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CILIATE-SYMBIONT SPECIFICITY OF FRESHWATER ENDOSYMBIOTIC *CHLORELLA* (TREBOUXIOPHYCEAE, CHLOROPHYTA)[1]

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The nature of *Chlorella* symbioses in invertebrates and protists has attracted much interest, but the uncertain taxonomy of the algal partner has constrained a deeper ecological understanding of this symbiosis. We sequenced parts of the nuclear 18S rDNA, the internal transcribed spacer (ITS)-1 region, and the chloroplast 16S rDNA of several *Chlorella* isolated from pelagic ciliate species of different lakes, *Paramecium bursaria* symbionts, and free-living *Chlorella* to elucidate phylogenetic relationships of *Chlorella*-like algae and to assess their host specificity. Sequence analyses resulted in well-resolved phylogenetic trees providing strong statistical support for a homogenous 'zoochlorellae' group of different ciliate species from one lake, but clearly different *Chlorella* in one of those ciliate species occurring in another lake. The two *Chlorella* strains isolated from the same ciliate species, but from lakes having a 10-fold difference in underwater UV transparency, also presented a distinct physiological trait, such as the ability to synthesize UV-absorbing substances known as mycosporine-like amino acids (MAAs). Algal symbionts of all *P. bursaria* strains of different origin resolved in one clade apart from the other ciliate symbionts but split into two distinct lineages, suggesting the existence of a biogeographic pattern. Overall, our results suggest a high degree of species specificity but also hint at the importance of physiological adaptation in symbiotic *Chlorella*.

Key index words: **ciliates; mixotrophy; mycosporine-like amino acids; *Paramecium;* phylogeny; symbiosis**

Abbreviations: **BI, Bayesian inference; GKS, Gossenköllesee; MAAs, mycosporine-like amino acids; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining; PIB, Piburger See**

Mutualistic symbioses are important ecological relationships that are generally defined as two or more species living together and providing benefit to each other (Margulis and Fester 1992, Douglas 1995). Several microalgal endosymbioses involving flatworms, cnidaria, and various protists contribute globally to the primary productivity of aquatic ecosystems and are important members of the trophic food web (Caron and Swanberg 1990). In particular, algal-bearing ciliates are a ubiquitous and abundant component of pelagic food webs in oceanic and freshwater systems (Dolan and Pérez 2000, Sonntag et al. 2006) of different trophic interactions (Berninger et al. 1986, Bienert et al. 1991). The mixotrophic nutrition mode of algal-bearing ciliates, combining both phagotrophy and phototrophy, is considered to be an adaptation allowing exploitation of oligotrophic environments (Dolan and Pérez 2000).

Most of our knowledge about the diversity of endosymbiotic algae is related to marine dinoflagellates in the genus *Symbiodinium*. These so-called zooxanthellae, were assumed to belong to a single species, *Symbiodinium microadriaticum* (Taylor 1974). However, infectivity, ultrastructural, behavioral, and more recently, molecular studies on cultured and freshly isolated algae have demonstrated that 'zooxanthellae' comprise a heterogeneous group of many species and strains (Rowan and Powers 1991, Baker 2003, Pochon et al. 2006). By contrast, little is known about the diversity of the freshwater counterparts, the so-called zoochlorellae, despite that the nature of *Chlorella* symbioses in invertebrates and protists has attracted scientific interest over the past decades (Karakashian and Karakashian 1973, Reisser and Häder 1984). For example, Schulze (1951) in investigating the 'zoochlorellae' of *P. bursaria* and other ciliates described several differences, particularly in their ability to infect the host. In *P. bursaria*, symbiosis only occurs with *Chlorella* species despite that other algae are efficiently ingested (Karakashian and Karakashian 1973).

Two factors have mainly hindered more detailed studies on the nature of *Chlorella* symbioses, namely, the impossibility of identifying species of *Chlorella*-like algae that are morphologically very similar and the difficulty of establishing aposymbiotic algal cultures (Huss et al. 1993). Indeed, molecular phylogenetic analyses of endosymbiotic *Chlorella* in ciliates are only available for *P. bursaria* (Huss et al. 1989, Nishihara et al. 1998, Hoshina et al. 2005). However, this species is rarely dominant in natural freshwater lakes, and consequently, information on

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endosymbiotic algal diversity of other 'zoochlorellate' ciliates is not available.

In this study, we performed phylogenetic analyses based on the partial nuclear 18S rDNA, the ITS-1 region, and the chloroplast 16S rDNA of symbiotic and free-living *Chlorella*-like algae and tested whether there were different algal symbionts among ciliate species from one freshwater habitat and if the same single ciliate species from different lakes harbored the same *Chlorella*. In the latter case, we also assessed a physiological trait of the symbiosis, such as the presence of UV-absorbing substances known as MAAs. Overall, these analyses indicate a high degree of host-specificity in *Chlorella* but also suggest the importance of physiological adaptation of the ciliate-*Chlorella* symbiosis to a key environmental factor, such as UV radiation.

MATERIALS AND METHODS

Sources of Chlorella*-bearing ciliates.* We selected two mountain lakes located at different altitudes and differing 10-fold in their UV transparency (Laurion et al. 2000). Gossenköllesee (GKS) is located in the Central Alps (47°13′N, 11°01′E; 2,417 m above sea level). The lake is ice covered from November through June–July. The second study site was the oligo-mesotrophic Piburger See (PIB), also located in the Central Alps (47°11′N, 10°53′E; 913 m above sea level). The ice cover in this lake lasts from December through April. Information about these lakes, including UV transparency, can be found elsewhere (Laurion et al. 2000).

Ciliate collection, species identification, and isolation. Samples were collected in both lakes during the ice-free season in the upper 6 m of the water column by vertical hauls with a plankton net of 10 μm mesh size (Uwitec, Mondsee, Austria). Ciliates were identified at the species level from live and protargol-stained (Pfister et al. 1999 and references therein) samples following the taxonomic keys of Foissner et al. (1999) under an Olympus microscope (model BX50, Vienna, Austria). *Chlorella*-bearing ciliates were isolated from freshwater samples with a fine glass pipette. Each individual ciliate was consecutively transferred at least five times to drops of sterile-filtered lake water (0.2 μm pore size) to minimize contamination by phytoplankton and microscopically checked for contamination (Sonntag et al. 2007).

