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Analysis of the Photobiont Population in Lichens Using a Single-Cell Manipulator

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Abstract

Lichen thalli are composed of at least two genetically different organisms; a fungus (the mycobiont) and an alga (the photobiont). However, little evidence exists for genetic homogeneity within the populations of the two bionts. The aim of our study was to assess the photobiont population of a lichen thallus in this context. To this end it is necessary to analyse a considerable number of photobiont strains, a task not easily achieved by the traditional micropipette method, usually applied for the isolation of photobionts. Instead a single-cell manipulator was used. This demonstrably facilitated the establishment of axenic cultures of lichen photobionts. Due to the higher precision in selection and a shorter handling time, the yield of algal strains is significantly higher in comparison with the micropipette method. A total of 24 photobiont strains were analysed, obtained from 3 different regions (the apothecial margin, the margin and the center of the thallus) of one and the same thallus of the foliose corticolous lichen *Pleurosticta acetabulum* (Necker) Elix & Lumbsch. The strains proved to be identical with respect to their morphology, belonging to *Trebouxia arboricola* Puymaly, and the absence of an intron at position 1512 in the 18S nrDNA (numbering based on the rDNA gene of *Escherichia coli*). The

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ITS-region of one representative per location was determined and resulted in identical sequences, again indicating a homogeneous photobiont population in this lichen thallus. In a second example, we analysed 15 photobiont clones isolated from a specimen of *Tremolecia atrata* (Ach.) Hertel, a crustose saxicolous lichen species. In this case the algal morphology, the absence of the 18S nrDNA intron at position 1512, and identical ITS nrDNA-sequences of all 15 clones revealed a homogeneous photobiont population. Further applications of the micromanipulator in the studies of lichens are briefly discussed.

Keywords: Lichen symbionts, photobiont, *Trebouxia*, micromanipulator, culture, *Pleurosticta acetabulum*, *Tremolecia atrata*, *Physconia distorta*, *Physconia grisea*

1. Introduction

Some mycobiont species are able to form lichen thalli with different photobiont genera, e.g. *Chaenotheca chrysocephala* (Tibell, 1980) or *C. chlorella* (Tibell, 1987). But also clones of the same photobiont species, differing in the possession of a group I intron and therefore being genetically different have been isolated from one and the same lichen thallus (Friedl et al., 2000). If this intron-loss were to be accompanied by other genetic differences, these findings could help to explain growth variation among individual lobes of *Parmelia* species as observed by Armstrong and Smith (1992). Nevertheless, little is known about the variation in the photobiont population of lichens on a larger scale, such as that concerning different parts of a thallus or studies based on several isolated clones. The reason for this lack of knowledge is that lichen photobionts have to be cultured – at best axenically – in order to determine them at the species level on the basis of morphology (Ettl and Gärtner, 1984; Friedl, 1989b). The most frequently used method is the micropipette method (Ahmadjian, 1967). Other techniques include filtration (e.g. Yamamoto et al., 1993) or centrifugation steps (e.g. Richardson and Smith, 1968). The main advantage of the micropipette method is that individual algal cells can be selected under microscopical control, therefore assuring the selection of the photobiont and excluding ubiquitous aerophytic algae. The main disadvantages, however, are that the procedures are time consuming and only 10–20% of the isolated cells subsequently form colonies. This disadvantage can be avoided by using a micromanipulator (Schweiger et al., 1987), which allows a faster and a more selective gathering of the algal cells and a shorter handling time of the cells under the microscope.

In order to investigate the photobiont population of a lichen thallus and to obtain photobiont strains from a foliose and a crustose lichen species, namely *Pleurosticta acetabulum* and *Tremolecia atrata*, we used the micromanipulator.

These strains were analysed with respect to morphological and molecular characteristics. Additionally, 131 clones obtained from 25 thalli of 7 lichen species were analysed with respect to their morphology in order to get an estimate of their photobiont inventory.

2. Material and Methods

Lichen samples

The algal clones examined from *Pleurostica acetabulum* were taken from a single lichen thallus growing on an ash tree near Fürstenfeldbruck, Bayern, Germany (520 m alt.). The investigated thallus of *Tremolecia atrata* was collected on Utön, Södermanland, Sweden (4 m alt.). All other lichen samples were taken from tree bark in locations around München, Germany. The lichen specimens collected and examined during this study have been deposited in the lichen herbarium of the Botanische Staatssammlung München (M).

