</i> (L.f.) Engl. var. <i>aloides</i>	HIRO 5762-LPCGS	248	154	248	AB304972	14, 28	Hancke & Liebenberg 1990, Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Lachenalia aloides</i> var. <i>aurea</i> (L.f.) Engl.	HIRO 5763-LPCGS	248	154	248	AB304973	14	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2004
<i>Lachenalia aloides</i> var. <i>quadricolor</i> (Jacq.) Engl.	HIRO 5764-LPCGS	248	154	248	AB304974	14	Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Lachenalia aloides</i> var. <i>vanzyliae</i> W. F. Barker	HIRO 5765-LPCGS	248	154	248	AB304975	28	Hamatani <i>et al.</i> 1998
<i>Lachenalia aloides</i> 'Lutea'	HIRO 5758-LPCGS	248	154	248	AB304976	14	Hamatani <i>et al.</i> 2007
<i>Lachenalia aloides</i> 'Pearsonii'	HIRO 5760-LPCGS	248	154	248	AB304977	-	
<i>Lachenalia arbutnothiae</i> W. F. Barker	HIRO 5766-LPCGS	249	154	248	AB304978	14	Johnson & Brandham 1997, Hamatani <i>et al.</i> 1997
<i>Lachenalia bachmannii</i> Bak.	HIRO 5767-LPCGS	248	154	248	AB304979	16	de Wet 1957, Johnson & Brandham 1997, Hamatani <i>et al.</i> 2004
<i>Lachenalia bulbifera</i> (Cyrillo) Engl.	HIRO 5768-LPCGS	248	154	248	AB304980	28, 42, 49, 56	Johnson & Brandham 1997, Hamatani <i>et al.</i> 1997, Kleynhans & Spies 1999
<i>Lachenalia carnososa</i> Bak. (Clone1)	HIRO 5805-LPCGS	248	154	249	AB439269	16	Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Lachenalia carnososa</i> Bak. (Clone2)	HIRO 5805-LPCGS	248	154	249	AB439270		
<i>Lachenalia carnososa</i> Bak. (Clone3)	HIRO 5805-LPCGS	248	154	249	AB439271		
<i>Lachenalia contaminata</i> Ait.	HIRO 5769-LPCGS	248	154	248	AB304981	14, 16, 32	de Wet 1957, Gouws 1965, Johnson & Brandham 1997, Hamatani <i>et al.</i> 2004
<i>Lachenalia hirta</i> (Thunb.) Thunb. 'TypeA'	HIRO 5770-LPCGS	249	154	248	AB304982	18, 22, 24	de Wet 1957, Johnson & Brandham 1997, Hancke 1991, Hamatani <i>et al.</i> 2004
<i>Lachenalia hirta</i> (Thunb.) Thunb. 'TypeB' (Clone1)	HIRO 5809-LPCGS	249	154	248	AB439272		
<i>Lachenalia hirta</i> (Thunb.) Thunb. 'TypeB' (Clone2)	HIRO 5809-LPCGS	250	154	248	AB439273		
<i>Lachenalia hirta</i> (Thunb.) Thunb. 'TypeB' (Clone3)	HIRO 5809-LPCGS	249	154	248	AB439274		
<i>Lachenalia juncifolia</i> Bak.	HIRO 5771-LPCGS	251	154	248	AB304983	22	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Lachenalia latifolia</i> Tratt.	HIRO 5772-LPCGS	248	154	246	AB304984	24	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Lachenalia latimeriae</i> W. F. Barker	HIRO 5773-LPCGS	248	154	247	AB304985	18	Hamatani <i>et al.</i> 2007
<i>Lachenalia liliflora</i> Jacq.	HIRO 5774-LPCGS	248	154	248	AB304986	16	Moffett 1936, de Wet 1957, Fernandes & Neves 1962, Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Lachenalia longibracteata</i> Phillips	HIRO 5775-LPCGS	248	154	248	AB304987	14	Hamatani <i>et al.</i> 2007
<i>Lachenalia muirii</i> W. F. Barker	HIRO 5777-LPCGS	248	154	248	AB304988	14	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Lachenalia mutabilis</i> Sweet.	HIRO 5778-LPCGS	248	154	249	AB304989	10, 14, 24, 56	de Wet 1957, Hancke & Liebenberg 1990, Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998