Isolation of algal symbionts. We isolated in hospite *Chlorella* from the most dominant ciliate species in terms of abundance/biomass found in those lakes (Table S1 in the supplementary material). For isolation, single ciliate individuals were pipetted into microtiter-wells and starved for some days in sterile Woods Hole MBL medium (Guillard and Lorenzen 1972) to avoid possible contamination from ingested algae. Then, single ciliates were washed, transferred into sterile microcentrifugation tubes, and gently sonicated for 20 s at 7 W (Bandelin Sonoplus HD 2070; Bandelin Electronic, Berlin, Germany). Symbiotic algae were first washed with sterile Woods Hole MBL medium through several centrifugation steps to exclude the rest of the host cell and then transferred into liquid Woods Hole MBL medium for cultivation. Successfully growing algal cultures were plated onto the same medium plus agar for further purification. To obtain axenic *Chlorella* cultures, Rifampicin (Sigma-Aldrich, Vienna, Austria) was added to a final concentration of 100 $mg \cdot L^{-1}$ to prevent bacterial growth. After some purification steps, algal microcolonies were selected and inoculated into sterile Woods Hole MBL medium for mass culture. In this way, aposymbiotic *Chlorella* cultures of *Askenasia chlorelligera* from GKS as well as of six pelagic ciliate species isolated from PIB, including *A. chlorelligera* were established (Table S1). We also established clonal cultures of *Chlorella* isolated from two *P. bursaria* strains, including one originated from PIB (strain PbPIB). Cultures were regularly controlled microscopically for axenic conditions.

Cultivation of ciliates and Chlorella. All *P. bursaria* strains were permanently cultured in Woods Hole MBL medium in addition to 30 $mg \cdot L^{-1}$ yeast extract. *Uroleptus* sp., *Stentor polymorphus*, and *Stokesia vernalis* were kept in culture for several months in Woods Hole MBL medium on a *Cryptomonas* sp. diet. We were not able to establish cultures of ciliates, except for those species mentioned above, but we kept them in the laboratory for some days to weeks in the original sample or in sterile-filtered lake water. Cultivation of ciliates and symbiotic algae was performed in light/temperature controlled growth chambers under a 16:8 light:dark (L:D) photoperiod and at 17°C–20°C. PAR was provided by five cool-white lamps (L36/W20; Osram, Vienna, Austria) delivering 180 μmol quanta $\cdot m^{-2} \cdot s^{-1}$.

DNA extraction and sequencing. DNA sequences were obtained from 12 symbiotic ciliate *Chlorella* strains and three free-living *Chlorella* species (Table S1). For DNA extraction, amplification, and sequencing, 30 mL of dense *Chlorella* cultures ($\approx 3 \times 10^7$ cells $\cdot mL^{-1}$) was harvested from liquid cultures by centrifugation. Then, the algal pellet was frozen in liquid nitrogen. Total DNA was extracted using a commercial DNA extraction kit (DNeasy® Plant Mini Kit; Qiagen, Vienna, Austria) according to the manufacturer's instructions, with the following modifications: 600 μL cold P1-buffer and 20 μL RNaseA (20 $mg \cdot mL^{-1}$) were added to the frozen algal pellet, and the mixture was sonicated on ice for 3 min at 42 W to ensure the rupture of the algal cell walls. After this step, we followed the standard extraction protocol. We amplified both strands of a nuclear small subunit (18S) rRNA gene segment and a chloroplast small subunit (16S) rRNA gene using the NS1/NS2 and CS1/CS2 primers reported by Wu et al. (2001), respectively, and the ITS-1 of the rDNA using the following primers cited in Huss et al. (2002): 5′-GGAGAAGTCGTAACAAGGTTTCCG-3′ and 5′-ATCCTGCAATTCACACCAAGTATCG-3′. Amplification by PCR was performed on a Techne PHC-3 Thermal Cycler (Staffordshire, UK). An initial denaturation step of 94°C for 5 min was followed by 35 cycles under the following conditions: 45 s denaturation at 94°C, 45 s annealing at 58°C for 18S (at 46°C for 16S, 45 s at 53°C for ITS-1), and 60 s extension at 72°C, followed by a final extension step of 72°C for 5 min. To check the density of amplified DNA fragments, 2 μL aliquots of the PCR products were separated on a 1% Tris acetate EDTA (TAE)–agarose gel (Sigma-Aldrich) in TAE buffer (Sigma-Aldrich) and stained with ethidium bromide (Invitrogen, Lofer, Austria). The amplification products were purified with ExoSAP-IT® (USB Corporation, Staufen, Germany) prior to direct sequencing with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit using an ABI 373A and ABI 3100 DNA sequencer (Applied Biosystems, Brunn am Gebirge, Austria), according to the manufacturer's instructions.

Phylogenetic analysis of Chlorella. DNA sequences were individually checked by eye; alignment of sequences was done using CLUSTALX (Thompson et al. 1994) and was improved by eye for the ITS-1 region. For phylogenetic inference, maximum-parsimony (MP; Farris 1970), neighbor-joining (NJ; Saitou and Nei 1987), and maximum-likelihood (ML; Felsenstein 1985) analyses were performed using PAUP*4.0b10 (Swofford 2002). Three data sets of sequences were analyzed. The first set comprised 558 bp gene segments of the 18S rDNA of 29 Trebouxiophyceae taxa; the second set was the same 18S rDNA segment combined with a 412 bp gene segment

