J. Eukaryot. Microbiol., 54(4), 2007 pp. 381–387

DOI: 10.1111/j.1550-7408.2007.00277.x

Differentiation of Two Very Similar Glaucomid Ciliate Morphospecies (Ciliophora, Tetrahymenida) by Fluorescence in Situ Hybridization with 18S rRNA Targeted Oligonucleotide Probes

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ABSTRACT. Conventional, morphological identification of ciliates and other protozoa needs considerable experience and often is difficult as various staining methods must be applied. New molecular techniques, such as fluorescence in situ hybridization (FISH) with gene probes, are powerful means to overcome this problem. As a test case, the morphology of two very similar, and thus difficult to differentiate ciliate morphospecies, *Glaucoma scintillans* and *Glaucomides bromelicola*, were compared. They were then distinguished by applying the Ciliate-FISH technique with a set of eight 18S rRNA targeted oligonucleotide probes, four of which have been developed for specific detection of *G. scintillans*. The remaining four probes were designed to detect *G. bromelicola* in order to prove probe specificities by binding to the homologous target region of the probes mentioned before. The tests resulted in a clear and easy differentiation of the two species by fluorescence signals of three of the four tested probe pairs. Thus, FISH techniques are very useful for the identification and detection of protozoa and might be of great help studying geographical distributions of known taxa.

Key Words. Fluorescence microscopy, gene probes, identification, morphology, rRNA.

IDENTIFICATION of ciliates often needs considerable experience because many species look alike to untrained eyes. Only when various staining techniques and/or sophisticated microscopical methods (e.g. interference contrast microscopy, scanning electron microscopy) are applied can the distinguishing features be revealed. This is a main drawback in using ciliates as bioindicators (Aescht and Foissner 1991). However, new molecular techniques are now available, especially fluorescence in situ hybridization (FISH), overriding such problems, provided that the probe target species are identified correctly, and that the probe has sufficient specificity.

Fried et al. (2002) presented a set of oligonucleotide probes to specifically identify *Glaucoma scintillans*. More recently, Foissner et al. (2003) reported on ciliates from tank bromeliads. In this latter study, one of the newly discovered species, *Glaucomides bromelicola*, looks so similar to *G. scintillans* that it appeared ideal to re-evaluate the specificity of the *G. scintillans* probes designed by Fried et al. (2002), and to demonstrate the potential usefulness of FISH for identifying common ciliate species.

MATERIALS AND METHODS

The figures of *G. scintillans* are based on various populations from Austria and Germany studied over the years. The oligonucleotide probes were developed on an isolate from a sewage plant in Ingolstadt, Germany (Fried et al. 2000). All populations were identified according to Foissner, Berger, and Kohmann (1994). *Glaucomides bromelicola* was discovered in tank bromeliads from the botanical garden on the Pico Isabel de Torres, Dominican Republic. The description of this species is in preparation; a brief description is found in Foissner et al. (2003), under the name ''new genus 1.''

Pure cultures of both species were established either as described in Fried et al. (2002) or on Eau de Volvic enriched with some sterilized, crushed wheat grains to stimulate growth of bacteria and small heterotrophic flagellates from the collection site. The morphology of both species was studied with the methods described in Foissner (1991), that is, in vivo, with various silver impregnation techniques, and by scanning electron microscopy.

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Using the ARB probe match tool (Ludwig et al. 2004; http://www.arb-home.de) four oligonucleotide probes, glsc407, glsc413, glsc651, and glsc1129 were developed to identify *G. scintillans*. These probes, intended to specifically bind to the 18S rRNA of *G. scintillans* (EMBL/GenBank sequence Accession number: AJ511861) during FISH (Fried et al. 2002), were re-evaluated against an updated dataset of more than 30,000 full and partial 18S rDNA sequences from prokaryotic and eukaryotic organisms. This dataset now comprises the new sequence from *G. bromelicola* (EMBL/Genbank sequence Accession number: AJ810077). For further differentiation of *G. scintillans* and *G. bromelicola*, a set of four new probes was designed: glbr407, glbr413, glbr651, and glbr1129 (Table 1). They served as a positive control for detection of *G. bromelicola* and bind to the same (homologous) target regions as the probes targeting the 18S rRNA of *G. scintillans*.

