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Authors: Grande, Francesco Dal, Beck, Andreas, Singh, Garima, and Schmitt, Imke

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PRIMER NOTE

MICROSATELLITE PRIMERS IN THE LICHEN SYMBIOTIC ALGA *TREBOUXIA DECOLORANS* (TREBOUXIOPHYCEAE)[1]

FRANCESCO DAL GRANDE[2], ANDREAS BECK[3,6], GARIMA SINGH[4], AND IMKE SCHMITT[2,5]

[2]Biodiversity and Climate Research Centre (BiK-F), Senckenberg Gesellschaft für Naturforschung, 60325 Frankfurt am Main, Germany; [3]Department of Lichenology and Bryology, Botanische Staatssammlung München, 80638 Munich, Germany; [4]Biodiversity and Conservation Biology, WSL Swiss Federal Research Institute, Zürcherstrasse 111, 8903 Birmensdorf, Switzerland; and [5]Institute of Ecology, Evolution and Diversity, Goethe Universität, Max-von-Laue-Str. 13, D-60438 Frankfurt, Germany

- *Premise of the study:* Polymorphic microsatellite markers were developed for the symbiotic green alga *Trebouxia decolorans* to study fine-scale population structure and clonal diversity.
- *Methods and Results:* Using Illumina pyrosequencing, 20 microsatellite primer sets were developed for *T. decolorans*. The primer sets were tested on 43 individuals sampled from four subpopulations in Germany. The primers amplified di-, tri-, and tetranucleotide repeats with three to 15 alleles per locus, and the unbiased haploid diversity per locus ranged from 0.636 to 0.821.
- *Conclusions:* The identified microsatellite markers will be useful to study the genetic diversity, dispersal, and reproductive mode of this common lichen photobiont.

Key words: *Anaptychia ciliaris*; photobiont; population genetics; selectivity; symbiosis; *Xanthoria parietina*.

Green algae of the genus *Trebouxia* constitute a significant portion of terrestrial algal diversity as they are the most common photobionts in lichens (Friedl and Büdel, 2008). Population biology of lichen photobionts is currently poorly understood, especially due to the lack of appropriate molecular markers. To date, highly variable markers such as microsatellites have only been developed for the photobionts of two lichen species, namely *Lobaria pulmonaria* (L.) Hoffm. (Dal Grande et al., 2010) and *Parmotrema tinctorum* (Delise ex Nyl.) Hale (Mansournia et al., 2012).

Trebouxia decolorans Ahmadjian is a common haploid lichen photobiont that has been reported from several continents and was found in association with both widespread (e.g., *Xanthoria parietina* (L.) Th. Fr.) and locally endangered (e.g., *Anaptychia ciliaris* (L.) Körb.) fungal species (Helms et al., 2001). Our goal was to develop microsatellite loci to be used in high-resolution population studies in *T. decolorans*. This is a key step in understanding reproductive mode and fine-scale spatial genetic structure and diversity in trebouxioid algae.

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[6]Author for correspondence: beck@bsm.mwn.de

