Genetic combinations of symbionts in a vegetatively reproducing lichen, *Parmotrema tinctorum*, based on ITS rDNA sequences

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ABSTRACT. The genetic combinations between mycobionts and photobionts in *Parmotrema tinctorum* collected from ca. 60 km^2 of the Shimizu district, of Shizuoka City in Japan was investigated based on ITS rDNA sequences. This lichen produces apothecia quite rarely, and in principle propagates vegetatively by isidia. The genetic diversity of the mycobiont comprised four types, while that of the photobiont comprised 21 types. There were 28 different combinations between mycobiont and photobiont. All the photobionts were identified as *Trebouxia corticola* (s. lat.), based on both molecular phylogenetic results and morphological observation of culture strains obtained in this study. Therefore, *P. tinctorum* is considered to be highly selective toward the photobiont. The 28 combinations from the small area represent an unexpectedly high diversity, because *P. tinctorum* is thought to propagate vegetatively. Four possible mechanisms to account the high genetic combinations are suggested: i.e., photobiont exchange, fusion of thalli, and long-distance dispersal of isidia or ascospores. The genetic diversity of photobionts was poor in the urban area, but rich in suburbs and mountainsides. This might be caused by a bottleneck or founder effect in the population recovering from former damage by heavy air pollution, or variable selectivity of *P. tinctorum* depending on the environments.

KEYWORDS. Diversity, ITS rDNA, mycobiont, *Parmotrema tinctorum*, photobiont, selectivity, *Trebouxia corticola*, vegetative reproduction.

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Lichens propagate by either ascospore-based sexual reproduction, which involves the acquisition of an appropriate alga by the germinating ascospore in the field (Ott 1987), or by vegetative reproduction via diaspores, such as isidia or soredia. The photobiont from the original lichen thallus is replaced in the sexual mode of reproduction and, consequently, genetic diversity in the form of various combinations between mycobiont and photobiont may occur. In contrast, vegetative reproduction is thought to

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produce a new thallus that is genetically identical with the original (Beck et al. 1998). Only a few studies have investigated genetic variation of the photobiont in vegetatively reproducing lichens (Beck et al. 2002; Opanowicz & Grube 2004; Romeike et al. 2002). Although several variations were found among the species studied, they were considered to be derived from different geographical or geohistorical origins. However, there has been no thorough genetic analysis of photobiont variation within a single population of a vegetatively reproducing lichen, and the nature of vegetative reproduction in lichens is still poorly understood.

The purpose of this study was to discuss the reproductive mode in a vegetatively reproducing lichen based on the genetic combinations between mycobiont and photobiont from a small geographical area. Sequences of ITS rDNA were used to analyze intraspecific variation of both mycobionts and photobionts, as done in previous studies (Beck et al. 1998, 2002; Opanowicz & Grube 2004; Romeike et al. 2002). *Parmotrema tinctorum* (Nyl.) Hale (**Fig. 1A**) was used in this study as a representative of a vegetatively reproducing species; it usually propagates by isidia (**Fig. 1B**), and only quite rarely produces apothecia.

Materials and Methods

Lichen samples. Sixty-nine specimens of *P. tinctorum* were collected from ca. 60 km^2 of the Shimizu district (34°57'40"N, 138°27'56"E to 35°02'55"N, 138°31'00"E) within the city of Shizuoka, Japan in 2003 (**Table 1; Fig. 2**). All specimens were collected from tombstones or stone-poles in cemeteries (37 sites), in order to ensure a uniform habitat. All the collected specimens were sterile. No fertile thalli were found in the investigated area, even on other substrates (e.g., tree bark). In some urban collection sites, the abundance of this species was low, and only one specimen was collected from such places. At sites where the species was abundant, several specimens were collected. The following specimen sets were collected from the same tombstones: [*Ohmura 5361A*, *5361B*], [*5364A*, *5364B*], [*5375A*, *5375B*], [*5385A*, *5385B*], [*5399*, *5400*, *5401*, *5402*], and [*5415*, *5416*] (see **Table 1** for specimen numbers). The voucher specimens are currently deposited at the National Institute for Environmental Studies, Tsukuba.

DNA extraction, PCR, and sequencing. DNA was extracted from each lichen thallus using a FastDNA Kit (Qbiogene) with SPIN module (Qbiogene), with the following modifications to the manufacturer's protocol. Ca. 20 mg of lichen thallus was put in a 1.5 ml tube along with 1 ml of sterile MilliQ water and then vortexed to rinse the thallus. The cleaned thallus was transferred to a new kit tube, and 1 ml of 'CLS-Y' solution was added. One more ceramic sphere, in addition to that already present in the kit tube, was added and the sample homogenized using a Mini-BeadBeater-1 (BioSpec Products) at 4,200 rpm for 30 sec. After homogenization, DNA extraction was carried out according to the manufacturer's instructions.

PCR amplification of the ITS rDNA regions of the mycobiont and photobiont was performed using the following primer sets. For mycobionts, ITS1F (Gardes & Bruns 1993) was used as the 5' primer and LR1 (Vilgalys & Hester 1990) as the 3' primer. For photobionts, ITS1T (Kroken & Taylor 2000) was used as the 5' primer and LR1 as the 3' primer. In some cases, when multiple non-specific PCR bands were produced by the ITS1T/LR1 primer pair, AL1500bf (Helms et al. 2001) was used as the 5' primer and LR3 (Friedl & Rokitta 1997) as the 3' primer for the amplification of photobiont ITS rDNA. PCR was performed using Ready-To-Go PCR Beads (Amersham Biosciences), with 1.0 µl of extracted DNA solution, 1.25 µl each of 10 pmol/µl primer and 21.5 µl of sterile MilliQ water (total volume 25µl) in each reaction tube. For high quality PCR amplification, the stepdown PCR method (Hecker & Roux 1996) was applied. PCR was carried out in a thermal cycler (TP240, Takara Bio) using the following program: 3 cycles of 95°C for 1 min, 68°C for 1 min, and 72°C for 1 min 30 sec; 3 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min 30 sec; 4 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min 30 sec; and 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec. The products of PCR amplification were checked electrophoretically alongside a molecular weight standard (λ/*Sty*I digest; Nippon Gene) in 1.5% agarose gels stained with ethidium bromide (0.5 µg/ml). 5.0 µl of PCR product was purified using 2.0 µl of ExoSAP-IT (Amersham Biosciences) according to the manufacturer's instructions.