Set-up of the micromanipulator

The instrumental set-up is shown in Fig. 1. The main components used are an inverted microscope (IM 35 Zeiss microscope with Nomarski optics, C. Zeiss, Oberkochen, Germany), a computer controlled microscope stage (EK8b-S4 microscope stage and control unit MCC 13 JS RS232, Gebr. Märzhäuser OHG, Wetzlar, Germany), driven by x, y and z-stepmotors, and a computer controlled nanoliter pump (modified diluter Microlab M, Hamilton, Bonaduz, Switzerland), which can be programmed for volume, speed and direction of pumping. A hand-pulled selection microcapillary, fixed to a holding device driven by the z-axis stepmotor, is connected via a Teflon tubing filled with mineral oil to the nanoliter pump. By use of a contering device, the capillary is fixed at the optical axis of the microscope. Thus, a cell that has been adjusted to the crosshairs position by microscope stage movement can easily be taken up into the capillary, since this is then directly above the cell. A personal computer is programmed such that any given position and sequence of steps (which may vary with the culture vessel used) can be adjusted. An optional useful feature is a TV-system which improves visibility of cells by projecting the image to a screen. The instrumental set-up is assembled in an air-flow cabinet in order to fulfil sterility requirements.

Photobionts

Photobionts were isolated using a micromanipulator and grown as described

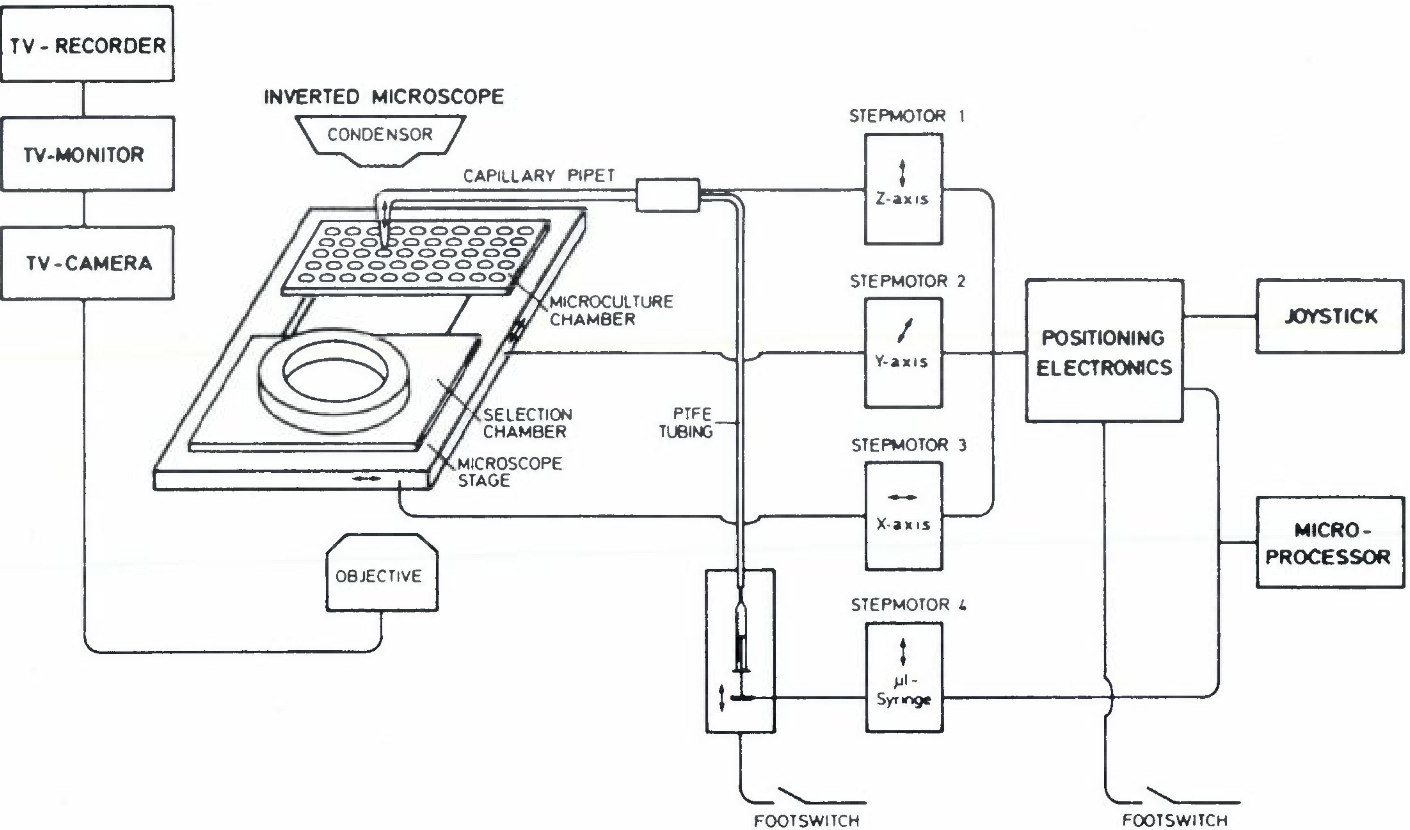

Figure 1. Instrumental set-up for the selection of single photobiont cells using a micromanipulator.

Parmelia sulcata photobionts have been noted; the significance of these is currently being investigated. The finding of morphologically identical photobionts being isolated from one lichen thallus can be taken as a first hint for the homogeneity of the photobiont population in this lichen thallus.

However, morphologically identical photobiont strains may vary slightly in their ITS-sequence (e.g. Beck, 1999). Therefore to confirm the homogeneity of the photobiont population a molecular analysis is needed in addition to the morphological investigations. Such analysis was carried out for *Pleurosticta acetabulum* and *Tremolecia atrata*, since the highest number of photobiont clones, derived from one and the same thallus, was obtained from these lichens.