including the ITS-1 region (total 970 bp) of 19 *Chlorella* taxa. The third data set comprised 424 bp of the chloroplast 16S rDNA of 15 *Chlorella* taxa. All MP trees were obtained via stepwise addition and then swapped using the tree-bisection-reconnection (TBR) algorithm. Sequences were unordered, characters equally weighted, and gaps were considered as a fifth base. MODELTEST 3.06 (Posada and Crandall 1998) was run to determine the appropriate model of molecular evolution for ML and NJ in a likelihood ratio test framework. ML analyses were performed with a heuristic search and random addition of sequences as implemented in PAUP*, with a starting tree obtained via stepwise addition of taxa, and then swapped using the TBR algorithm. For Bayesian inference (BI), we used the program MRBAYES V.3.0b4 (Huelsenbeck and Ronquist 2001). Four Monte Carlo Markov chains were run for 2,000,000 generations, sampling every 100 generations; the initial 25% of trees were discarded. The model of evolution used in Bayesian analysis was the symmetrical model plus gamma (SYM + G; Zharkikh 1994). Nodal support in MP, NJ, and ML was evaluated by 1,000 bootstrap pseudo-replications in PAUP* and by calculating posterior probabilities by preparing a 50% majority rule of the resulting trees in the Bayesian analysis. For the chloroplast 16S segment, a minimum spanning network was additionally performed using the program Network3.exe (Bandelt et al. 1999). Of the total 558 nucleotide positions in the 18S rDNA, 50 were variable and 18 were parsimony informative, excluding *Muriella terrestris*, which was used as outgroup taxon. MP analysis resulted in four most-parsimonious trees with the following scores: tree length = 60, consistency index (CI) = 0.852, retention index (RI) = 0. 868, and rescaled consistency index (RC) = 0.740. The SYM + G model (Zharkikh 1994) was selected by MODELTEST as the best-fitting evolutionary model with the following likelihood parameters: substitution rate matrix *R*(a) [A–C] = 0.8494, *R*(b) [A–G] = 1.2579, *R*(c) [A–T] = 0.8436, *R*(d) [C–G] = 1.2580, *R*(e) [C–T] = 3.0363, *R*(f) [G–T] = 1.0000; gamma distribution with the shape parameter (alpha) = 0.1406 with equal nucleotide frequencies. In the combined data set (18S 558 bp + ITS-1 412 bp), the sequence of the free-living *C. saccharophila* SAG 211-1a was defined as outgroup. MP analysis of 172 parsimony informative characters resulted in one most-parsimonious tree with a length of 387, a CI of 0.866, an RI of 0.933, and an RC of 0.808. MODELTEST proposed SYM + G (Zharkikh 1994) as the best-fitting evolutionary model with the following likelihood parameters: substitution rate matrix *R*(a) [A–C] = 0.8494, *R*(b) [A–G] = 1.2579, *R*(c) [A–T] = 0.8436, *R*(d) [C–G] = 1.2580, *R*(e) [C–T] = 3.0363, *R*(f) [G–T] = 1.0000; gamma distribution with the shape parameter (alpha) = 0.1406 and equal base frequencies. For the chloroplast 16S rRNA gene, the Hasegawa–Kishino–Yano plus gamma model (HKY + G; Hasegawa et al. 1985) was selected by MODELTEST as the best-fitting model, with a gamma shape correction of 0.0147; a transition/transversion ratio of 2.7917; and base frequencies of A = 0.2667, C = 0.1982, G = 0.3152, and T = 0.2199. The number of parsimony informative sites was three. The DNA sequences are available from GenBank under the accession numbers specified in Table S1.

MAAs. Analyses of MAAs in *A. chlorelligera* isolated from PIB and GKS were done by HPLC in aqueous methanol extractions as described elsewhere (Sommaruga et al. 2006, Sonntag et al. 2007).

RESULTS

Only one *Chlorella* genotype was detected simultaneously in single ciliate populations. This holds true for *P. bursaria* (strains KM2, PbW, and PbPIB), but also for extracts of at least twice independently established aposymbiotic *Chlorella* cultures from different planktonic ciliates.

The 18S rDNA sequence analyses resulted in a phylogenetic tree with identical *Chlorella* symbionts of the six pelagic ciliate species from PIB at the top (Fig. 1). This result is perhaps not surprising, because the analyzed first part of the 18S rDNA is slowly evolving. However, the sequence from *Chlorella* of *A. chlorelligera* isolated from GKS was different. *Chlorella* of all *P. bursaria* strains isolated from different regions of the world shared almost identical sequences. They clustered near the free-living *Chlorella* sp. Itas 2/24S-11w, *C. sorokiniana* Praha A14, *Chlorella* sp. FACHB31, *C. vulgaris* SAG 211-11b, and '*C. minutissima* CCAP' 211/52 (Fig. 1). However, the latter is apparently misclassified,

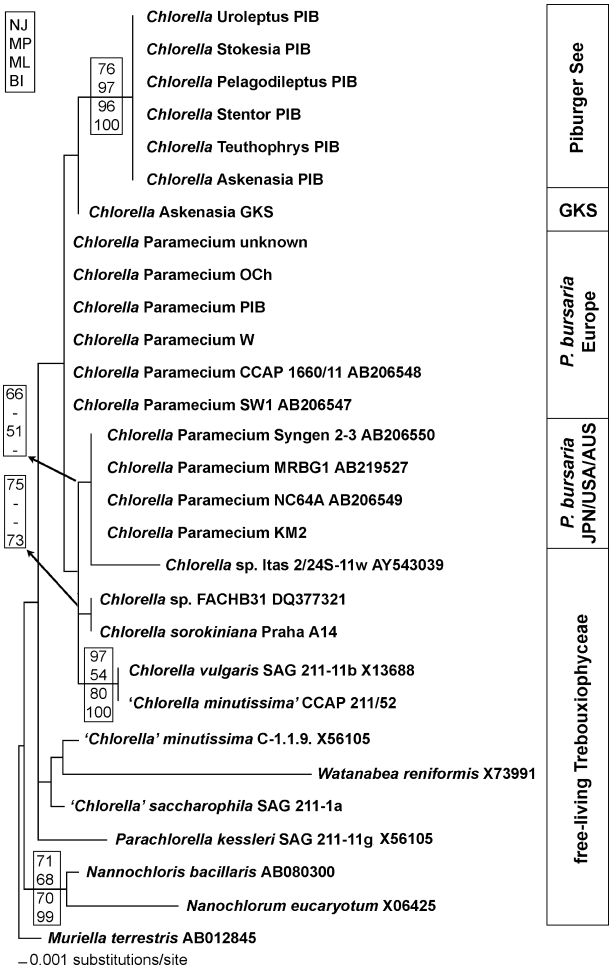

FIG. 1. Phylogenetic reconstruction of 29 Trebouxiophyceae taxa inferred from 558 bp of the 18S rRNA gene sequences. Neighbor-joining (NJ) phylogram of 17 symbiotic and seven free-living *Chlorella* taxa, four other Trebouxiophyceae taxa, and *Muriella terrestris* used as outgroup. The four numbers at nodes correspond from top to bottom to bootstrap values >50 in NJ (1,000 replicates), MP (1,000 replicates), and ML (1,000 replicates) analyses, and posterior probabilities >50% in Bayesian inference (BI) analysis. MP, maximum parsimony; ML, maximum likelihood.

because its 18S DNA sequence has been determined to be identical to that of *C. vulgaris* SAG 211-11b (Hepperle and Schlegel 2002).