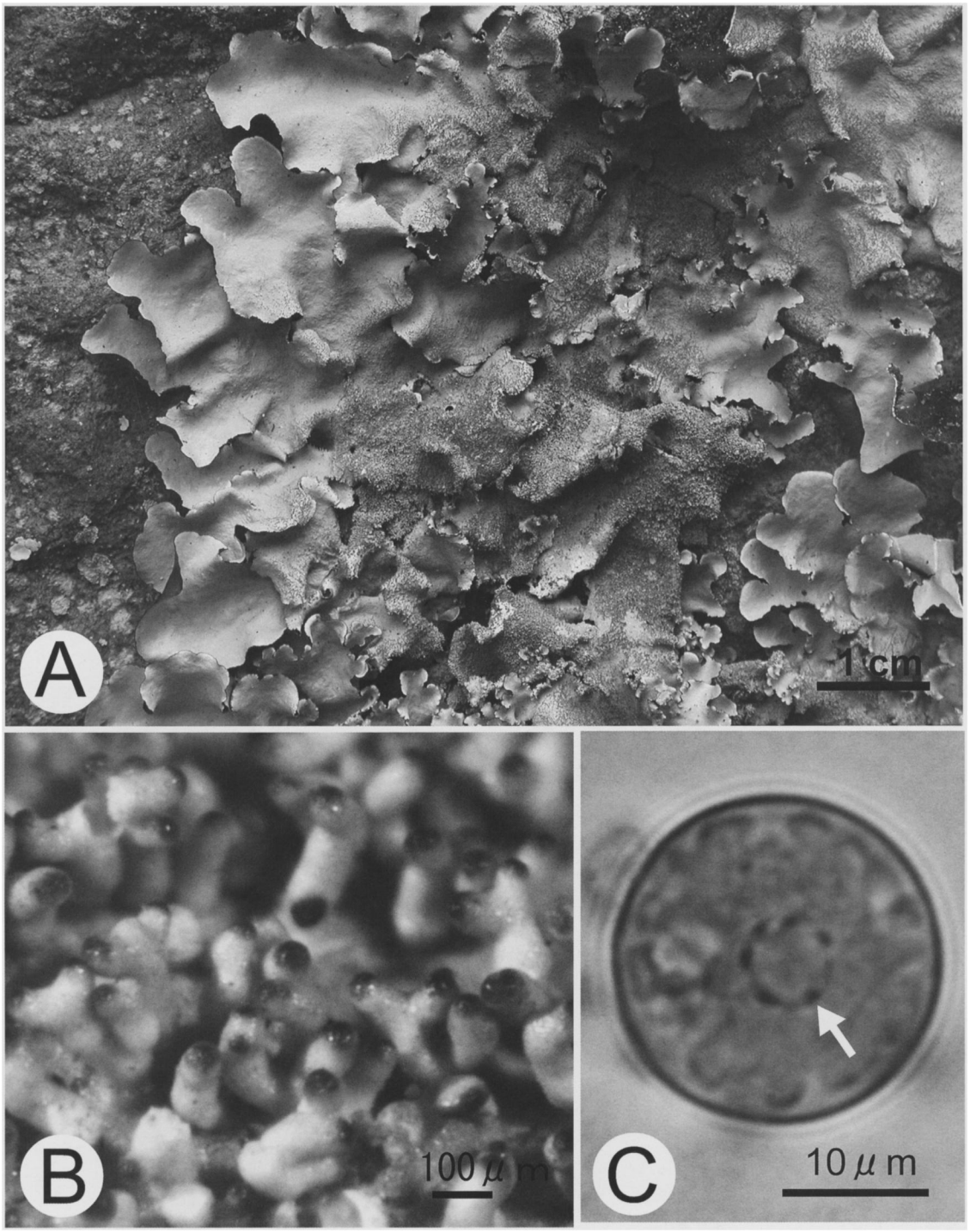

Figure 1. *Parmotrema tinctorum* and its photobiont. **A.** Sterile thallus. **B.** Isidia as diaspore of this lichen. **C.** *Trebouxia corticola* (s. lat.) characterized by distinct starch sheaths surrounding the pyrenoid (arrow). A & B: *Ohmura 5342*. C: isolated from *Ohmura 5375A*.

Sequencing was performed with a CEQ8000 Genetic Analysis System (Beckman Coulter) using a DTCS Quick Start Kit (Beckman Coulter) according to the manufacturer's instructions. The sequencing primers were ITS1F and LR1 for mycobionts; ITS1T and LR1, or AL1500bf, ITS2T (Kroken & Taylor 2000), ITS3T (Kroken & Taylor 2000) and LR3 for photobionts. The sequencing reaction was 50 cycles of

Table 1. Collection data for the investigation of combinations between mycobiont and photobiont in *Parmotrema tinctorum*. All voucher specimens are deposited at the National Institute for Environmental Studies, Tsukuba. *An asterisk after the specimen number indicates the photobiont was isolated from the specimen. **Numbers and letters indicate the sequence type of mycobiont and photobiont as shown in **Table 2** and **Fig. 3**, respectively. ***Only representative sequences were registered to DDBJ/EMBL/GenBank.

Site no.	Locality in Japan, Pref. Shizuoka, Shizuoka-city	Collection date	*Specimen voucher	**Combination between mycobiont (left) and photobiont (right) based on ITS rDNA sequence type	***DDBJ/EMBL/GenBank accession number of ITS rDNA sequence	
					Mycobiont	Photobiont
1	Sena, Koukyo-in Temple, 40 m alt.	21/10/2003	*Ohmura 5385A*	1–K	–	–
			Ohmura 5385B	4–D	–	–
			Ohmura 5386	1–K	–	–
		25/12/2003	*Ohmura 5406*	1–A	–	–
			Ohmura 5407	1–P	–	AB177834
			Ohmura 5408	1–K	–	–
2	Shimizu-torisaka, Daifuku-ji Temple, 20 m alt.	21/10/2003	*Ohmura 5383*	1–K	–	–
3	Shimizu-torisaka, Myogen-ji Temple, 35 m alt.	21/10/2003	*Ohmura 5381*	2–A	–	–
4	Shimizu-ohuchi, Tourin-ji Temple, 30 m alt.	21/10/2003	*Ohmura 5380* *	1–L	–	AB177832
5	Shimizu-ohuchi, Reizan-ji Temple, 40 m alt.	21/10/2003	*Ohmura 5390*	1–K	–	–
	ibid., 140m alt.		*Ohmura 5391* *	1–K	–	–
	ibid., 300m alt.		*Ohmura 5396*	3–K	–	–
6	Shimizu-ohuchi, Houkai-ji Temple, 15 m alt.	21/10/2003	*Ohmura 5378* *	1–K	–	–
7	Shimizu-umegaya, Gyuran-ji Temple, 20 m alt.	21/10/2003	*Ohmura 5375A* *	1–K	AB177401	AB177829
			Ohmura 5375B *	1–U	–	AB177830
			Ohmura 5376 *	1–Q	–	AB177831
		25/12/2003	*Ohmura 5409*	1–C	–	–
			Ohmura 5410	1–C	–	AB177835
			Ohmura 5411	1–G	–	–
			Ohmura 5412	1–G	–	–
8	Shimizu-oshikiri, Entsuu-ji Temple, 5 m alt.	21/10/2003	*Ohmura 5374*	1–A	–	–
9	Senagawa, Seson-ji Temple, 10 m alt.	28/7/2003	*Ohmura 5352*	3–K	–	–
10	Shimizu-noujima, Houon-ji Temple, 5 m alt.	28/7/2003	*Ohmura 5349*	2–K	–	–
11	Shimizu-takahashi, Kogen-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5364A*	1–K	–	–
			Ohmura 5364B	1–E	–	AB177827
12	Shimizu-yasaka-higashi, Shinpuku-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5362*	1–K	–	–
13	Shimizu-nishikubo-cho, Ryuun-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5361A* *	1–B	–	AB177825
			Ohmura 5361B *	1–R	–	AB177826

Table 1. Continued.