Cultivated cells of *G. scintillans* and *G. bromelicola* were fixed with 50% Bouin's solution and immobilized on microscope slides coated in such a manner formaldehyde-fixed cells bind covalent to the slide surface (SuperFrost Plus Gold; Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany). Therefore, each microscope slide was prepared with spots of cells of *G. scintillans*, *G. bromelicola*, and a mixture of both. After washing three times in tap water and then dehydration (30%–50%–80%–100% EtOH, each 5 min), the cells were hybridized (46 °C, 2 h) with the *G. scintillans*-specific probes glsc407, glsc413, glsc651, and glsc1129, the *G. bromelicola*-specific probes glbr407, glbr413, glbr651, and glbr1129, a universal Bacteria-specific probe mix consisting of the probes EUB338, EUB338-II, and EUB338-III as a negative control, and the universal Eukarya-specific probe EUK1195 as a positive control (see Table 1; for details see Fried et al. 2002). Furthermore, a second negative control was obtained by hybridizing the cells against a solution without any probe in order to assess autofluorescence of the hybridized cells. All tests were repeated by applying two probes labeled differently within the same hybridization: the *G. scintillans*- or *G. bromelicola*-specific probes were labeled with Cy3 and the EUK1195 probe, was labeled with the sulfoindocyanine dye Cy5 or fluorescein (MWG AG, Ebersberg, Germany).

Probe-related fluorescence signals were detected after FISH using epifluorescence microscopy (Axioplan, Carl Zeiss AG, Oberkochen, Germany) and confocal laser scanning microscopy (CLSM; LSM510, Carl Zeiss AG). The signals were recorded as digital images together with their respective images made with bright field illumination (for details see Fried et al. 2002).

Table **1.** 18S-rRNA-targeted oligonucleotide probes used for fluorescence in situ hybridization (FISH).

Probe	18S-rRNA binding site (5′–3′); references		5′-sequence-3′	References	Target organism(s)	EMBL sequence accession number	RF [%]
	Escherichia coli	1st target organism					
EUB338	338–355		GCTGCCTCCCGTAGGAGT	Amann et al. (1990)	Bacteria		≤60.5
EUB338-II	338–355		GCAGCCACCCGTAGGTGT	Daims et al. (1999)	Planctomycetes		47–67
EUB338-III	338–355		GCTGCCACCCGTAGGTGT	Daims et al. (1999)	Verrucomicrobia and others		≤50
EUK1195	1195–1209		GGGCATCACAGACCTG	Giovannoni et al. (1988)	Eukarya		nd
glsc407	407–425	437–454	ACTAAGTCTCCCGGTCTG	Fried et al. (2002)	*Glaucoma scintillans*	AJ511861	≤30
glsc413	413–429	443–460	GTAGAGACTAAGTCTCCC	Fried et al. (2002)	*Glaucoma scintillans*	AJ511861	<10
glsc651	651–651	651–677	AGTCGCACCAGTAAAGGC	Fried et al. (2002)	*Glaucoma scintillans*	AJ511861	≤20
glsc1129	1129–1142	1288–1305	CACCGTTGTTCACAATGC	Fried et al. (2002)	*Glaucoma scintillans*	AJ511861	≤20
glbr407	407–425	470–487	ACAGAGTTTCCCGGTCTG	This study	*Glaucomides bromelicola*	AJ810077	nd
glbr413	413–429	476–493	GTAGAAACAGAGTTTCCC	This study	*Glaucomides bromelicola*	AJ810077	nd
glbr651	651–651	693–710	AGTCGCACCAGTGAAGGC	This study	*Glaucomides bromelicola*, ''New genus 2 (Ecuador)''*	AJ810077 AJ810076	nd
glbr1129	1129–1142	1322–1339	GCTTGTTGTTCACAGAGC	This study	*Glaucomides bromelicola*, ''New genus 2 (Ecuador)''	AJ810077 AJ810076	nd

*Name as published in Foissner et al. (2003); respective organism name in EMBL = ''Ciliate sp. WFg2.''

RF, range of recommended formamide concentration in the hybridization buffer; underlined nucleotides are different regarding the homologous probes specific for detection of *Glaucoma scintillans*; nd, not determined.

To allow direct signal comparison, microscope settings, such as laser power, pinhole diameter, detection gain, amplifier offset, time exposure, and filter sets for fluorescence image acquisition were kept the same for all tests.