Analysis of the combined (i.e., 18S and ITS rDNA) data set (Fig. 2) resulted in a better resolution and indicated that the *Chlorella* symbionts of the ciliates from PIB were closely related, although their sequences were not identical (mean pair-wise distances: 0.565%). The position of the *A. chlorelligera* symbionts from GKS was different, but next to those from the symbionts isolated from PIB and apart from the *P. bursaria* symbionts. The latter split into two clusters, with all the ''European'' strains in the first group and the ''Japanese'' strain KM2 together with the Australian strain MRBG1 and the U.S. strain NC64A in the second group (mean pair-wise distances first cluster: 0.114%; mean pair-wise distances second cluster: 0.282%; mean pair-wise distances all *P. bursaria*: 3.771%). Phylogenetic analyses of the ITS-1 region alone (data not shown) resulted in the same tree topologies.

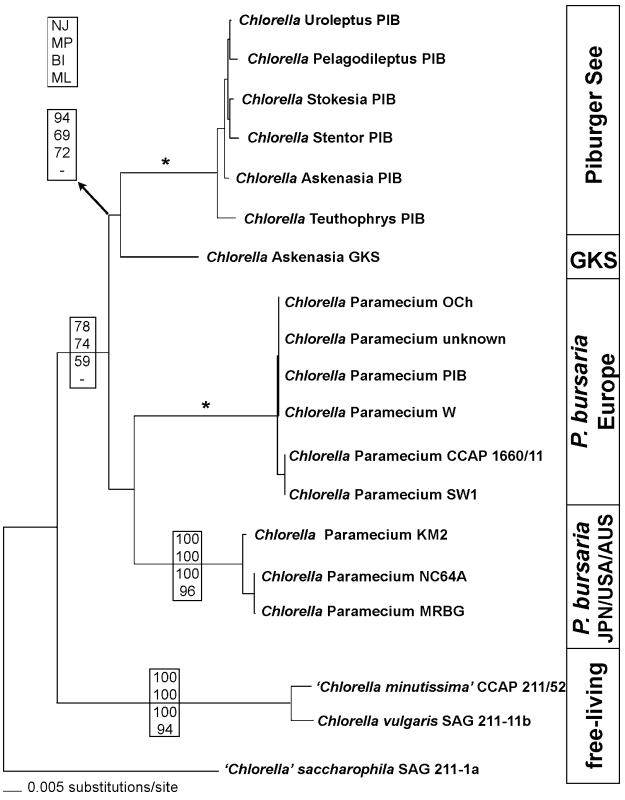

FIG. 2. Maximum-likelihood (ML) phylogram of 19 *Chlorella* taxa from a combined data set of 558 bp of the 18S rRNA gene and 412 bp including the ITS-1 region. The tree was obtained with the SYM + G evolutionary model as explained in the Materials and Methods section. The three numbers at nodes correspond from top to bottom to bootstrap values >50 in NJ (1,000 replicates) and MP (1,000 replicates) analyses, and posterior probabilities >50% in Bayesian inference (BI) analyses. Nodes with bootstrap support of 100 in all analyses are indicated with an asterisk. MP, maximum parsimony; NJ, neighbor joining; ITS, internal transcribed spacer.

Due to very little genetic variation in the chloroplast 16S rDNA data set, a minimum spanning network (Network3.exe, Bandelt et al. 1999) was performed in addition to MP, NJ, ML, and BI analyses (Fig. 3). The chloroplast gene segment of all five investigated *P. bursaria* symbionts was identical, and the free-living *C. sorokiniana* Praha A14 shared the same sequence. All six sequenced symbionts of pelagic ciliates from PIB had identical partial 16S sequences. *Chlorella* of *A. chlorelligera* from GKS was placed in the network between the *P. bursaria* group including *C. sorokiniana* Praha A14 and the PIB group with only one mutational step in each direction.

The HPLC analysis of MAAs revealed the presence of seven substances in *A. chlorelligera* from GKS (Fig. 4A), but none in the same ciliate species from PIB (Fig. 4B).

DISCUSSION

Some 125 years after Brandt introduced the term 'zoochlorella' to describe the symbiotic algae in freshwater hosts, including hydras, sponges, and protists (Sapp 1994), surprisingly little is known about the origin and diversity of these symbionts. In this study, we identified several phylogenetic types of in hospite *Chlorella* among different symbiotic ciliate species (Figs. 1–3).

Host specificity. Our results clearly showed the occurrence of different *Chlorella* symbionts in different hosts. Moreover, we never detected more than one *Chlorella* genotype simultaneously in a single host population. This was verified by several independent DNA extractions from aposymbiotic algal cultures and from symbiotic ciliate cultures, which always resulted in identical DNA sequences. Certainly, sequences of a minimal fraction of different symbionts could have remained undetected due to insufficient replication numbers in PCR in our study. However, our results are in agreement with those reported by Lewis and Muller-Parker (2004), where in five pooled sea anemones only one type of 'zoochlorellae' was detected. Studies on marine *Symbiodinium* symbioses, however, have shown that a given host can harbor more than one genotype simultaneously (Santos et al. 2004).