Site no.	Locality in Japan, Pref. Shizuoka, Shizuoka-city	Collection date	*Specimen voucher	**Combination between mycobiont (left) and photobiont (right) based on ITS rDNA sequence type	***DDBJ/EMBL/GenBank accession number of ITS rDNA sequence	
					Mycobiont	Photobiont
14	Shimizu-sodeshi-cho, Shinnyo-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5360*	1–K	–	–
15	Shimizu-okitsu-seikenji-cho, Zuiun-in Temple, 20 m alt.	20/10/2003	*Ohmura 5357A* *	1–G	–	AB177821
			Ohmura 5357B *	1–N	–	AB177822
			Ohmura 5357C *	2–A	AB177402	AB177823
		25/12/2003	*Ohmura 5399*	4–K	AB177404	–
			Ohmura 5400	1–G	–	–
			Ohmura 5401	1–G	–	–
			Ohmura 5402	1–G	–	–
			Ohmura 5403	1–K	–	–
			Ohmura 5404	1–O	–	AB177833
			Ohmura 5405	1–K	–	–
16	Shimizu-okitsu-seikenji-cho, Seiken-ji Temple, 20 m alt.	20/10/2003	*Ohmura 5358*	1–J	–	AB177824
17	Shimizu-yada, Toukou-ji Temple, 10 m alt.	28/7/2003	*Ohmura 5353*	2–A	–	–
18	Shimizu-kusanagi, Reisen-ji Temple, 10 m alt.	28/7/2003	*Ohmura 5354*	1–K	–	–
19	Shimizu-imaizumi, Ryogon-in Temple, 10 m alt.	28/7/2003	*Ohmura 5351*	1–K	–	–
20	Shimizu-utozaka, Seigan-ji Temple, 10 m alt.	28/7/2003	*Ohmura 5350*	1–A	–	–
21	Shimizu-irie-minami-cho, Hougan-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5368*	1–K	–	–
22	Shimizu-ejiri-cho, Houun-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5367*	1–K	–	–
23	Shimizu-kami-shimizu-cho, Keiun-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5371*	3–A	AB177403	–
24	Shimizu-kami-shimizu-cho, Zensou-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5370*	1–K	–	–
25	Shimizu-minami-yabe, Nouman-ji Temple, 15 m alt.	25/12/2003	*Ohmura 5421*	2–A	–	–
			Ohmura 5422	1–A	–	–
			Ohmura 5423	1–D	–	–
26	Shimizu-cho, Myokei-ji Temple, 5 m alt.	20/10/2003	*Ohmura 5372* *	1–D	–	AB177828
27	Hirasawa, Heitaku-ji Temple, 100 m alt.	28/7/2003	*Ohmura 5355*	1–G	–	–
28	Shimizu-muramatsu, Tessyu-ji Temple, 15 m alt.	27/7/2003	*Ohmura 5336*	1–M	–	AB177817
29	Shimizu-muramatsu, Ryuka-ji Temple, 15 m alt.	27/7/2003	*Ohmura 5337*	1–A	–	–
30	Shimizu-muramtsu, Kaicho-ji Temple, 5 m alt.	19/10/2003	*Ohmura 5373*	1–K	–	–
31	Ago, Houdai-in-betsu-in, 15 m alt.	27/7/2003	*Ohmura 5340*	1–F	–	AB177818

Table 1. Continued.

Site no.	Locality in Japan, Pref. Shizuoka, Shizuoka-city	Collection date	*Specimen voucher	**Combination between mycobiont (left) and photobiont (right) based on ITS rDNA sequence type	***DDBJ/EMBL/GenBank accession number of ITS rDNA sequence	
					Mycobiont	Photobiont
32	Mt. Kuno-zan, ca. 100 m alt.	27/7/2003	*Ohmura 5342*	1–G	–	–
		25/12/2003	*Ohmura 5413*	1–F	–	–
			Ohmura 5414	1–K	–	–
			Ohmura 5415	1–T	–	AB177836
			Ohmura 5416	1–S	–	–
			Ohmura 5418	2–S	–	AB177838
33	Shimizu-komakoshi-nishi, Bansyo-ji Temple, 15 m alt.	27/7/2003	*Ohmura 5339*	1–K	–	–
34	Shimizu-orido, 5 m alt.	28/7/2003	*Ohmura 5347*	1–A	–	–
35	Shimizu-miho, Miho Shrine, 5 m alt.	28/7/2003	*Ohmura 5356*	1–I	–	AB177820
36	Shimizu-miho, Myofuku-ji Temple, 5 m alt.	28/7/2003	*Ohmura 5344*	2–H	–	AB177819
37	Shimizu-miho, Tokai-ichiko-mae, 5 m alt.	28/7/2003	*Ohmura 5346*	1–K	–	–

96°C for 20 sec, 50°C for 20 sec, and 60°C for 4 min. The forward and reverse sequences were assembled and manually checked using ATGC ver. 4 (Genetyx), by which the final sequence was compiled.

Alignment and phylogenetic analyses. Alignment of sequences was performed using ClustalW ver. 1.82 (Thompson et al. 1994). From the differences in the mycobiont and photobiont sequences, their combinations were determined. The frequencies of the combinations were shown in **Fig. 4**.

Pairwise distance of photobiont genotypes was calculated using MEGA ver 3.0 (Kumar et al. 2004). In order to treat the gap as an informative site, the "Pairwise-Deletion" option was selected. A total of 677 aligned sites with 21 recognized genotypes from the photobiont were used for the analysis. Selection of the nucleotide substitution model and the estimation of most parameters were done with hierarchical likelihood ratio testing using Modeltest 3.04 (Posada & Crandall 1998). The Kimura 2-parameter model (Kimura 1980) was selected with equal base frequencies; gamma distribution shape parameter $\alpha = 0.2599$.

Photobiont sequences were compared with all the available sequences of *Trebouxia* retrieved from DDBJ/EMBL/GenBank for the molecular phylogenetic analyses. Only representatives of reference *Trebouxia* sequences (16 OTUs from 15 taxa) were considered in the final analyses [i.e., *T. arboricola* Puym. (Z68703, Bhattacharya et al. 1996); *T. asymmetrica* Friedl & G. Gärtner (AJ249565, Friedl et al. 2000); *T. corticola* (P. A. Archibald) G. Gärtner (AJ249566, Friedl et al. 2000); *T. flava* P. A. Archibald (AF242467, Kroken & Taylor 2000); *T. galapagensis* (Hildreth & Ahmadjian) G. Gärtner (AJ249567, Friedl et al. 2000); *T. gelatinosa* P. A. Archibald (Z68698, Bhattacharya et al. 1996); *T. gigantea* (Hildreth & Ahmadjian) G. Gärtner (AJ249577, Friedl et al. 2000); *T. higginsiae* (Hildreth & Ahmadjian) G. Gärtner (AJ249574, Friedl et al. 2000); *T. impressa* Ahmadjian (AJ249570, Friedl et al. 2000); *T. incrustata* G. Gärtner (AJ293795, Helms et al. 2001); *T. jamesii* (Hildreth & Ahmadjian) G. Gärtner (Z68700, Bhattacharya et al. 1996); *T. potteri* G. Gärtner (AF242469, Kroken & Taylor 2000); *T. showmanii* (Hildreth & Ahmadjian) G. Gärtner (AF242470, Kroken & Taylor 2000); *T.*