Table 1. --continued

<i>Lachenalia namaquensis</i> Schltr. ex W. F. Barker	HIRO 5776-LPCGS	249	154	249	652	AB304990	16	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Lachenalia orchoides</i> (L.) Ait.	HIRO 5779-LPCGS	248	154	248	650	AB304991	28	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
var. <i>glaucina</i> (Jacq.) W. F. Barker								
<i>Lachenalia orchoides</i> (L.) Ait. var. <i>orchoides</i>	HIRO 5780-LPCGS	248	154	248	650	AB304992	16, 17, 28, 29	Moffett 1936, de Wet 1957, Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Lachenalia pallida</i> Ait.	HIRO 5781-LPCGS	248	154	248	650	AB304993	16	Moffett 1936, Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Lachenalia peersii</i> Marloth ex W. F. Barker	HIRO 5782-LPCGS	248	154	248	650	AB304994	12, 14	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2004
<i>Lachenalia purpureoerulea</i> Jacq.	HIRO 5794-LPCGS	248	154	249	651	AB304995	16	Moffett 1936, Johnson & Brandham 1997, Hamatani
<i>Lachenalia pusilla</i> Jacq. 'TypeA'	HIRO 5783-LPCGS	248	149	243	640	AB304996	14, 16	Nordenstam 1982, Johnson & Brandham 1997, Hamatani <i>et al.</i> 1997
<i>Lachenalia pusilla</i> Jacq. 'TypeB'	HIRO 5795-LPCGS	248	154	245	647	AB304997		
<i>Lachenalia pustulata</i> Jacq. 'TypeA'	HIRO 5784-LPCGS	248	154	249	651	AB304998	16	Moffett 1936, Johnson & Brandham 1997, Hamatani <i>et al.</i> 2004
<i>Lachenalia pustulata</i> Jacq. 'TypeB'	HIRO 5785-LPCGS	248	154	249	651	AB304999		
<i>Lachenalia reflexa</i> Thunb. (Clone1)	HIRO 5806-LPCGS	248	154	248	650	AB439275	14, 16	Moffett 1936, de Wet 1957, Hance & Liebenberg 1990, Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Lachenalia reflexa</i> Thunb. (Clone2)	HIRO 5806-LPCGS	248	154	248	650	AB439276		
<i>Lachenalia reflexa</i> Thunb. (Clone3)	HIRO 5806-LPCGS	248	154	248	650	AB439277		
<i>Lachenalia rosea</i> Andrews	HIRO 5788-LPCGS	246	154	250	650	AB305000	14	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Lachenalia rubida</i> Jacq.	HIRO 5796-LPCGS	248	154	248	650	AB305001	14	Moffett 1936, Hamatani <i>et al.</i> 1998
<i>Lachenalia splendida</i> Diels	HIRO 5789-LPCGS	248	154	249	651	AB305002	16	Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Lachenalia unicolor</i> Jacq. 'TypeA' (Clone1)	HIRO 5808-LPCGS	248	154	248	650	AB439278	16	Moffett 1936, de Wet 1957, Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Lachenalia unicolor</i> Jacq. 'TypeA' (Clone2)	HIRO 5808-LPCGS	248	154	248	650	AB439279		
<i>Lachenalia unicolor</i> Jacq. 'TypeA' (Clone3)	HIRO 5808-LPCGS	247	154	248	649	AB439280		
<i>Lachenalia unicolor</i> Jacq. 'TypeB' (Clone1)	HIRO 5807-LPCGS	248	154	248	650	AB439281		
<i>Lachenalia unicolor</i> Jacq. 'TypeB' (Clone2)	HIRO 5807-LPCGS	248	154	248	650	AB439282		
<i>Lachenalia unifolia</i> Jacq.	HIRO 5790-LPCGS	250	154	248	652	AB305003	21, 22, 24, 26, 44	Moffett 1936, de Wet 1957, Johnson & Brandham 1997, Hamatani <i>et al.</i> 2004
<i>Lachenalia variegata</i> W. F. Barker	HIRO 5797-LPCGS	248	154	248	650	AB305005	12, 28	Spies <i>et al.</i> 2002, Hamatani <i>et al.</i> 2004
<i>Lachenalia viridiflora</i> W. F. Barker	HIRO 5798-LPCGS	248	154	248	650	AB305006	14	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Lachenalia zeyheri</i> Bak.	HIRO 5791-LPCGS	251	154	248	653	AB305004	22, 23	Johnson & Brandham 1997, Hamatani <i>et al.</i> 1998
<i>Massonia depressa</i> Houtt.	HIRO 5799-LPCGS	250	154	248	652	AB305007	26	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Massonia pustulata</i> Jacq.	HIRO 5793-LPCGS	249	154	248	651	AB305008	22, 28	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Polyxena ensifolia</i> (Thunb.) Schönland	HIRO 5801-LPCGS	249	154	249	652	AB305010	24, 26	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Polyxena longituba</i> A. M. van der Merwe	HIRO 5802-LPCGS	248	154	249	651	AB305011	28	Hamatani <i>et al.</i> 2007
<i>Polyxena paucifolia</i> (W. F. Barker) A. M. van der Merwe & Manning	HIRO 5792-LPCGS	247	154	246	647	AB305012	24, 26	Johnson & Brandham 1997, Hamatani <i>et al.</i> 2007
<i>Ornithogalum umbellatum</i> L. Outgroup	HIRO 5803-LPCGS	263	154	227	644	AB305009		