Twenty-four clones were obtained from the 30 selected photobiont cells of the foliose lichen *P. acetabulum*, which corresponds to a yield of 80%. The photobiont cells were isolated from three different thallus areas of *P. acetabulum* (Fig 3). From two locations (1 and 2 in Fig. 3), the margin of the thallus and the apothecial margin, all 15 cultured cells gave rise to axenic clones, with 8 clones (98.068A1–98.068B3) from spot 1 (margin of the thallus) and 7 clones (98.068B4–98.068C5) from spot 2 (apothecial margin). From the third location (3 in Fig. 3), in the centre of the thallus, only 60% (9 out of 15 cells) gave rise to clones (98.099A1–98.099C5). This may be due to the fact that the cells in the centre of the thallus do not grow as readily as cells of the lobe margins or the apothecium. With respect to morphology, all clones belong to *Trebouxia arboricola*. This photobiont has already been recorded from *P. acetabulum* (Friedl, 1989b; Friedl et al., 2000).

All of the 15 cultured algal cells of the crustose lichen *Tremolecia atrata*, which were selected from three different areoles, gave rise to colonies. The morphology of the cells was that of *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner. The chloroplast was broadly lobed and had an indistinct pyrenoid. Maximum cell size tended to be slightly smaller than expected. This is the first photobiont record for this lichen. Interestingly, it is the same photobiont species which has been recorded for several other lichens, which also grow on rocks containing heavy metals (Beck, 1999).

Introns have previously been used as markers in population studies (e.g. DePriest, 1993). The existence of introns is an easily accessible marker, because a simple agarose gel electrophoresis of PCR reaction products is sufficient to differentiate between strains with or without this intron. Friedl et al. (2000) reported the existence of algal strains containing an intron in the 18S nrDNA at position 1512 with respect to the *Escherichia coli* rDNA (1512-intron) and another strain, from the same thallus of *P. acetabulum*, without it. Therefore, we used this marker as a first test for the homogeneity of the algal population in the investigated thalli of *P. acetabulum* and *T. atrata*. It was shown that in the thallus of *T. atrata* all 15 photobiont strains were identical with respect to the length of the PCR product obtained. The same was true for the 24

Table 1. Percentages of clones obtained from different tested lichen specimens when using the micropipette method or a single-cell manipulator. When using the micropipette method 24 cells were selected and grown, whereas with the single-cell manipulator only 15 cells were cultured. Strain numbers listed in different lines represent photobiont clones obtained from different lichen samples.