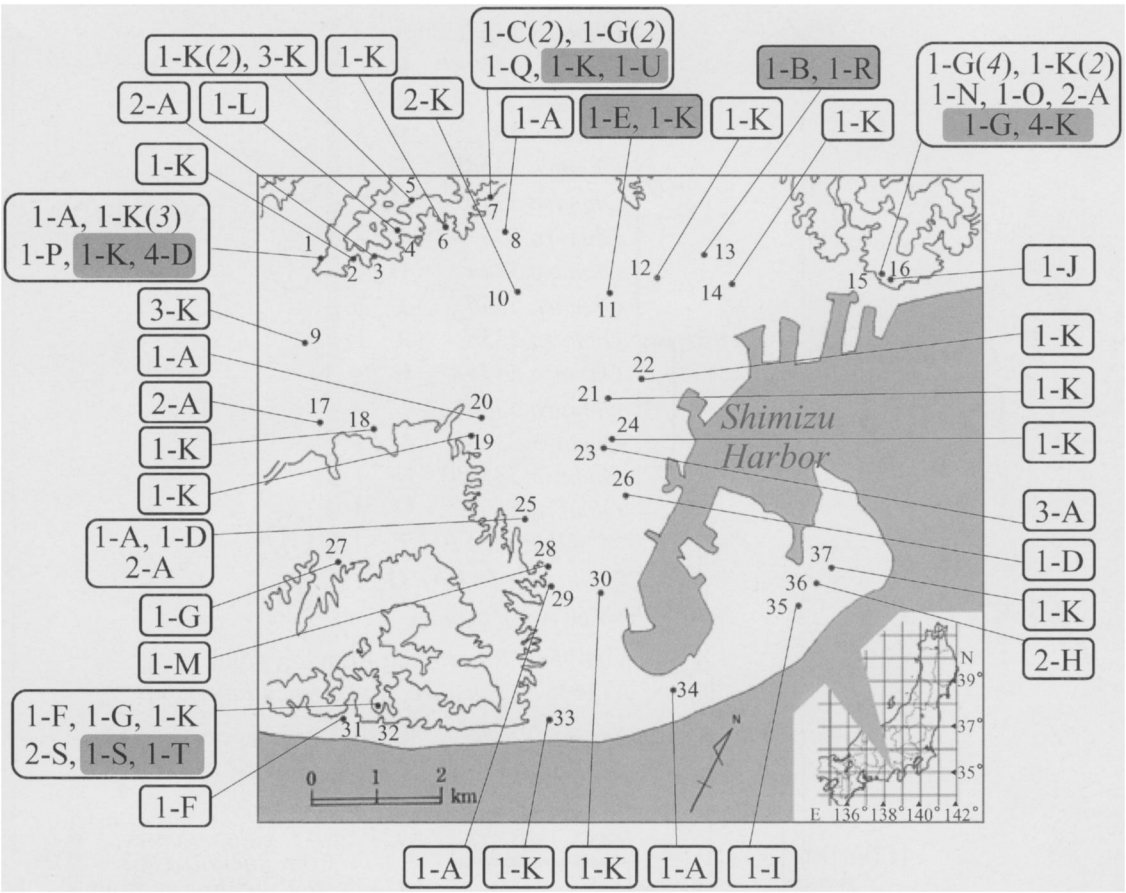

Figure 2. Distribution map of genetic combination types between mycobiont (left number) and photobiont (right letter) in *Parmotrema tinctorum*. Italic numbers in parentheses indicate how often these combinations have been found. The numbers are only shown when they were found more than twice. Highlighted combination types show the lichen thalli were collected from the same stones. Each collection site is marked with a number that corresponds to an entry in **Table 1**. The interval between contour lines is 100 m.

usneae (Hildreth & Ahmadjian) G. Gärtner (AJ249573, Friedl et al. 2000; Z68702, Bhattacharya et al. 1996); and *Trebouxia* sp. (AJ293792, Helms et al. 2001)]. After removing sites with gaps, missing data and ambiguous data, the resulting alignment of 557 sites was used for the molecular phylogenetic analyses of photobionts.

Maximum likelihood (ML) analysis was performed with optimization criteria using PAUP* ver. 4.0b10 (Swofford 2002). Selection of the nucleotide substitution model and the estimation of most parameters for ML analysis were done with hierarchical likelihood ratio testing using Modeltest 3.04. The Tamura-Nei substitution model (Tamura & Nei 1993) was selected with equal base frequencies; substitution rate matrix A-C=1.0000, A-G=3.7876, A-T=1.0000, C-G=1.0000, C-T=4.7016, G-T=1.0000; gamma distribution shape parameter α=0.2802; proportion of invariable sites I=0. ML analysis was started with a Neighbor-Joining tree and subsequent heuristic search using the tree bisection-reconnection branch-swapping algorithm. All other parameters were as the default settings of PAUP. For the bootstrap analysis of the ML tree, the heuristic setting was used with 100 resamplings.

Bayesian inference with the Markov chain Monte Carlo (B/MCMC) method (Larget & Simon 1999) was also performed using MrBayes ver. 3.0b4 (Huelsenbeck & Ronquist 2001) to assess the level of confidence for relationships revealed by the ML search. The same model and number of rate categories as used in the ML search was used for the B/MCMC