CTAB method (Doyle and Doyle 1987). The nucleotide sequences of ITS 1, 5.8S, and ITS 2 were amplified from the total DNA using ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') PCR primers (White *et al.* 1990). The PCR amplifications were performed using a GeneAmp PCR system 2700 (Perkin-Elmer, Boston, Massachusetts, U.S.A.) with a thermal cycler program for an initial 1 min denaturation at 97°C, and then, 27 cycles at 97°C for 1 min, at 52°C for 1 min, and at 72°C for 2 min, followed by an extension step at 72°C for 7 min.

The ITS PCR products were subjected to agarose gel (1%) electrophoresis. The ITS PCR products were subjected to agarose gel (1%) electrophoresis. The resulting PCR products were, then, purified with the QIA quick gel extraction kit (Qiagen, Calif., U.S.A.). The ITS regions were sequenced with an automated sequencer (3130x Genetic Analyzer; ABI Prism, Foster City, CA, USA) using the Big Dye Terminator Kit (ABI) according to the manufacturer's protocol.

Some of PCR products such as *Lachenalia carnosa*, *L. hirta* cv. 'Type B', *L. reflexa* and *L. unicolor* 'Type A' and 'Type B' which showed difficulty in obtaining clean sequences from direct PCR sequencing were cloned using pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) following the manufacture's protocol. *Escherichia coli* strain JM109 (TaKaRa Bio, Otsu, Japan) were used for transformation. Then blue/white selection was performed and plasmid DNA were extracted using Wizard miniprep (Promega, Madison, WI, U.S.A.).

Data analysis The sequences obtained were aligned using the Clustal W program (Thompson *et al.* 1994) on the DNA Data Bank of Japan (DDBJ) website (<http://www.ddbj.nig.ac.jp>) with the default settings followed by adjustment by eye. The phylogenetic tree was constructed with two program packages such as (1) PAUP 4.0b (Swofford 2000) for the maximum parsimony (MP) method (Fitch 1971) with a heuristic search of 1,000 random addition replicates performed by tree bisection-reconnection (TBR) and MulTree and (2) MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with the Bayesian inference (BI) method using the GTR+G model with 50,000,000 generations, prior to phylogenetic reconstruction; MrModeltest 2.2 (Nylander 2004) was implemented in hierarchical likelihood ratio tests to allow us to make a rational decision regarding the nucleotide-based substitution model that the best fitted data used for the BI method. The bootstrap (BS) analyses with the MP method was conducted on 1,000 replicates using the heuristic search option in PAUP (Felsenstein 1985), and the Bayesian posterior probabilities (PPB) were calculated using MrBayes. Each of the two methods were executed on data without indels.