Lichen species	Strain-number	Percentage of successful cultures	
		Micropipette method n=24	Single-cell manipulator n=15
Physconia distorta	96.037 / 97.016	4	27
Physconia grisea	96.038 / 97.015	4	33
Lecidella elaeochroma	96.013	13	
	96.014	8	
	96.019	8	
	96.021	13	
	96.032	13	
	98.107		27
Parmelia sulcata	96.001	13	
	98.106		47
Phaeophyscia orbicularis	96.009	13	
	96.026	8	
	98.083		80
	98.105		60
	99.055		93
Physcia adscendens	96.006	13	
	96.008	17	
	96.012	13	
	96.027	8	
	98.098		40
	99.007		93
Xanthoria parietina	96.004	13	
	96.007	8	
	96.025	17	
	98.104		80
Mean value		11	58

species, in all other lichens the photobionts isolated in this study belonged to the same species as already reported for these lichens by Beck et al. (1998; for *Phaeophyscia orbicularis, Physcia adscendens, Xanthoria parietina* and *Lecidella elaeochroma*) and Friedl (1989b; for *Parmelia sulcata*). Some differences from the typical *T. impressa* chloroplast morphology of the

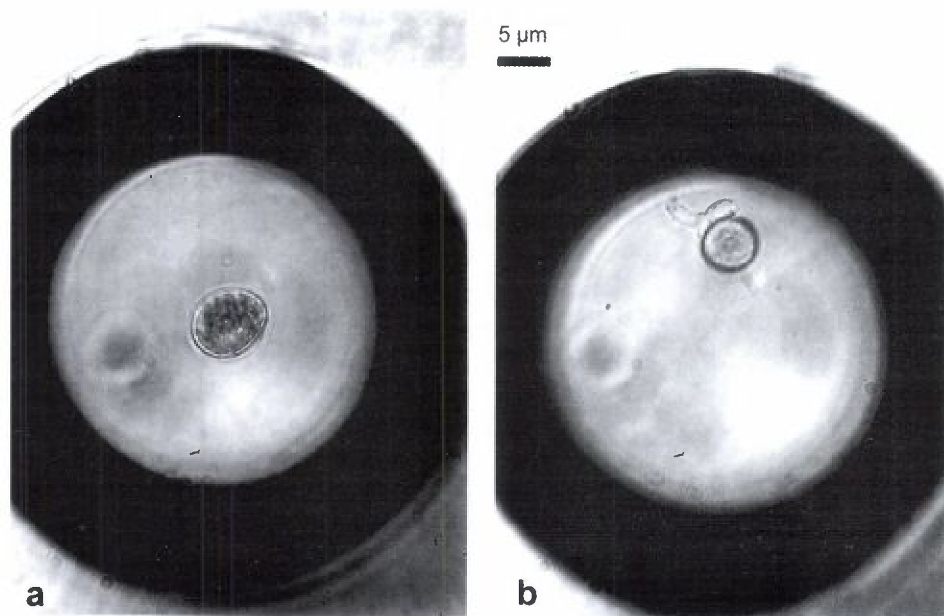

Figure 2. Selected photobiont cell lying in the capillary. Note the high magnification which allows a careful evaluation of the selected cell, e.g. for chloroplast damage or attached mycobiont-hyphae (as seen in b). Bar = 5 µm.

isolation of photobionts by both methods. In the case of the other lichens each isolation was done from a different thallus. In all lichen species the number of cells which subsequently formed colonies was much higher when the single-cell manipulator was used (Table 1; on average about 5 times higher). Several reasons may contribute to this, for example the better and easier selection of undamaged photobiont cells (due to the higher magnification when using an inverted microscope) and the shorter handling time of the photobiont cells. But the variation in the percentages of obtained photobiont clones was still quite high (e.g. in the case of *Physcia adscendens* (Fr.) Oliv.), probably reflecting different physiological or ontogenetic states of the photobiont cells in the different lichen specimens.