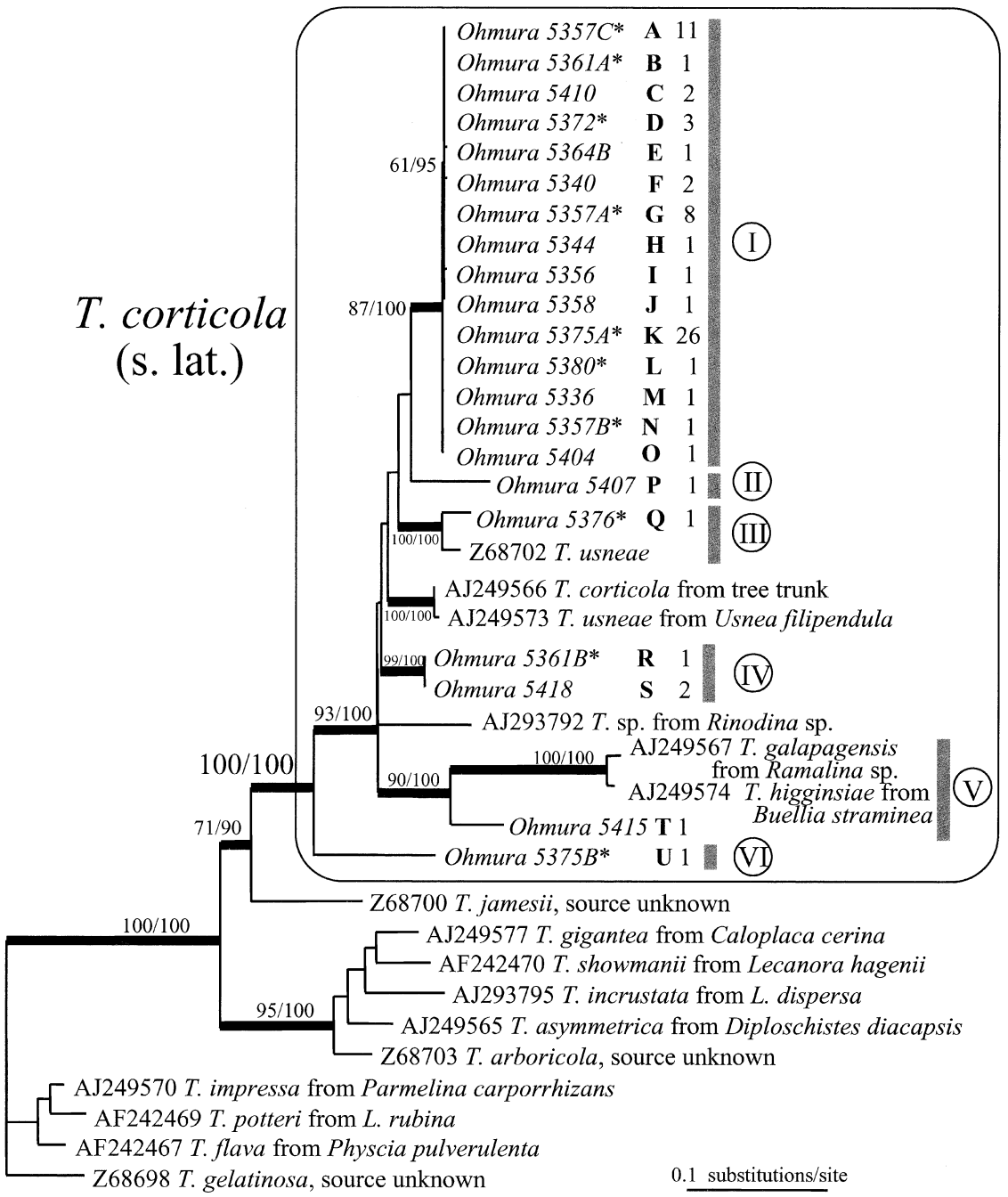

Figure 3. ML tree of ITS rDNA sequences from photobionts in *Parmotrema tinctorum* and other strains of *Trebouxia* from various lichens. Unless indicated, the photobionts are derived from *P. tinctorum*. The voucher specimen number of *P. tinctorum* and sequence types of the photobionts (A–U) are given as OTU names. Numbers of samples are indicated after the OTU names. DDBJ/EMBL/GenBank accession numbers and taxon names are indicated for the reference OTUs of *Trebouxia*. Six phylogenetic groups of photobionts of *P. tinctorum* in *Trebouxia corticola* (s. lat.) clade are marked with gray bars (groups I–VI). Asterisks at the right side of voucher specimen numbers indicate that the photobiont of the specimen was isolated and cultured. Numbers at thicker branches indicate bootstrap values (left) and B/MCMC posterior probabilities (right) (numbers are shown only when ≥50% in both). The tree was rooted by the branch leading to *T. impressa-T. potteri-T. flava-T. gelatinosa*. The DDBJ/EMBL/GenBank accession numbers of photobionts sequenced in this study are shown in **Table 1**.

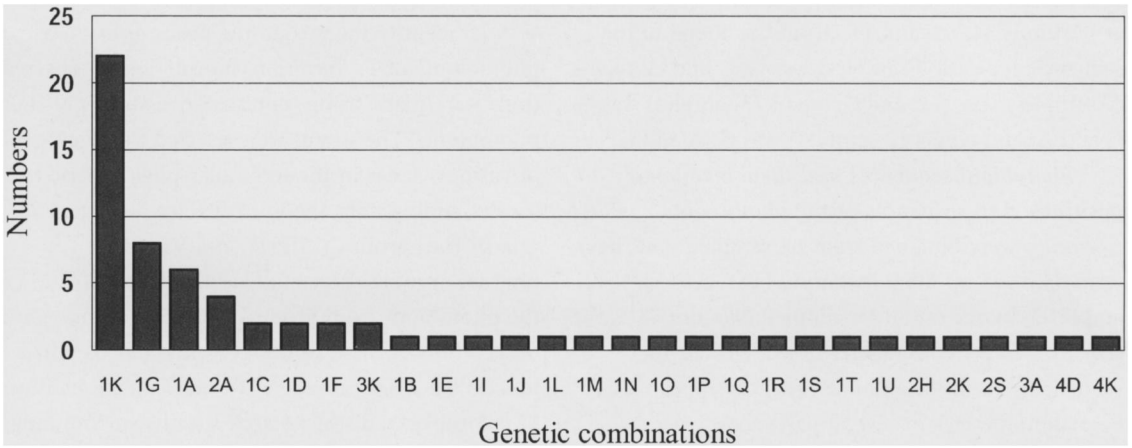

Figure 4. Frequencies of genetic combinations between mycobiont and photobiont in *Parmotrema tinctorum* collected from Shimizu district.

analysis. One of every 100 trees was sampled for a total of 2,000,000 generations with DNA substitution parameters estimated during the search. The first 4,000 trees from 20,000 sampled trees were discarded to avoid trees that might have been sampled prior to convergence of the Markov chains. Posterior probabilities of $\geq$95% are considered to be statistically significant.

Photobiont culture. Photobionts were isolated and cultured axenically. They were selected from various genotypes (see **Table 1** and **Fig. 3**). A 2–3 mm^2 fragment, including the algal layer, was cut from near the edge of the lichen thallus. The fragment was placed between sterile glass slides along with 1–2 drops of sterile MilliQ water, and ground gently with the slides to separate the fungus and alga. A single algal cell was isolated by micropipette, and rinsed three times with sterile 1×N Bold's Basal Medium (BBM, Bischoff & Bold 1963). The cell was cultured in BBM for ca. one month at 20°C under 20 $\mu mol/m^2/s$ photon irradiance with 12 hr light and 12 hr dark cycles. After one month, 100 µl of the culture suspension was spread uniformly on the surface of a BBM plate (solidified with 1.5% agar) using a spreader. The plate was cultured under the same conditions as above until colonies were visible (ca. one month). When contamination was found on a plate, a relatively uncontaminated colony was carefully picked and transferred to a new BBM plate. Before spreading, 100 µl of sterile MilliQ water was added to the colony to separate algal cells from contaminants and facilitate spreading. This procedure was repeated until no further contamination was found. A colony was picked from the plate and streaked with an inoculating loop onto a slant in a screw-cap tube containing 10 ml medium. After one month of subculture, identification was made morphologically with BBM under a bright field microscope. The obtained culture strains are currently maintained as private collections of the first author at the National Institute for Environmental Studies, Tsukuba.

Results

Mycobiont sequences. ITS sequences of *Parmotrema tinctorum* were successfully obtained from 69 samples, and they were all 524-bp long, including ITS1, 5.8S rDNA, and ITS2. There were no gaps in the alignment of these sequences. Although all sequences were identical in the 5.8S rDNA and ITS2 regions, sequence differences in the ITS1 region allowed them to be classified into four types. Each of the four types differed by only one residue from the others (**Table 2**):

Table 2. Variations of mycobiont ITS sequence of *Parmotrema tinctorum.*

Type	Position (5'-3')			Number of samples
	34	65	115	
1	G	C	G	57
2	T	C	G	7
3	G	C	A	3
4	G	A	G	2

at positions 34, 65, and 115 from the 5' end of the sequence, type 1 (57 samples) was G, C and G; type 2 (7 samples) was T, C and G; type 3 (3 samples) was G, C and A; and type 4 (2 samples) was G, A and G.

Photobiont sequences and their taxonomic positions. ITS sequences of the photobionts in *P. tinctorum* were obtained from 68 samples, and they were 637–668-bp long, including ITS1, 5.8S rDNA, and ITS2. In one out of 69 samples, *Ohmura 5419*, the sequence could not be determined because the sequencing result in an autosequencer appeared as overlapping waves in several parts.

The photobiont sequences fell into at least 21 types (genotypes A–U in **Table 3** and **Fig. 3**), based on differences in residues and gaps. These genotypes could be divided into six groups, based on pairwise distances (P) among 21 genotypes (**Table 3**) and the phylogenetic analyses with representative *Trebouxia* species (**Fig. 3**). The grouping was performed depending on whether the OTUs formed a distinct clade or not. Although the group V could also be two separate groupings, the group was kept as a single group in this study in order to consider the unidentified photobiont sequence of *Ohmura 5415* in relation to known photobiont sequences. Most genotypes (A–O) belong to group I, and 61 of 68 samples are this group. The genetic difference among the genotypes is $0.002 \leq P \leq 0.008$. Genotypes R and S belong to group IV, in which their difference is $P = 0.002$. Other groups (groups II, III, V and VI) consisted of one genotype. The genetic differences among these six groups are $0.087 \leq P \leq 0.260$.