Species-specific infection in the case of *P. bursaria* has been proposed in the literature (Schulze 1951, Karakashian and Karakashian 1973). In our study, the only ciliate in PIB, whose symbiont strain was largely different from the other six investigated taxa in the lake was *P. bursaria*. The *Chlorella* sequence from this ciliate grouped within the sequences of all other *Paramecium* symbionts isolated from various habitats around the world (Table S1 and Fig. 2), indicating a strong species-specificity in *Paramecium-Chlorella* symbiosis. This specificity has also been confirmed by several reinfection experiments (Summerer et al. 2007).

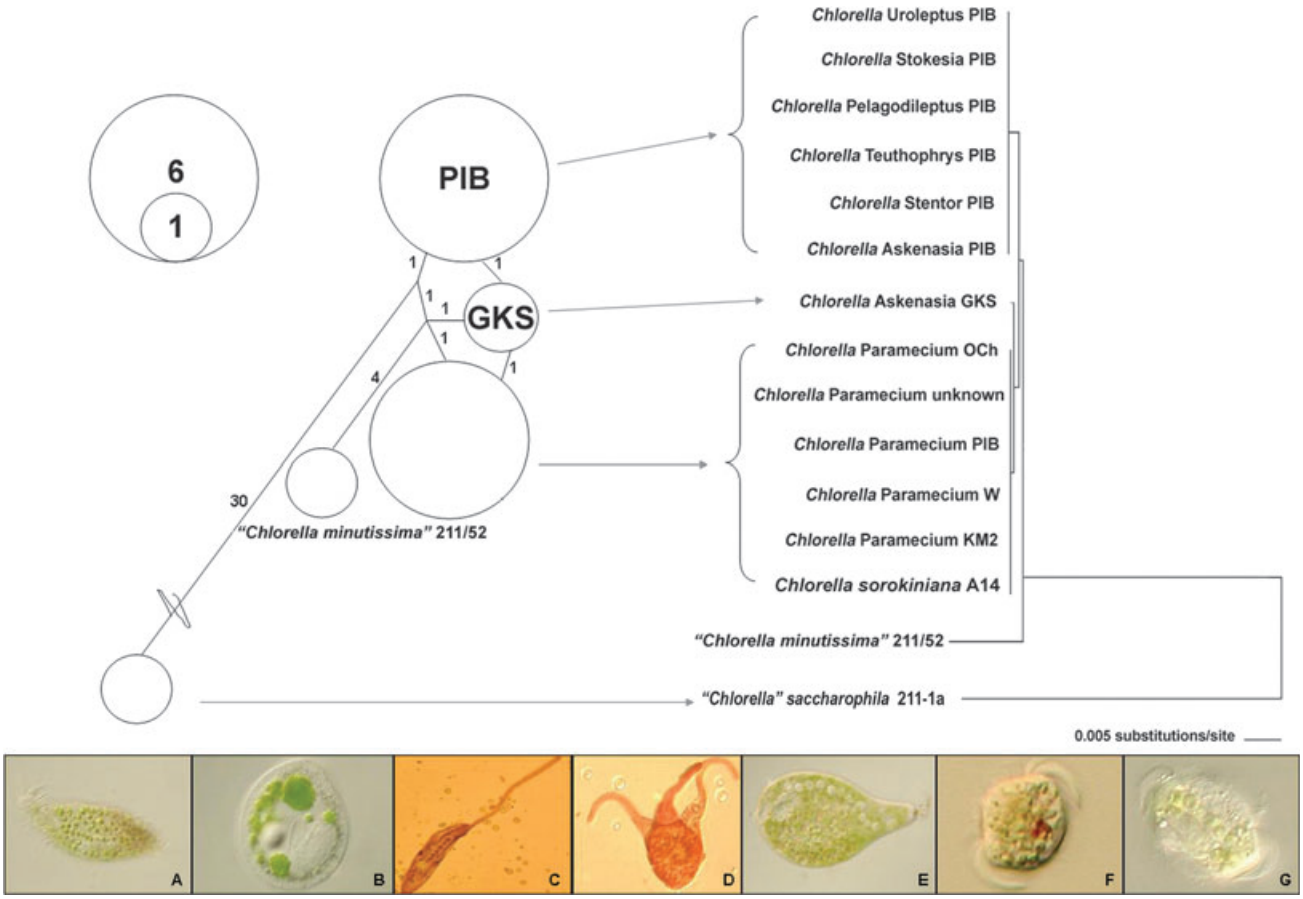

FIG. 3. Minimum spanning network (left) in combination with a neighbor-joining (NJ) phylogram (right) of 15 *Chlorella* taxa calculated from a 424 bp chloroplast 16S rRNA gene segment. The NJ tree was calculated according to the HKY + G model (see Materials and Methods). In the minimum spanning network, identical 16S rDNA sequences are represented by a circle whose size is proportional to the number of taxa showing that sequence (big circle, six taxa; small circle, one taxon); numbers on each line indicate the mutational steps. Note that the sequences of the pelagic Piburger See (PIB) taxa are identical, as well as those of the *Paramecium bursaria* symbionts (including *Chlorella sorokiniana*). *Chlorella* of *Askenasia chlorelligera* from Gossenköllesee (GKS) lies between the two clusters with only one mutational step in each direction. Micrographs of live individuals of *Uroleptus* sp. (A), *Stokesia vernalis* (B), *Stentor polymorphus* (E), *A. chlorelligera* PIB (F), *A. chlorelligera* GKS (G), and protargol-stained individuals of *Pelagodileptus trachelioides* (C) and *Teuthophrys trisulca trisulca* (D).