All photobiont clones isolated from the same lichen species have been found to be morphologically identical and by this criterion to belong to the following species: The algal partner of *Physconia distorta, P. grisea, Parmelia sulcata, Phaeophyscia orbicularis* and *Physcia adscendens* was *Trebouxia impressa* Ahmadjian, whereas *Lecidella elaeochroma* and *Xanthoria parietina* had *T. arboricola* as photobiont. This is the first photobiont report for the *Physconia*

by Friedl (1989a). To make sure that the isolated cells indeed were the photobiont of the lichen and not aerophytic algae all lichen thalli have been washed carefully with distilled water before the isolation procedure and the identity of the photobiont genus has been determined in the lichenized state. Culture strains of the isolated photobionts are maintained in the algal collection at the University of Bayreuth, Lehrstuhl für Pflanzensystematik. The photobionts were examined both in the lichenized and cultured state by standard light microscopy techniques. For identification, the isolated strains were compared with cultures of all known species of *Trebouxia* (Gärtner, 1985; Ettl and Gärtner, 1995) obtained from culture collections (SAG, Schlösser, 1994; UTEX, Starr and Zeikus, 1993) or were kindly provided by Dr. E. Tschermak-Woess (Vienna, Austria).

DNA extraction, PCR, and sequencing

For DNA extraction, PCR reactions, cleaning of the PCR products and sequencing of the ITS and the 5' part of the 18S nrDNA (to confirm the absence of a 1512-intron) primers and conditions have been used as previously described (Beck, 1999).

3. Results and Discussion

Using the micromanipulator we were able to select specific algal cells (Fig. 2). Due to the high magnification (320×) it was easily possible to differentiate between healthy and damaged cells and to exclude contaminating material. Therefore no additional cleaning steps (transfer to drops of sterile water), as used with the micropipette method, were necessary. This not only saves time but allows for a shorter handling time of the algal cells in the intensive irradiation conditions of a microscope. This may be a critical step during the isolation and cultivation procedures, particularly for light sensitive algal strains such as the *Trebouxia* photobiont of *Letharia columbina* (Nutt.) Thomson (S. Kroken, Berkeley, pers. comm.).

In order to compare the percentages of successful cultures obtained with the micropipette method and our new method respectively, the following lichen species were investigated: *Physconia distorta* (With.) Laundon (1 thallus), *P. grisea* (Lam.) Poelt (1 thallus), *Lecidella eleochroma* (Ach.) M. Choisy (6 thalli), *Parmelia sulcata* Taylor (2 thalli), *Phaeophyscia orbicularis* (Neck.) Moberg (5 thalli), *Physcia adscendens* (Fr.) H. Olivier (6 thalli) and *Xanthoria parietina* (L.) Th. Fr. (4 thalli). Table 1 lists the efficiencies of all isolation experiments, giving also the corresponding strain numbers. In the case of the two *Physconia* species one and the same thallus has been used for