RESULTS

Nucleotide sequence analysis and comparison. The data matrix of ITS included 659 base pairs (bp). Amplification of the ITS regions using the primer pair described above gave PCR products of approximately 650 bp (647-653) in length, except 640 bp in *L. pusilla* 'Type A' and 644 bp in *O. umbellatum* (outgroup). The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank databases under accession numbers AB304971 to AB305012 and AB439269 to AB439282. Then, the boundaries of ITS 1 and ITS 2 and the nuclear rDNA coding regions were determined by comparing them with a sequence of 18S-26S nrDNA cistron of *Tulipa clusiana* (Liliaceae) (Accession No. AM180460). The length of the amplified ITS regions is as follows: the length of ITS 1 varied from 246 to 251 bp (except 263 bp in *O. umbellatum*), the length of the 5.8S rRNA was 154 bp (except 149 bp in *L. pusilla* 'Type A'), and the length for ITS 2 varied from 243 to 249 bp (except 227 bp in *O. umbellatum*) (Table 1).

Phylogenetic analysis. Total number of rearrangements tried in MP analysis of the ITS data was 49,706,079 and score of best trees found was 351, with a consistency index, including uninformative characters (CI), of 0.809 and a retention index (RI) of 0.856 for the data without indels. A total of 135 distinct data patterns under the best-fit model indicated the GTR + G substitution model by MrModeltest for the dataset.

All the analyses and supporting values for each method are shown in Fig. 1. BP indicates BS values as percentages based on 1,000 replications using the MP method, and PPB indicates Bayesian posterior probabilities as percentages.

In the consensus tree based on the ITS sequences, two species of *Massonia* was made one clade and distinguished from the clade constructed by *Lachenalia* and *Polyxena* (BS=100%, BPP=1.00) (Fig. 1).

In the clade constructed by *Lachenalia* and *Polyxena* (99.9%, 0.99), two clades were made with high values; one was Clade I (90.0%, 1.00) made from three species of *Polyxena* and another was Clade II (97.6%, 1.00) made from *Lachenalia unifolia*, *L. juncifolia* and *L. zeyheri*, and one clade (Clade III) with low values (68.8%, 0.89) made from 25 species, four varieties and five cultivars (41 samples) which were from *L. bachmannii* to *L. rosea*. Four samples of *L. hirta* made one clade (99.4%, 1.00) from one species, and *L. latimeriae* and *L. latifolia* were not included in clades.

In Clade III included one major clade (Clade IV; 83.3%, 1.00) consisting of twelve species and three cultivars from *L. bachmannii* to *L. pusilla*, and included other 13 species, four varieties and two cultivars ("Others"). In Clade IV, eleven species and two cultivars (except for *L. pusilla*) made smaller clade 78.0%, 1.00). In this smaller clade, moreover *L. unicolor* and *L. pallida* made smaller clade (90.9%, 1.00), and other taxa did not

Fig. 1. The strict consensus tree based on the internal transcribed spacer (ITS) sequence data without indels. Numbers listed along the branch show bootstrap values based on 1,000 replications using MP method by PAUP (BP), and Bayesian posterior probabilities by MrBayes (PPB) (BP in % / PPB). Supporting values more than 50% were overlaid (less than 50%, but with the most support in each method, were indicated as ≤ 50).