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METHODS AND RESULTS

We established an algal culture of *T. decolorans* (strain AB05019B2, Botanische Staatssammlung München) from the lichen *X. parietina* collected in Maising, Germany (47°58′19″N, 11°16′34″E, 635 m a.s.l.), using a micromanipulator (Beck and Koop, 2001). Approximately 10 µg of total DNA from the algal culture (ITS sequence GenBank JF831923) was used to construct a library (sheared DNA fragments were of 500 bp length) for 100 bp × 100 bp paired-end sequencing using an Illumina GAIIx and standard Illumina protocols (Illumina, San Diego, California, USA). The Illumina sequencing of this sample was done in the laboratory of D. Bhattacharya (Rutgers University, New Brunswick, New Jersey, USA) and is described in more detail in Beck et al. (unpublished). All 244 958 contigs, totaling 64.2 Mbp of genome data with an average coverage of 3.8×, were screened in fasta files using MSATCOMMANDER 1.0.8 (Faircloth, 2008) accepting dinucleotide repeats of ≥10, trinucleotide repeats of ≥8, and tetranucleotide repeats of ≥8. One hundred out of 244 958 contigs screened contained repeats consisting of 58 di-, 20 tri-, and 22 tetranucleotide repeats. Primers were developed using Primer3 (Rozen and Skaletsky, 2000). Forward primers were appended with an M13 tag (5′-TGTAAAACGACGGCCAGT-3′). Nine sequences were discarded because the flanking regions of the repeat sequences were too short in length and therefore not suitable for primer design. Primers could be designed for 91 contigs containing repeats, including 54 di-, 19 tri-, and 18 tetranucleotide repeats. Primers were checked for amplification with the original *T. decolorans* culture, and with DNA isolated from the same algal taxon of the locally endangered lichen *A. ciliaris* collected in Pähl, Germany, on *Tilia* sp. (47°55′N, 11°11′E, 662 m a.s.l., M-0102896; ITS sequence GenBank JX444960). PCR was performed in a 10 µL reaction volume containing ~1–5 ng genomic DNA, 1× Type-it Multiplex Master Mix (QIAGEN, Hilden, Germany), 0.15 µM reverse primer, 0.01 µM M13-tailed forward primer, and 0.15 µM of 6FAM–M13-labeled primer (Schuelke, 2000). PCR was carried out with an initial 5-min denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 45 s, eight cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s, and a final extension of 72°C for 30 min. Primer pairs that either failed to amplify in either one or both photobiont strains, or produced multiple, spurious bands during PCR were discarded. Primers that worked on both photobionts and provided clear electropherograms were selected, which left 24 loci worth further testing, comprising 13 di-, four tri-, and seven tetranucleotide repeats.

TABLE 1. Characteristics of 20 microsatellite primers developed in *Trebouxia decolorans.*

Locus	Primer sequences (5′–3′)	Repeat motif	Dye	Size range (bp)[a]	T_a (°C)	GenBank accession no.
Tde01	F: *GGTGTCTCCATCAAGCACCT R: TGACTGGCCGAAACATGTAA	$(AAG)_{13}A(AAG)$	FAM	93–114	57	JX437011
Tde02	F: *ACCCTCGGGACTCCATCTAA R: AGCAGGAAAGGACCTGACAA	$(AC)_{11}$	VIC	410–425	57	JX437012
Tde03	F: *CACACGCACTGTCTCATCAA R: GCAAGCATCTGAGCAAACAA	$(ATA)_{14}$	PET	341–385	57	JX437013
Tde04	F: *GGAAGGTTTCAAGGGTTTCC R: AGTGACGCGCTGCTATGAC	$(TG)_{13}$	FAM	125–156	57	JX437014
Tde05	F: *CTTGATGATATCGCCCCTGT R: CTGCAGCACTTCATCACGTT	$(AAT)_8$	VIC	374–401	57	JX437015
Tde06	F: *AATGCCATCGTCATCAAGGT R: CCTGCAGGCATTCAAACATA	$(TGTC)_9$	NED	214–272	57	JX437016
Tde07	F: *TGGACGCATTGCTTGAGTAG R: CTGGGCAGATGTGATCATTG	$(CAGG)_9$	PET	302–345	57	JX437017
Tde08	F: *GTTTCCCTGCAACCCTGTAA R: AAGCAGCTGTTCCTGTCTCC	$(GA)_{12}$	VIC	395–419	57	JX437018
Tde09	F: *CTCATTTGCACAGCCTTTGA R: CCTCTTTGCTTGCCACATTT	$(AC)_{10}$	PET	363–404	57	JX437019
Tde10	F: *GTCTGCAGCCATCACCTGT R: CCTGTGTTTGAACCCCATGT	$(CA)_{11}$	FAM	165–177	57	JX437020
Tde11	F: *AGGCTGCTCAAGGCAAGTAA R: TGCCTGACATTGATCTCTGC	$(TG)_{12}$	VIC	362–371	57	JX437021
Tde12	F: *TTCCATGATGCCAAAGTCAC R: TTGCAGCCATTCACTACGTC	$(GTCT)_8$	NED	208–256	57	JX437022
Tde13	F: *GTGCAGCTTGTCACTGAAGG R: GCGCCCATACACTTTTGAAT	$(TG)_{12}$	PET	313–321	57	JX437023
Tde14	F: *GGTGACAGAGCACAGCAAGA R: CGTTGCAGGTGTGTTTTCAT	$(AC)_{28}$	FAM	266–307	57	JX437024
Tde15	F: *ATGTGTCCACGGACAGACAA R: ACACCTTTGGTCCTGACAGC	$(TG)_{13}$	VIC	359–409	57	JX437025
Tde16	F: *GTTGCTGTGCGAGTGGTGTA R: CCTGGAATGCCATATGTTTGT	$(TTCG)_9$	NED	120–450	57	JX437026
Tde17	F: *TAGGTGGGCCTCTGAACATT R: TCAGTTGTTGCTCCGCTATG	$(AG)_{13}$	PET	304–386	57	JX437027
Tde18	F: *ATCATGCAACGGAATAAACCA R: CCTGCCTTATGTACTGCCTCA	$(TGTA)_{13}$	VIC	226–386	57	JX437028
Tde19	F: *TGTTTGAGTACGGCCAACAG R: GAACACGATGCACAAGGCTA	$(AC)_{12}$	NED	286–290	57	JX437029
Tde20	F: *TGACCTTCCTCACTCCTGTTC R: AGCACCATGAGAACCAAAGG	$(TTC)_8$	PET	304–316	57	JX437030