The molecular phylogenetic tree for *Trebouxia*, including photobionts of *P. tinctorum* and other representative strains of *Trebouxia*, based on ITS rDNA sequences, is shown in **Fig. 3**. The OTUs of all the genotypes formed a monophyletic clade together with the known sequences of *T. corticola*, *T. galapagensis*, *T. higginsiae*, *T. usneae*, and *Trebouxia* sp. from DDBJ/EMBL/GenBank with highly reliable support values for the branch (100% in both ML and B/MCMC). At least eight distinct groups were included in the clade, among which six groups were from the photobiont of *P. tinctorum*. This clade formed a sister clade with the OTU of *T. jamesii*, but support value for the branch was relatively lower (71% in ML and 90% in B/MCMC).

To identify the taxonomic position of these photobionts of *P. tinctorum*, morphological observations were made using representative strains of the photobionts. The strains were selected from genotypes of various clades in the molecular phylogenetic tree. Twelve strains from the four distinct phylogenetic groups (i.e., groups I, III, IV and VI) of the photobiont were successfully isolated, but isolation of the photobiont from group II failed. Morphological observation of these strains revealed that they had unicellular spherical vegetative cells, simple incisions of chloroplasts, distinct starch sheaths surrounding the pyrenoid, and autosporangia (**Fig. 1C**). These characteristics are a close match with the features of the *Trebouxia corticola* group, which includes, e.g., *T. corticola*, *T. galapagensis*, *T. higginsiae* and *T. usneae*. This group still has taxonomic problems in the identification at the species level (see discussion). Therefore, the strains obtained in this study were all tentatively identified as *T. corticola* (s. lat.).

Genetic combinations between mycobiont and photobiont. Between the four types of mycobiont and 21 types of photobiont, 28 different combinations were detected in *P. tinctorum* from the investigated area (see **Table 1** and **Figs. 2, 4**). As shown in **Fig. 4**, the most common combination was 1-K (22 samples), followed by 1-G (8 samples), 1-A (6 samples), 2-A (4 samples), and 1-C, 1-D, 1-F and 3-K (2 samples in each). The remaining combinations were found in one sample each.

Different genetic types or combinations were found not only within a single collection site (i.e., site numbers 1, 5, 7, 11, 13, 15, 25 and 32), but also from the same tombstone (**Fig. 2**). The genetic combinations found in sets of specimens collected from the same tombstones are as follows: [(specimen numbers)=(combinations of genotypes)]; [(*Ohmura 5361A*, *5361B*)=(1-B, 1-R)], [(*5364A*, *5364B*)=(1-K, 1-E)], [(*5375A*, *5375B*)=(1-K, 1-U)], [(*5385A*, *5385B*)=(1-K, 4-D)], [(*5399*, *5400*, *5401*, *5402*)=(4-K, 1-G, 1-G, 1-G)] and [(*5415*, *5416*)=(1-T, 1-S)].

Discussion

Identifications of photobionts of *Parmotrema tinctorum* collected in the investigated area were made based on the results of molecular phylogenetic analyses and the morphology of the culture strains in

Table 3. ITS rDNA pairwise distance among 21 genotypes of photobiont in *Parmotrema tinctorum*. *Genotypes (A–U) or groups (I–VI) are identical in **Table 1** and **Fig. 3**.

Genotype*	Group	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
A	I	—																				
B	I	0.003	—																			
C	I	0.003	0.003	—																		
D	I	0.003	0.003	0.003	—																	
E	I	0.005	0.005	0.005	0.002	—																
F	I	0.003	0.003	0.003	0.003	0.005	—															
G	I	0.002	0.002	0.002	0.002	0.003	0.002	—														
H	I	0.000	0.003	0.003	0.003	0.005	0.003	0.002	—													
I	I	0.002	0.005	0.005	0.005	0.006	0.005	0.003	0.002	—												
J	I	0.002	0.005	0.005	0.005	0.006	0.005	0.003	0.002	0.003	—											
K	I	0.005	0.002	0.005	0.005	0.006	0.005	0.003	0.005	0.006	0.006	—										
L	I	0.006	0.003	0.006	0.006	0.008	0.006	0.005	0.006	0.008	0.008	0.002	—									
M	I	0.006	0.003	0.006	0.006	0.008	0.006	0.005	0.006	0.008	0.008	0.002	0.003	—								
N	I	0.006	0.003	0.006	0.006	0.008	0.006	0.005	0.006	0.008	0.008	0.002	0.003	0.003	—							
O	I	0.006	0.003	0.006	0.006	0.008	0.006	0.005	0.006	0.008	0.008	0.002	0.003	0.003	0.003	—						
P	II	0.116	0.110	0.113	0.116	0.118	0.116	0.113	0.114	0.119	0.122	0.107	0.105	0.105	0.110	0.113	—					
Q	III	0.100	0.097	0.099	0.099	0.102	0.099	0.097	0.100	0.103	0.103	0.094	0.097	0.097	0.097	0.097	0.148	—				
R	IV	0.095	0.092	0.094	0.092	0.094	0.094	0.092	0.095	0.098	0.096	0.089	0.092	0.092	0.092	0.092	0.152	0.139	—			
S	IV	0.090	0.087	0.089	0.087	0.089	0.089	0.087	0.090	0.093	0.091	0.085	0.087	0.087	0.087	0.089	0.145	0.133	0.002	—		
T	V	0.159	0.152	0.158	0.158	0.161	0.158	0.155	0.159	0.163	0.167	0.148	0.151	0.152	0.152	0.155	0.223	0.195	0.159	0.155	—	
U	VI	0.209	0.213	0.217	0.213	0.216	0.217	0.213	0.203	0.213	0.201	0.210	0.213	0.205	0.214	0.213	0.226	0.224	0.218	0.214	0.260	—

light microscopy. *Trebouxia corticola* and its related species, *T. galapagensis*, *T. higginsiae* and *T. usneae*, share the same features as recorded in our results, and they are characterized from other *Trebouxia* species by having distinct starch sheaths surrounding the pyrenoid ("*corticola*-type pyrenoid" in Friedl 1989a). The monophyly of this group was supported by molecular phylogenetic analyses (**Fig. 3**). These species have been distinguished mainly by shape of the chloroplasts, number of starch sheathes, and cell cycle. However, some species of the group have been considered within the variation of *T. corticola*. Friedl (1989b) treated *T. higginsiae* as a synonym of *T. corticola*, and Takeshita (2001) treated *T. galapagensis* as a synonym of the species. In addition, the OTUs of *T. usneae* did not form a monophyletic clade (**Fig. 3**). The present phylogenetic analyses show the clade of the *T. corticola* group includes several phylogenetic groups, suggesting their genetic diversification. Further study such as ultrastructural observation as well as phylogenetic analyses beyond the present study is needed to clarify the taxonomic problems of the *T. corticola* group. A taxonomic treatment of *T. corticola* is not the purpose of this study, and for our strains we confirmed the presence of a *T. corticola*-type pyrenoid. For this reason, we are treating our strains as *T. corticola* (s. lat.). The OTUs of the photobionts from *P. tinctorum* form a monophyletic clade together with those of the *T. corticola* (s. lat.) strains. Therefore, all photobionts of *P. tinctorum* collected in the investigated area were identified as *T. corticola* (s. lat.).