Data of "CB" show classifications referred from Manning *et al.* (2002). Data of "GD" show classifications referred from Duncan (1988). Data of "2n" are their chromosome numbers referred from previous reports. Data of "x" are their basic chromosome numbers and "Ha" show classifications both referred from Hamatani *et al.* (1998, 2004 and 2007).

made clear clades.

DISCUSSION

Lachenalia, *Massonia*, and *Polyxena* in the Liliaceae are considered to be closely related to each other (Manning *et al.* 2002, Pfosser *et al.* 2003). Based on the results in the ITS, *Massonia* was clearly distinguished from *Lachenalia* and *Polyxena* by forming a clade separate from that of *Lachenalia* and *Polyxena*. This result agrees with the analysis of *atpB* and *trnL-F* by Pfosser *et al.* (2003) and with the comparison of karyotypes by Hamatani *et al.* (2007).

The morphological differences between *Polyxena* and *Lachenalia* are very clear (Manning *et al.* 2002). In the study of karyotypes (Hamatani *et al.* 2007), differences in particular characteristics were observed; for example, the existence of three large chromosomes; and hence, this study supported the results of karyomorphological studies. Alternatively, Manning *et al.* (2004) suggested that five known species of *Polyxena* should be incorporated into the genus *Lachenalia*, according to their *trnL-F* and

rbcL sequences. In this study comparing ITS regions, it was confirmed that the *Polyxena* species had elements that distinguished it from the *Lachenalia* species. The three species of *Polyxena*, observed in this study, formed one clade (Clade I; 90.0%, 1.00) that was separate from the clade formed by the *Lachenalia* species.

Two methods, based on morphological information, were used to classify the *Lachenalia* species; one, based on the flower forms, was advocated by Duncan (1988) and the other, based on general plant forms, was advocated by Manning *et al.* (2002). In this study, we compared the phylogenetic tree constructed from ITS data with the morphological classifications; however, we could not identify particular mutual points (Fig. 1). These two classification methods were useful for horticultural purposes and general identification of plants, but they did not reflect the genetic similarities.

In the 31 species, four varieties and six cultivars of *Lachenalia* studied, *L. unifolia*, *L. juncifolia*, and *L. zeyheri* formed Clade II, supported by high values (97.6%, 1.00). The chromosome numbers of these three

species were reported to be $2n=16$, 21–24, 26, and 44, respectively (Moffett 1936, de Wet 1957, Johnson and Brandham 1997, Hamatani *et al.* 1998, 2004, 2007), and the basic chromosome numbers were considered to be $x=11$ or 12. However, *L. latifolia* and *L. hirta* ($x=11$ or 12) (Johnson and Brandham 1997, Hamatani *et al.* 2004, 2007) did not form a clade with other species. They differed in the comparison of chromosomes and ITS regions.

The 25 species, four varieties and five cultivars of *Lachenalia* (from *L. bachmannii* to *L. rosea*) formed Clade III, supported by low values (68.8%, 0.89). The chromosome numbers of species included in this clade were reported as $2n=10$, 12, 14–18, 21, 24, 28, 32, 42, and 56 (Moffett 1936, de Wet 1957, Gouws 1965, Nordenstam 1982, Hancke 1991, Johnson and Brandham 1997, Kleynhans and Spies 1998, Hamatani *et al.* 1998, 2004, 2007, Spies *et al.* 2002), their basic chromosome numbers being $x=7$ and 8. Thus, it was considered that the relationship between species with $x=7$ and 8 were

stronger than the relationship between species with $x=7$ or 8 and $x \neq 7$ and 8.

Lachenalia latimerae ($2n=18$; $x=9$) had a unique chromosome number and did not show strong relationships with any other species in the comparison of ITS data. It did not show notable morphological differences from the *Lachenalia* species (Duncan 1988, Duncan *et al.* 2005, Manning *et al.* 2002); however, in the eastern region, it was one of the most widely distributed species of *Lachenalia* (Fig. 2).