Note: T_a = annealing temperature when run individually.

[a] Fragment size range based on 43 samples collected from four subpopulations located in Frankfurt, Germany.

* M13 tail (TGTAAAACGACGGCCAGT).

These 24 loci were tested for variability in 43 samples from four subpopulations of photobionts of the lichen *X. parietina* occurring on branches of four trees of *Juglans regia* L. in Frankfurt, Germany (subpopulation 1: 50°9′9.98″N, 8°45′56.38″E, 110 m a.s.l.; subpopulation 2: 50°9′18.90″N, 8°46′45.41″E, 108 m a.s.l.; subpopulation 3: 50°9′14.73″N, 8°46′25.79″E, 112 m a.s.l.; subpopulation 4: 50°9′27.01″N, 8°46′28.50″E, 158 m a.s.l.). We extracted total genomic DNA using the cetyltrimethylammonium bromide (CTAB) method (Cubero and Crespo, 2002). PCR was carried out as described above, using 0.15 µM of either 6FAM, NED, PET, or VIC–M13-labeled primer in each reaction. Cross-species amplification of all microsatellite loci was performed in five other congeneric species: *T. asymmetrica* Friedl & Gärtner, *T. corticola* (Archibald) Gärtner, *T. gigantea* (Hildreth & Ahmadjian) Gärtner, *T. impressa* Ahmadjian, and *T. simplex* Tscherm.-Woess (Appendix 1). The same PCR conditions were used as described above except that an annealing temperature gradient of 50°C to 57°C was used in the first 30 cycles. For all taxa, DNA quality was confirmed by the successful PCR amplification of algal ITS region with ITS1T and ITS4T primers (Kroken and Taylor, 2000). The PCR reactions (25 µL), containing 0.65 U *Ex Taq* polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan), 1× reaction buffer, 100 µM of each dNTP, 0.4 µM of each primer, and 1–5 ng of genomic DNA template, were performed with initial denaturation at 95°C for 4 min, followed by 38 cycles of 95°C for 30 s, 50°C for 40 s, 72°C for 1 min, and final elongation at 72°C for 5 min. For microsatellite testing, PCR products were multiplexed: 0.5 µL of each labeled amplicon were added to 98 µL H_2O and were run on a 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using LIZ-500 as internal size standard. Alleles were sized with Geneious version 5.6 (Drummond et al., 2011). The variability of each microsatellite locus was measured by counting the number of alleles and calculating unbiased haploid diversity using GenAlEx version 6.41 (Peakall and Smouse, 2006).

Cross-species amplification failed in all congeneric species tested, supporting what seems to be a general trend of microsatellite development studies for lichen symbionts, that mycobiont-specific markers have higher intrageneric cross-species transferability than photobiont-specific markers (Dal Grande, 2011; Jones et al., 2012; Dal Grande et al., unpublished data). Four primer pairs did not amplify in the majority of samples tested and were therefore discarded. Twenty loci were polymorphic and consistently amplifiable in all samples of the four subpopulations of *T. decolorans* from the lichen *X. parietina*. Among the 20 microsatellite motifs, 11 were dinucleotide repeats, four were trinucleotide repeats, and five were tetranucleotide repeats. Sequences of the microsatellite loci as they appear in the original sample were deposited in GenBank (Table 1). The microsatellite loci produced three to 15 alleles per locus, and average haploid diversity over loci in four subpopulations varied from 0.636 to 0.821 (Table 2). A total of 36 unique multilocus genotypes were observed in the data set, suggesting that clonal diversity is high in this unicellular alga.