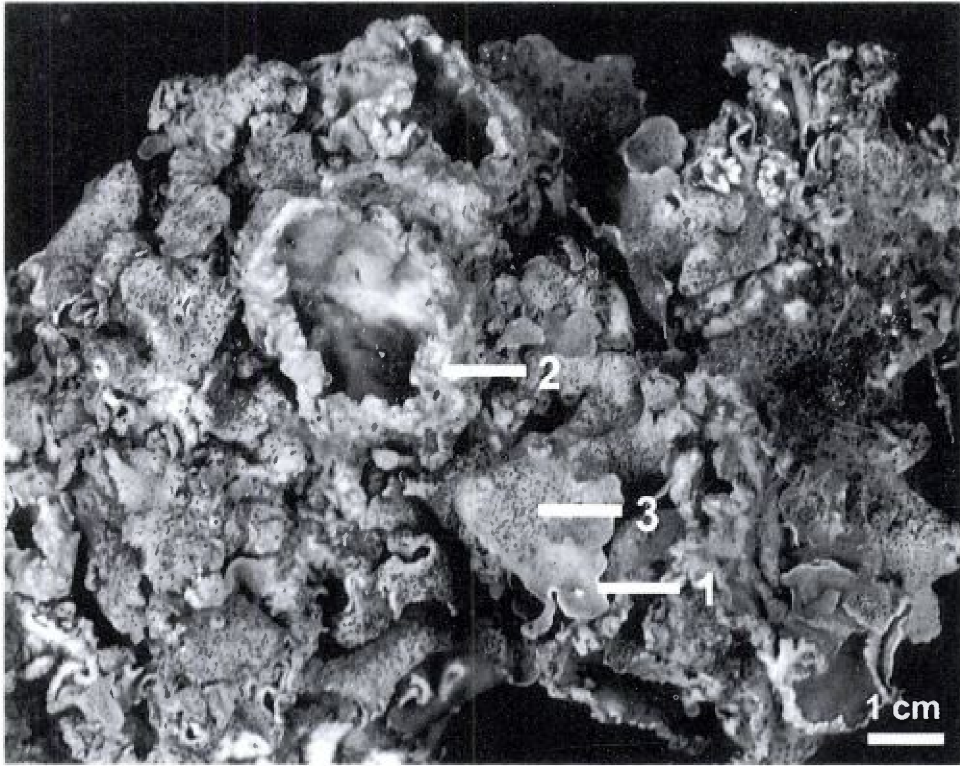

Figure 3. The three locations of *Pleurosticta acetabulum* from which photobiont cells have been taken (1, margin of a thallus-lobe; 2, margin of the apothecium; 3, centre of a thallus-lobe). Bar = 1 cm.

photobiont strains isolated from *P. acetabulum*. Due to the length of the PCR product (ca. 1,4 kb) it could be shown that no algal clone had a 1512-intron. This result was confirmed by sequencing the 5' part of the 18S nrDNA of the clones obtained from *P. acetabulum* and *T. atrata* mentioned below. In order to investigate the homogeneity of the algal population further, the ITS region of one representative of the clones from the three thallus spots of *P. acetabulum* was also sequenced (98.068A1, 98.068C5, 98.099B1). The ITS sequence is very similar (4 to 42 different nucleotides) to the known sequences of *T. arboricola*, and differs by only 4 base changes from the *T. arboricola* photobiont of *P. acetabulum* (Genbank accession number: Z68703) as reported by Bhattacharya et al. (1996). All three ITS sequences obtained in this investigation were identical, indicating a homogeneous photobiont population with respect to this molecular marker as well. All thalli of *P. acetabulum* investigated so far had

T. arboricola as their photobiont. This indicates a rather high selectivity of the fungal partner. This assumption is corroborated by the finding that the ITS sequence from strains obtained from the same thallus were identical. On the other hand, there are slight differences in the ITS sequences of the *T. arboricola* strains isolated from thalli collected in different regions of Germany. Analysis of a sample from near München, Germany (this study), and another collected near Bayreuth, Germany (Genbank accession number: Z68703.1), indicated that the ITS sequence is a suitable marker for the detection of genetic differences in photobiont populations as previously assumed (Beck et al., 1998). The ITS nrDNA has been sequenced for all 15 clones obtained from *T. atrata*. All sequences were identical, indicating a homogeneous photobiont population in the three areoles investigated. Our results indicate that the studied lichen mycobionts were associated with a single photobiont clone, resulting in a homogeneous photobiont population in the lichen thallus. Nevertheless it would be interesting to continue testing these results with other even more rapidly evolving markers (e.g. microsatellites).

Axenic cultures of photobionts – at least without any visible contamination by fungi or bacteria – are far more easily obtained using a micromanipulator than the micropipette method. This allows for less time-consuming work with photobiont cultures, which is a prerequisite for an extended examination of the correlation of morphological and molecular features. It will be necessary to investigate higher numbers of strains to learn more about their morphological variation.

Further applications of the micromanipulator will include the examination of the hymenial algae of some lichen taxa for comparison with algal cells isolated from the thallus. Identity of the algae occurring in the two parts could be regarded as a strong indication that they are usually dispersed together with the mycobiontal spores. Of course, the single-cell manipulator could also be applied for the isolation of mycobiontal mito- or meiospores or hyphal fragments.

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