The species forming Clade III had a basic chromosome number of $x=7$ or 8. Twelve species and three cultivars (from *L. bachmannii* to *L. pusilla*) formed Clade IV; among these, *L. pusilla* and *L. muirii* had a basic chromosome number of $x=7$, whereas the other ten species and three cultivars had a basic chromosome number of $x=8$. In species forming Clade IV, the relationship between the $x=8$ species was stronger than the relationship between the $x=8$ and $x=7$ species. Furthermore, karyomorphological studies by Hamatani *et*

Fig. 2. Habitats of *Lachenalia* and *Polyxena* studied.

A: Habitats of species of Clade IV in figure 1. B: Habitats of species of “Others” in figure 1.

C: Habitats of *Lachenalia* species which have basic chromosome numbers not as $x=7$ or 8.

D: Habitats of species of *Polyxena*.

Data were referred from website “African Flowering Plants Database”.

al. (1997, 2004, 2007) reported that the variation of karyotypes between the $x=8$ species was not as advanced as the variation in the $x=7$ species, because the $x=8$ species showed only one karyotype pattern as compared to four karyotype patterns shown by the $x=7$ species.

The 13 species, four varieties and two cultivars in Clade III called as "Others" in Fig. 1 (from *L. arbutnotiae* to *L. rosea*), which were not included in Clade IV, did not construct any clades. In the group "Others", it could not discover the relationships among the each species.

Clade IV had high confidence values (83.3%, 1.00) and consisted of all species with $x=8$ and two species with $x=7$ (*L. pusilla* and *L. muirii*). Based on ITS sequence data, these two $x=7$ species could be clearly distinguished from the other $x=7$ species ("Others" in Fig. 1), that is 13 species, four varieties and two cultivars. Although, some varieties and cultivars had their elementary species included in "Others", there were no notable combinations that gave close relationships on comparing ITS sequence data. The results did not support the grouping suggested by Hamatani *et al.* (1997, 2003, 2007) in karyomorphological studies.

Each species in Clade IV and those included in the group "Others" are distributed in a long and narrow area in the south-western part of southern Africa; the distribution of many species and varieties overlapped (Fig. 2). According to Duncan (1988), *Lachenalia* could hybridize easily. Thus, it was suggested that the speciation of the $x=7$ and 8 *Lachenalia* species resulted not only from mutation, but also from hybridizations between species with the same distribution. There are many reports discussing polyploidy and aneuploidy (for example, Hancke and Liebenberg 1990, Hancke 1991, Johnson and Brandham 2007) among the $x=7$ species. Hamatani *et al.* (2007) showed several variations in the patterns of karyotypes among the $x=7$ species; however, polyploidy and aneuploidy were reported in only one (*L. contaminata*) $x=8$ species (Gouws 1965, Johnson and Brandham 1997, Hamatani *et al.* 2004). There were no variations in the karyotype patterns of $x=8$ species (Hamatani *et al.* 2007). It was suggested that the $x=7$ species, speciated from the mutual ancestral species of Clade IV and the "Others" group, expanded their distribution areas through speciation. These new species then independently expanded their distribution areas through further speciation; for example, *L. pusilla*, *L. muirii*, and the $x=8$ species studied. Hereafter, the speciation of $x=7$ and $x=8$ species may be clarified by research, using *L. pusilla*, *L. muirii*, and so forth.

Alternatively, the *Lachenalia* species with the basic chromosome numbers of $x=9$ and $x=11$ or 12 ($x \neq 7$ and 8), showed strong relationships between their ITS data, but did not show the general unity exhibited by the $x=7$ and 8 species. Moreover, the $x \neq 7$ and 8 species were considered to have speciated from multiple ancestors since they were not included in the clade formed by the

$x=7$ and 8 species (Clade III). This implies that the ancestors of $x \neq 7$ and 8 and those of $x=7$ and 8 speciated from mutual ancestral species of *Lachenalia*. According to Johnson and Brandham (1997), these species were the ones with basic chromosome numbers $x=5$, 6, 10, 13, and 15, and except for the chromosome numbers considered in this study, the histories of these species will be clarified future studies.

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