When considering species specificity in mutualistic symbioses, it is important to distinguish between host specificity (symbiont-specific host) and symbiont specificity (host-specific symbiont). In general, hosts appear to be more specific than symbionts (Baker 2003 and references therein). Taking into account genetic consistencies and distances of symbionts, and the congruency of host-symbiont relationships, Hoshina et al. (2005) concluded that the types of *Chlorella* symbionts depend on their *Paramecium* hosts' lineages. Margulis and Chapman (1998) differentiated between cyclical and permanent symbiosis. In cyclical or open associations, the symbiosis has to be reestablished at some stage of each generation; hence, they are much more sensitive to environmental conditions than permanent symbioses. In this regard, ciliate-*Chlorella* symbioses, at least those of *P. bursaria,* appear as permanent associations with hereditary symbionts (Siegel 1960). By contrast, associations between reef-building corals and *Symbiodinium* are typical and well-studied examples for cyclical symbioses (Margulis and Chapman 1998 and references therein). Yet, *Symbiodinium*-symbioses also show specificity and nonrandom patterns of association (Baker 2003). Garcia-Cuetos et al. (2006) suggested that the strong host-symbiont specificity observed in Soritinae (foraminiferans) is a combined effect of biogeographical isolation, a selective recognition mechanism, and vertical transmission of symbionts. The latter two mechanisms apply equally to *P. bursaria* symbioses. In mutualistic *Chlorella* associations, acid tolerance seems to be correlated with their symbiotic capabilities. Thus, in contrast to nonsymbiotic taxa, symbiotic *Chlorella* species are able to grow at or below pH 3.5 (Huss et al. 1993 and references therein). Moreover, Stabell et al. (2002) reported that the host:symbiont ratio might be regulated by pH in perialgal vacuoles. The more food-limited and, therefore, slowly growing the host is, the lower the pH value becomes, and the more

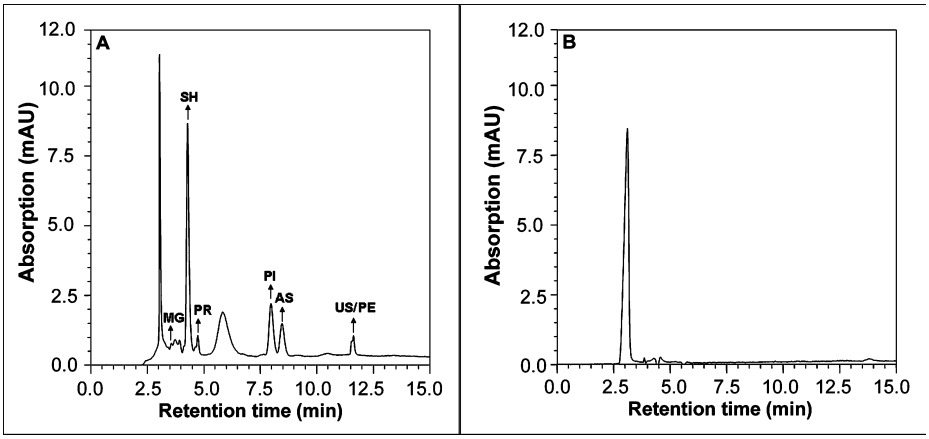

FIG. 4. HPLC-chromatograms (detection at 320 nm) of an aqueous methanolic extract of *Askenasia chlorelligera* from Gossenköllesee (A) and from Piburger See (B). Mycosporine-like amino acids detected were mycosporine-glycine (MG), shinorine (SH), porphyra-334 (PR), palythine (PI), asterina-330 (AS), usujirene (US), and palythene (PE).

photosynthetically fixed carbon is transported to the host until algal net growth is almost stopped completely (Stabell et al. 2002).

One important result of our study was that there were differences among symbiont populations of the same host species from different lakes/regions. In our data set based on the combined 18S and ITS rDNA, all *P. bursaria* 'zoochlorellae' were merged into a monophyletic clade, but within this clade, they split into two distinct lineages (Fig. 2). Kvitko et al. (2001) proposed one *Chlorella paramecii* group subdivided into two ecotypes differing by their sensitivity to elevated temperature, surface antigens, and sensitivity to two types of viruses. The two *Paramecium-Chlorella* groups in our study were congruent with the so-called northern and southern ecotypes proposed by Kvitko et al. (2001). Thus, the 'zoochlorellae' of all European *Paramecium* strains (Table S1) would fall within the ''northern'' ecotype, and the U.S. (NC64A, Syngen 2-3), Japanese (KM2), and Australian (MRBG1) *Paramecium* symbionts would represent the ''southern'' type.

Over the past years, there have been intensive discussions on the biogeography of microorganisms. For example, Finlay (2002) has claimed that there is no biogeography (the ''everything is everywhere'' hypothesis) for free-living species smaller than 2 mm in size. He proposed that 'protozoa' were sufficiently abundant to have worldwide distribution and that consequently there existed no endemic species and no biogeography at all (Finlay 2002). By contrast, a large body of research supports the idea that free-living microorganisms exhibit biogeographical patterns (Fierer and Jackson 2006, Hughes-Martiny et al. 2006). Our data on *Chlorella* symbionts from *P. bursaria* also support the existence of a biogeographical pattern. Moreover, biogeographic trends have been observed among dinoflagellate symbionts of reef-building corals (Baker 2003, LaJeunesse et al. 2003, 2004) and foraminiferans (Garcia-Cuetos et al. 2006).

*Physiological adaptation of the ciliate-*Chlorella *symbiosis.* Another interesting result of our study was that the DNA-sequences of symbionts isolated from the same single ciliate species (i.e., *A. chlorelligera*) but isolated from two different lakes indicated different strains of *Chlorella.* Moreover, the *Askenasia* symbionts from PIB were closer related to the 'zoochlorellae' of the other five pelagic ciliate species in the lake than to the symbionts of *Askenasia* in GKS. We hypothesize that this divergence could be related to the adaptation of this symbiosis to different environmental conditions prevailing in these two lakes. Although it is difficult to pinpoint one single environmental factor, alpine lakes such as GKS are ecosystems generally characterized by some of the highest UV exposure conditions of any freshwater system. This trend is because incident UV fluxes increase with altitude and underwater UV transparency is high due to the low concentration of chromophoric or colored dissolved organic matter (Sommaruga 2001). Indeed, GKS is 10 times more UV transparent than PIB (Laurion et al. 2000).