RESULTS

Brief description of *Glaucoma scintillans* (for detailed accounts, see the reviews by Corliss 1971 and Foissner et al. 1994). *Glaucoma scintillans* is an ellipsoidal to slightly ovate, holotrichous ciliate with a size of 35–75 × 25–45 μm (Fig. 1, 2, 7). The globular macronucleus is in or near the body center. A contractile vacuole with a single excretory pore occurs in the posterior dorsal quarter. Mucocysts are scattered through the cortex, but are minute and thus inconspicuous. There are 30–40 longitudinal ciliary rows, which form a preoral suture, left of midline. The first row right of the oral apparatus is semicircularly curved anteriorly, that is, surrounds the anterior mouth margin and almost touches the first ciliary row left of the oral apparatus. Six to nine (postoral) ciliary rows abut the posterior mouth margin (Fig. 1, 2, 7). The oral apparatus is subapically located and has an elliptical entrance. The three adoral membranelles are in a rather deep buccal cavity, and membranelles 1 and 2 form a conspicuous, scintillating ciliary tuft. The inconspicuous undulating membrane is on the right side buccal lip and ciliated only in the anterior two-thirds (Fig. 1, 2, 7). Primary silverline meridians connect the basal bodies of the cilia within a row, and a secondary silverline meridian each occurs between two primary meridians (Fig. 8).

Brief description of *Glaucomides bromelicola*. This species strongly resembles *G. scintillans* in vivo, in silver preparations, and the scanning electron microscope because it has a very similar size (30–80 μm length) and shape, as well as somatic and oral ciliary pattern; even the minute patch (''X-group'') of basal bodies occurs at the anterior end of adoral membranelle 2 (compare Fig. 3, 4 with Fig. 1, 2, 7). Only by careful examination can it be recognized that the bromeliad tank ciliate is different from *G. scintillans*: it lacks cilia (not basal bodies, and thus both species look very similar in silver preparations!) on the posterior dorsal half (Fig. 5). Further investigation revealed two other subtle differences: the silverline pattern and the ability to form macrostomes. The primary silverline meridians of *G. bromelicola* have short, transverse outgrowths (Fig. 9) lacking in *G. scintillans* (Fig. 8). When bacterial food is depleting, *G. bromelicola* produces macrostomes, which are up to 80 μm long and feed on small

Fig. **1–9.** *Glaucoma scintillans* (Fig. 1, 2, 7, 8) and *Glaucomides bromelicola* (Fig. 3–6, 9) in the scanning electron microscope (Fig. 1, 4–6) and after protargol (Fig. 2, 3), Chatton–Lwoff silver nitrate (Fig. 7, 9) and Klein–Foissner silver nitrate (Fig. 8) impregnation. Both species have a very similar size and shape as well as somatic and oral ciliary pattern, and are thus easily confused. However, they can be distinguished with various sophisticated morphological features (shown here), rRNA gene sequences (Foissner et al. 2003), and oligonucleotide probes (see ''Results''). EP, excretory pore of contractile vacuole; OA, oral apparatus; PM, paroral membrane. Scale bars = 20 μm. **1–4**, **7**. Ventral views of trophonts. Arrowheads mark the curved anterior portion of the first ciliary row right of the oral apparatus. Arrow denotes a minute patch of basal bodies (''X-group'') at anterior end of adoral membranelle 2. **5, 6.** *Glaucomides bromelicola* has an unciliated patch (Fig. 5, asterisk) on the dorsal side and can form macrostomes (Fig. 6, compare with ordinary specimen shown in Fig. 4). **8, 9.** Silverline pattern of ventral and left side. Arrowheads mark primary meridians, that is, the silverline associated with the basal bodies within a row. Note lack of transverse outgrowths from primary meridians in *G. scintillans* (Fig. 8).

heterotrophic flagellates (Fig. 6). *Glaucoma scintillans* does not form macrostomes under the same conditions, and there is no evidence of macrostomes in the vast literature on that species (for reviews, see Corliss 1971; Foissner et al. 1994). Last but not least, 18S rRNA gene sequence analysis revealed that the bromeliad tank ciliate is more closely related to *Bromeliophrya brasiliensis*, a glaucomid ciliate from Brazilian tank bromeliads (Foissner 2003), than to *G. scintillans* (Foissner et al. 2003, new genus 2).