Other reports of the photobiont from *P. tinctorum* from other areas have called the material *T. higginsiae* from Ibaraki and Hiroshima in Japan (Kasai et al. 2004) or *T. usneae* from Florida, U.S.A. (Friedl 1989b). As mentioned in this study, these taxa also belong to *T. corticola* (s. lat.). Our results and these facts suggest that the "selectivity" of the mycobiont toward the photobiont in *P. tinctorum* is considered to be relatively high, compared with previous reports, e.g., the photobionts of *Chaenothecopsis consociata* (Nádv.) A. F. W. Schmidt, *Diploschistes muscorum* (Scop.) R. Sant., and *Umbilicaria* species from Antarctica (Friedl 1987; Romeike et al. 2002; Tschermak-Woess 1980). The term "selectivity" was differentiated from the term "specificity" in Beck et al. (2002), and this paper follows the meaning of their terminology. That is, "selectivity" is for the characterization of interaction between organisms viewed from the perspective of one biont only, while "specificity" is for the symbiotic association as a whole, therefore depending on the degree of selectivity of the partaking bionts which then has to be mutually exclusive. Although the mechanism of selectivity in lichens is poorly understood, crustose species seem to have a low degree of selectivity and foliose species seem to be highly selective (Helms et al. 2001). *Parmotrema tinctorum*, a foliose lichen, may also be an example of this tendency.

A large number of genetic combinations between mycobiont and photobiont were detected in *P. tinctorum*. The 28 combinations from the small area represent an unexpectedly high diversity, because *P. tinctorum* is thought to propagate vegetatively. In fact, no fertile individuals were found in the investigated area. Finding the same several combinations between mycobiont and photobiont in the area suggests that vegetative reproduction of *P. tinctorum* might occur and produce the same genetic combinations as that of the original thallus. However, the number of the most common combination ('1-K') was only 22 of 68 samples (**Fig. 4**), and various genetic combinations were detected from the same cemetery, and even from the same tombstone (**Fig. 2**). Therefore, the original thallus of *P. tinctorum* may not always produce the same combination through vegetative reproduction.

To account for the high diversity of genetic combinations in *P. tinctorum*, the following four possibilities are suggested (**Fig. 5**): (1) photobiont exchange between original photobiont and compatible alga on the substrate may occur in the redifferentiation stage of isidia (**Fig. 5A**); (2) photobiont or mycobiont exchange by the result of fusion between genetically different individuals, and various genetic types of isidia are produced from the same thallus and dispersed (**Fig. 5B**); (3) genetically diverse isidia are brought into the area through long-distance dispersal from various geographic origins (**Fig. 5C**); and (4) large numbers of ascospores are widely distributed through long-distance dispersal from the few individuals that produce apothecia, and then photobiont exchange occurs at various local sites (**Fig. 5D**).

First of all, the possibility of photobiont exchange in a vegetatively reproducing lichen has already been

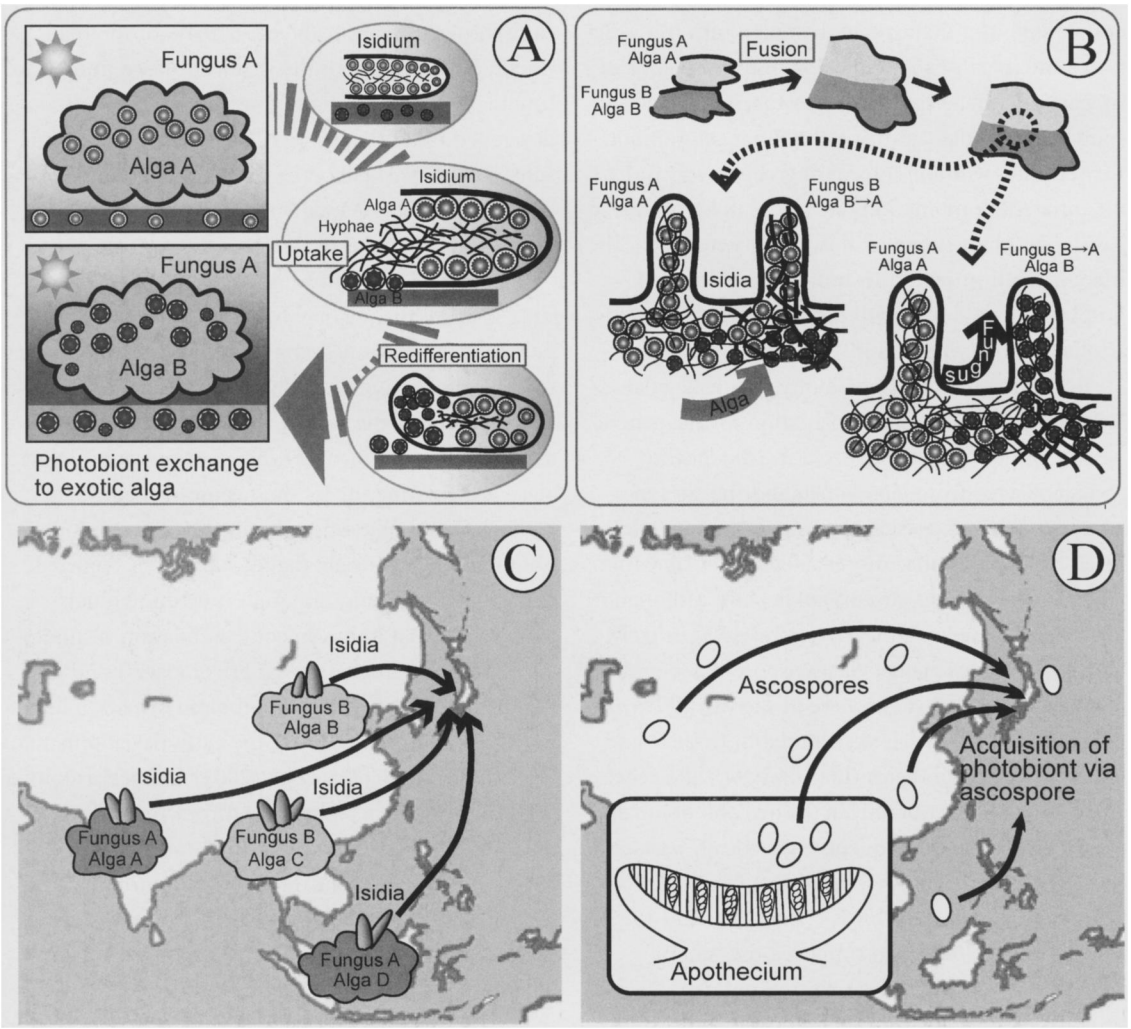

Figure 5. Possibilities of the diversification of genetic combination between mycobiont and photobiont in *Parmotrema tinctorum*. **A.** Photobiont exchange between original photobiont and compatible alga on the substrate or photobiont from other lichen thallus. **B.** Photobiont or mycobiont exchange by the result of fusion between genetically different individuals. **C.** Genetically diverse individuals are dispersed from various geographic origins. **D.** Large numbers of ascospores are widely distributed through long-distance dispersal from the few individuals that produce apothecia.

pointed out by Beck et al. (1998). Photobiont exchange in vegetatively reproducing lichens may occur between the original photobiont and a compatible alga growing on the substrate or from a photobiont from other lichen thallus. That is, during redifferentiation from isidium to a mature thallus, the photobiont in the isidium of *P. tinctorum* might be replaced with an alga more adaptive to the environment. The original photobiont may be reduced or eliminated through competition with the exotic photobiont.

The second possible explanation for photobiont diversity is that there may be photobiont or mycobiont exchange through the result of fusion between genetically different individuals. Fusion of lichen thalli has been reported in various species (Armstrong 1984; Asta & Letrouit-Galinou 1995; Jahns 1972; Moxham 1981) and, for example, variability of thallus in the genus *Cladina* was explained by fusion (Jahns 1972). As seen in various lichens, the fusion of *P. tinctorum* thalli between genetically different individuals may occur. Along the border part of the fusion, photobionts and mycobionts from different thalli may be interwoven with each other. The genetically different photobiont from

another side of a thallus may be taken into the isidia of a counterpart of the thallus, or the mycobiont of one part may penetrate into the other part and produce new isidia capturing the other photobiont. Theoretically, without considering chimerical isidia, four different combinations at most can be produced in isidia within a single thallus, when two genetically different thalli in regard to both mycobiont and photobiont would fuse with each other. Subsequently, various genetic types of isidia may be dispersed.