Our understanding of the interaction between solar UV radiation and mutualistic associations is mainly based on studies in algal-invertebrate symbiosis, particularly in corals and their 'zooxanthellae' of the genus *Symbiodinium* (Shick and Dunlap 2002). In those mutualistic associations, UV protection is an advantage provided by the symbionts through the synthesis of intracellular water-soluble UV-absorbing substances known as MAAs. These natural sunscreen substances protect important cell targets from harmful effects of UV radiation and increase the overall resistance of phototrophic and heterotrophic organisms (Shick and Dunlap 2002). The role of MAAs in other mutualistic associations is poorly known, but these substances have been reported for the marine heterotrich ciliate *Maristentor dinoferus* hosting *Symbiodinium* symbionts (Sommaruga et al. 2006) and, recently, for several freshwater

Chlorella-bearing ciliates (Sonntag et al. 2007). The latter study provides the first evidence for the symbiotic origin of MAAs in ciliates. The positive detection of MAAs in the *Askenasia-Chlorella* symbiosis from the UV-transparent alpine lake GKS, but not in that from the less UV-transparent PIB (Fig. 4) suggests a physiological adaptation of this symbiosis to an extreme environment. The existence in *Chlorella*-bearing ciliates of UV protecting substances is an important strategy that probably determined their abilities to thrive in sunlit UV-exposed waters.

In summary, species-specific symbioses, which may benefit from long-term optimized adaptations of both partners, seem to be common, as can be seen in the case of *P. bursaria*. Nevertheless, adaptation of the symbiosis to different environmental conditions, such as UV radiation, but probably also to other factors, like viral pressure on the algae (Van Etten et al. 1982), are involved in the selection of symbiotic partners. Our results raise several interesting issues for further research, among them, whether the appearance of *Chlorella* living as symbionts was a single event or occurred several times independently. Our data are in line with previous studies presuming several infection events, but perhaps within lineages of algae that are predisposed for symbiosis (Douglas and Huss 1986, Huss et al. 1993). Moreover, which strains of *Chlorella* are abundant in different aquatic environments and, therefore, which are actually available for symbiosis needs to be assessed.

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Baker, A. C. 2003. Flexibility and specificity in coral-algal symbiosis. Diversity, ecology, and biogeography of *Symbiodinium*. *Annu. Rev. Ecol. Evol. Syst.* 34:661–89.

Bandelt, H. J., Forster, P. & Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16:37–48.

Berninger, U. G., Finlay, B. J. & Canter, H. M. 1986. The spatial distribution and ecology of zoochlorellae-bearing ciliates in a productive pond. *J. Protozool.* 33:557–63.

Bienert, R. W., Beaver, J. R. & Crisman, T. L. 1991. The contribution of zooplankton biomass in an acidic, subtropical lake. *J. Protozool.* 38:352–4.

Caron, D. A. & Swanberg, N. R. 1990. The ecology of planktonic sarcodines. *Rev. Aquat. Sci.* 3:147–80.

Dolan, J. R. & Pérez, M. T. 2000. Costs, benefits and characteristics of mixotrophy in marine oligotrichs. *Freshw. Biol.* 45:227–38.

Douglas, A. E. 1995. The ecology of symbiotic microorganisms. *Adv. Ecol. Res.* 26:69–103.

Douglas, A. E. & Huss, V. A. R. 1986. On the characteristics and taxonomic position of symbiotic *Chlorella*. *Arch. Microbiol.* 145:80–4.

Farris, J. S. 1970. Methods for computing Wagner Trees. *Syst. Zool.* 19:83–92.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–91.

Fierer, N. & Jackson, R. B. 2006. The diversity and biogeography of soil bacterial communities. *Proc. Natl. Acad. Sci. U. S. A.* 103:626–31.

Finlay, B. J. 2002. Global dispersal of free-living microbial eukaryote species. *Science* 296:1061–3.

Foissner, W., Berger, H. & Schaumburg, J. 1999. *Identification and Ecology of Limnetic Plankton Ciliates*. Informationsbericht 3/99 Bayerisches Landesamt für Wasserwirtschaft, Munich, Germany, 793 pp.

Garcia-Cuetos, L., Pochon, X. & Pawlowski, J. 2006. Molecular evidence for host-symbiont specificity in Soritid Foraminifera. *Protist* 156:399–412.

Guillard, R. R. L. & Lorenzen, C. J. 1972. Yellow-green algae with chlorophyllide c. *J. Phycol.* 8:10–4.

Hasegawa, M., Kishino, H. & Yano, T. 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 22:160–74.

Hepperle, D. & Schlegel, I. 2002. Molecular diversity of eucaryotic picoalgae from three lakes in Switzerland. *Int. Rev. Hydrobiol.* 87:1–10.

Hoshina, R., Kato, Y., Kamako, S. & Imamura, N. 2005. Genetic evidence of ''American'' and ''European'' type symbiotic algae of *Paramecium bursaria* Ehrenberg. *Plant Biol.* 7:526–32.

Huelsenbeck, J. P. & Ronquist, F. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–5.

Hughes-Martiny, J. B., Bohannan, B. J. M., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L., Horner-Devine, M. C., et al. 2006. Microbial biogeography: putting microorganisms on the map. *Nat. Rev. Microbiol.* 4:102–12.

Huss, V. A. R., Ciniglia, C., Cennamo, P., Cozzolino, S., Pinto, G. & Pollio, A. 2002. Phylogenetic relationships and taxonomic position of *Chlorella*-like isolates from low pH environments (pH < 3.0). *BMC Evol. Biol.* 2:1–3.

Huss, V. A. R., Holweg, C., Seidel, B., Reich, V., Rahat, M. & Kessler, E. 1993. There is an ecological basis for host/symbiont specificity in *Chlorella/Hydra* symbioses. *Endocytobiosis Cell Res.* 10:35–46.

Huss, V. A. R., Huss, G. & Kessler, E. 1989. Deoxyribonucleic acid reassociation and interspecies relationships of the genus *Chlorella* (Chlorophyceae). *Plant Syst. Evol.* 168:71–82.

Karakashian, M. W. & Karakashian, S. J. 1973. Intracellular digestion and symbiosis in *Paramecium bursaria*. *Exp. Cell Res.* 81:111–9.

Kvitko, K. V., Migunova, A. V., Karelov, D. V. & Prokosheva, M. J. 2001. Molecular taxonomy of virus-sensitive *Chlorella* sp. – symbionts of *Paramecium bursaria*. *Protistology* 2:96–104.