TABLE 2. Results of initial primer screening in four German subpopulations of *Trebouxia decolorans*.

	Subpopulation 1 (N = 10)		Subpopulation 2 (N = 11)		Subpopulation 3 (N = 13)		Subpopulation 4 (N = 9)	
Locus	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*
Tde01	4	0.71	6	0.91	4	0.78	4	0.69
Tde02	5	0.82	5	0.86	5	0.85	3	0.64
Tde03	7	0.91	6	0.89	8	0.92	4	0.81
Tde04	5	0.8	5	0.84	3	0.71	4	0.81
Tde05	5	0.76	7	0.87	5	0.81	2	0.39
Tde06	5	0.76	6	0.86	6	0.86	4	0.58
Tde07	5	0.76	5	0.78	5	0.85	3	0.56
Tde08	4	0.73	4	0.75	4	0.62	3	0.56
Tde09	6	0.89	7	0.89	5	0.76	5	0.86
Tde10	3	0.64	3	0.71	4	0.68	2	0.22
Tde11	2	0.36	4	0.67	4	0.62	2	0.39
Tde12	5	0.8	5	0.82	6	0.83	3	0.75
Tde13	3	0.73	4	0.78	4	0.81	2	0.5
Tde14	8	0.93	9	0.96	8	0.94	5	0.72
Tde15	3	0.64	5	0.86	3	0.72	2	0.5
Tde16	3	0.38	6	0.84	4	0.68	5	0.81
Tde17	5	0.84	5	0.78	7	0.89	4	0.69
Tde18	6	0.78	7	0.91	6	0.83	5	0.72
Tde19	3	0.6	3	0.66	3	0.71	3	0.75
Tde20	4	0.73	4	0.78	3	0.41	4	0.78

Note: *A* = number of alleles; *h* = unbiased haploid diversity; *N* = sample size for each subpopulation.

CONCLUSIONS

This set of novel polymorphic microsatellite markers can provide insights into fine-scale population structure and transmission mode of the common symbiotic alga *T. decolorans*. They are currently being used to analyze clonal diversity and photobiont selectivity in lichen communities with *X. parietina* and *A. ciliaris*.

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Appendix 1. Voucher information for *Trebouxia* species and lichen samples screened for 20 microsatellite loci.

Trebouxia asymmetrica Friedl & Gärtner strain AB99023C2 ex *Fulgensia fulgida* (Botanische Staatssammlung München, lichen voucher 'Rambold 6254/2-7'; ITS Sequence GenBank: AF344175).

Trebouxia corticola (Archibald) Gärtner strain UTEX 909 free-living (ITS Sequence GenBank: AJ249566).

Trebouxia decolorans Ahmadjian strain AB05019B2 ex *Xanthoria parietina* (Botanische Staatssammlung München, lichen voucher M-0102151; ITS Sequence GenBank: JF831923).

Trebouxia gigantea (Hildreth & Ahmadjian) Gärtner strain UTEX 2231 ex *Caloplaca cerina* (ITS Sequence GenBank: AJ249577).

Trebouxia simplex Tscherm.-Woess strain AB97017A2 ex *Lecidea silacea* (Botanische Staatssammlung München, lichen voucher M-0039557; ITS Sequence GenBank: AF128270).

Anaptychia ciliaris (L.) Körb. (Botanische Staatssammlung München, lichen voucher M-0102896; ITS Sequence GenBank: JX444960).

Xanthoria parietina (L.) Th. Fr. (Herbarium Senckenbergianum Frankfurt/Main, FR, Germany): Germany, Hesse, Frankfurt, leg. Dal Grande, Singh, Schmitt, 11 July 2012: subpopulation 1: G0101–G0110, subpopulation 2: G0201–G0211, subpopulation 3: G0301–G0313, subpopulation 4: G0401–G0409.