Regarding the third possibility, Romeike et al. (2002) proposed a similar explanation for the genetic diversity of photobionts detected in *Umbilicaria* species collected from closely neighboring sites in Antarctica. They suggested that the diversity might be caused by long-distance dispersal of *Trebouxia* within the dry diaspores (e.g., soredia) of lichens. Although *P. tinctorum* produces apothecia very rarely, it is likely that photobiont exchange via ascospore-based sexual reproduction occurs somewhere in the world. *Parmotrema tinctorum* is widely distributed in tropical and temperate regions of the world (Elix 1994), and genetic variations may arise not only in the mycobiont but also in the photobiont. Subsequently, genetically variant isidia may be distributed by wind (Muñoz et al. 2004) or other vectors, such as birds (Bailey & James 1979). Romeike et al. (2002) also supposed the genetic variations of the *Umbilicaria* photobiont could be caused by emergence from different geohistorical periods, depending on deglaciation processes. In the case of *P. tinctorum*, however, this assumption could not apply for the diversification of the photobiont.

Regarding the fourth possible explanation for photobiont diversity, Harada (2003) discussed the possibility of ascospore-based dispersal of *P. tinctorum*, based on the discovery of several fertile specimens in Japan. He supposed that isidia of this species may not be easily broken from the thallus, and it is not clear how the isidia are transported or whether isidia really grow into mature thalli. However, ascospores may be easily dispersed by wind, thus facilitating long-distance dispersal. Even if the occurrence of fertile individuals is rare, large amounts of ascospores might be distributed whenever apothecia are produced on the thallus. Resultingly, ascospore-based dispersal may commonly occur in the field. Nevertheless, from our field observations of *P. tinctorum*, isidia seem to be easily broken off from the older parts of the thallus, usually at the center of the thallus. A large number of tiny thalli are occasionally found around a well-developed older thallus of *P. tinctorum* on the same substrate. So, we believe the dispersal of *P. tinctorum* is usually via isidia or fragmentation of the thallus, at least within the investigated area.

The occurrence of multiple photobionts inhabiting a single thallus may be involved in the possibilities mentioned above. Multiple photobionts might be taken into a thallus during the early development of the lichen, for example by isidia or ascospores on a substrate where various compatible algae are growing, or by the fusion of thalli that contain genetically different photobionts. Multiple photobionts in a single thallus have been reported from various lichens, although most individuals probably have a homogeneous population of photobiont within a single thallus. Friedl (1987) and Ott (1987) reported different photobiont species inhabiting the same thallus during the early developmental stage of lichens. Helms et al. (2001) also reported two genetically distinct photobionts from thalli of *Rinodina atrocinerea* (Dicks.) Körb., *R. tunicata* H. Mayrhofer & Poelt, and *Rinodinella controversa* (A. Massal.) H. Mayrhofer & Poelt. In such cases, in theory, various diaspores having various genetic types of photobiont could be produced within a single thallus. Overlapping waves from an autosequencer for the sample of *Ohmura 5419* may be caused by multiple photobionts in a single thallus. Examinations of two different parts of the thallus confirmed this hypothesis.

All these hypotheses are plausible, and they all may be more or less involved in the dispersal of *P. tinctorum*. When the mycobiont sequence of an individual is different from a genetically homogeneous population, it can easily be imagined that the thallus may have come from another locality. This situation is exemplified by the following collections from single tombstones: [(specimen numbers)=(combinations of genotypes)]; [(*Ohmura 5385A*, *5385B*)=(1-K, 4-D)] and [(*5399*, *5400*, *5401*, *5402*)=(4-K, 1-G, 1-G, 1-G)]. However, when the photobiont sequence differs from that of an adjacent thallus in which the mycobiont sequence is identical, the possibility of photobiont exchange through

vegetative reproduction may be more plausible. This situation is exemplified by following collections: [(*Ohmura 5361A*, *5361B*)=(1-B, 1-R)], [(*5364A*, *5364B*)=(1-K, 1-E)], [(*5375A*, *5375B*)=(1-K, 1-U)], and [(*5415*, *5416*)=(1-T, 1-S)].

It should be noted that the genetic diversity of photobionts was poor in the urban area around Shimizu Harbor, where all the genotypes of photobionts belonged to group I (see **Figs. 2, 3**). On the other hand, the diversity was rich in suburbs and mountainsides, and the genetic distances were also far among the genotypes especially at the Sites 1, 7, 13 and 32 (see **Table 3** and **Figs. 2, 3**). Namely, the photobiont genotypes belonging to groups II–VI were found only in suburbs or mountainsides. The urban area around Shimizu Harbor was formerly damaged by heavy SO_2 air pollution in the 1960s and 70s. *Parmotrema tinctorum* was completely eliminated from the area where the SO_2 concentration was more than 0.02 ppm (Sugiyama 1973). SO_2 air pollution has drastically decreased after enforcement of the Basic Law for Environmental Pollution of 1967, and its concentration in this area has been under 0.01 ppm for the last twenty years. Populations of *P. tinctorum* in the area have recovered as shown in our results, although they are still poorly developed. The recovery of populations in a short period usually results in poor genetic diversity in an area, and the phenomenon can be explained as a bottleneck or founder effect. Many individuals of *P. tinctorum* in the urban area belong to "1-K" type, which may suggest the possibility of this hypothesis. Heibel et al. (1999) reported no founder effect was found in recovering populations of *Usnea filipendula* Stirt. based on RAPD analysis of the mycobiont. In contrast, Crespo et al. (1999) found in *Parmelia sulcata* Taylor a lower genetic diversity in recolonizing sites compared with long-established sites, consistent with a bottleneck or founder effect. In regard to lichen photobionts, no attempt has been made to study the bottleneck or founder effect based on air pollution levels. Another possibility to explain the low genetic diversity of photobionts in the urban area is that the selectivity of *P. tinctorum* could vary depending on differences in the environment. In other words, only a strictly compatible alga could be the photobiont of the lichen in a severe environment, while various allied algae might be acceptable as a photobiont in a more favorable environment. In contrast to the distinct tendency of photobiont diversity, there seems to be less correlation between the mycobiont diversity and environmental differences. These observations could be explained by the photobiont exchange to the more appropriate algal strain (**Fig. 5A**). When isidia having an unstably related photobiont disperse into a severe environment, the isidia may die. But, if photobiont exchange to the strictly compatible alga were to occur, then the isidia might be able to survive and grow. Resultingly, only particular phylogenetic groups of photobionts may distribute in a severe environment, while the mycobiont having different genotypes from the local population may possibly distribute. The matter of selectivity in lichens is one of the ongoing and interesting fields in lichenology, and further studies are needed to solve the problem.

To clarify the dispersal mode of *P. tinctorum*, the following experimental approaches may be helpful: analysis of the relationship between the photobionts and the algae on the substrate; analysis of the genetic diversity of the mycobionts and photobionts in *P. tinctorum* from various localities in the world, or from several spots within a single thallus; and analysis of the population structure in a small area using more detailed genetic methods for detection of individual differences, such as Amplified Fragment Length Polymorphism analysis or microsatellite analysis.

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