LaJeunesse, T. C., Bhagooli, R., Hidaka, M., Done, T., deVantier, L., Schmidt, G. W., Fitt, W. K. & Hoegh-Guldberg, O. 2004. Closely-related *Symbiodinium* spp. differ in relative dominance within coral reef host communities across environmental, latitudinal, and biogeographic gradients. *Mar. Ecol. Prog. Ser.* 284:147–61.

LaJeunesse, T. C., Loh, W. K. W., van Woesik, R., Hoegh-Guldberg, O., Schmidt, G. W. & Fitt, W. K. 2003. Low symbiont diversity in southern Great Barrier Reef corals, relative to those of the Caribbean. *Limnol. Oceanogr.* 48:2046–54.

Laurion, I., Ventura, M., Catalan, J., Psenner, R. & Sommaruga, R. 2000. Attenuation of ultraviolet radiation in mountain lakes: factors controlling the among- and within-lake variability. *Limnol. Oceanogr.* 45:1274–88.

Lewis, L. A. & Muller-Parker, G. 2004. Phylogenetic placement of ''zoochlorellae'' (Chlorophyta), algal symbiont of the temperate sea anemone *Anthopleura elegantissima*. *Biol. Bull.* 207:87–92.

Margulis, L. & Chapman, J. 1998. Endosymbioses: cyclical and permanent in evolution. *Trends Microbiol.* 6:342–5.

Margulis, L. & Fester, R. [Eds.] 1992. *Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis*. MIT Press, Cambridge, Massachusetts, 454 pp.

Nishihara, N., Horiike, S., Takahashi, T., Kosaka, T., Shigenaka, Y. & Hosoya, H. 1998. Cloning and characterization of endosymbiotic algae isolated from *Paramecium bursaria*. *Protoplasma* 203:91–9.

Pfister, G., Sonntag, B. & Posch, T. 1999. Comparison of a direct live count and an improved quantitative protargol stain (QPS) in determining abundance and cell volumes of pelagic freshwater protozoa. *Aquat. Microb. Ecol.* 18:95–103.

Pochon, X., Montoya-Burgos, J. I., Stadelmann, B. & Pawlowski, J. 2006. Molecular phylogeny, evolutionary rates, and divergence timing of the symbiotic dinoflagellate genus *Symbiodinium*. *Mol. Phylogenet. Evol.* 38:20–30.

Posada, D. & Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14:817–8.

Reisser, W. & Häder, D.-P. 1984. Role of endosymbiotic algae in photokinesis and photophobic responses of ciliates. *Photochem. Photobiol.* 39:673–8.

Rowan, R. & Powers, D. A. 1991. A molecular genetic classification of zooxanthellae and the evolution of animal-algal symbioses. *Science* 251:1348–51.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–25.

Santos, S. R., Shearer, T. L., Hannes, A. R. & Coffroth, M. A. 2004. Fine-scale diversity and specificity in the most prevalent lineage of symbiotic dinoflagellates (*Symbiodinium*, Dinophyceae) of the Caribbean. *Mol. Ecol.* 13:459–69.

Sapp, J. 1994. *Evolution by Association: A History of Symbiosis*. Oxford University Press, Oxford, UK, 255 pp.

Schulze, K. L. 1951. Experimentelle Untersuchungen über die Chlorellen-Symbiose bei Ciliaten. *Biol. Gen.* 19:281–98.

Shick, J. M. & Dunlap, W. C. 2002. Mycosporine-like amino acids and related gadusols: biosynthesis, accumulation, and UV-protective functions in aquatic organisms. *Annu. Rev. Physiol.* 64:223–62.

Siegel, R. W. 1960. Hereditary endosymbiosis in *Paramecium bursaria*. *Exp. Cell Res.* 19:239–52.

Sommaruga, R. 2001. The role of solar UV radiation in the ecosystem of alpine lakes. *J. Photochem. Photobiol. B Biol.* 62:35–42.

Sommaruga, R., Whitehead, K., Shick, J. M. & Lobban, C. S. 2006. Mycosporine-like amino acids in the zooxanthella-ciliate symbiosis *Maristentor dinoferus*. *Protist* 157:185–91.

Sonntag, B., Posch, T., Klammer, S., Teubner, K. & Psenner, R. 2006. Phagotrophic ciliates and flagellates in an oligotrophic, deep, alpine lake: contrasting variability with seasons and depths. *Aquat. Microb. Ecol.* 43:193–207.

Sonntag, B., Summerer, M. & Sommaruga, R. 2007. Sources of mycosporine-like amino acids in planktonic *Chlorella*-bearing ciliates (Ciliophora). *Freshw. Biol.* 52:1476–85.

Stabell, T., Andersen, T. & Klaveness, D. 2002. Ecological significance of endosymbionts in a mixotrophic ciliate – an experimental test of a simple model of growth coordination between host and symbiont. *J. Plankton Res.* 24:889–99.

Summerer, M., Sonntag, B. & Sommaruga, R. 2007. An experimental test of the symbiosis specificity between the ciliate *Paramecium bursaria* and strains of the unicellular green alga *Chlorella*. *Environ. Microbiol.* 9:2117–22.

Swofford, D. L. 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4*. Sinauer Associates, Sunderland, Massachusetts.

Taylor, D. L. 1974. Symbiotic marine algae: taxonomy and biological fitness. *In* Vernberg, W. B. [Ed.] *Symbiosis in the Sea*. University of South Carolina Press, Columbia, South Carolina, pp. 245–62.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673–80.

Van Etten, J. L., Meints, R. H., Kuczmarski, D., Burbank, D. E. & Lee, K. 1982. Viruses of symbiotic *Chlorella*-like algae isolated from *Paramecium bursaria* and *Hydra viridis*. *Proc. Natl. Acad. Sci. U. S. A.* 79:3867–71.

Wu, H.-L., Hseu, R.-S. & Lin, L.-P. 2001. Identification of *Chlorella* spp. isolates using ribosomal DNA sequences. *Bot. Bull. Acad. Sin.* 42:115–21.

Zharkikh, A. 1994. Estimation of evolutionary distances between nucleotide sequences. *J. Mol. Evol.* 39:315–29.

Supplementary Material

The following supplementary material is available for this article:

Table S1. Origin of investigated and reference algal strains used in the phylogenetic analysis.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2007.00455.x

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