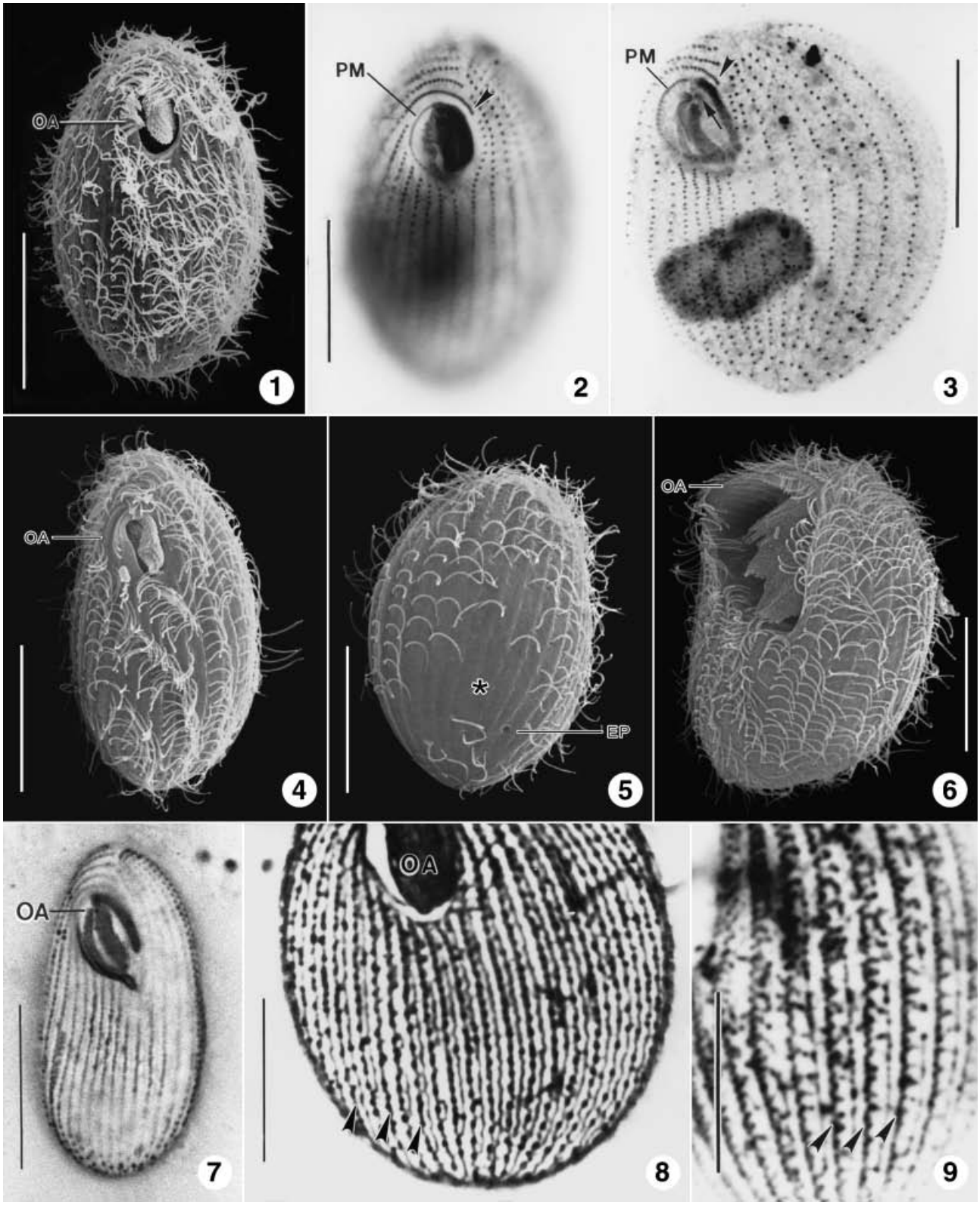

Table **2.** Target sequences of the four probes specifically binding to the 18S-rRNA of *Glaucoma scintillans* in relation to some homologous sequences from other members of the ciliate subclass Hymenostomatia (reference organisms for which the probes have been tested are marked in bold).

Organism GenBank/EMBL sequence accession number	Probe target sequence (5′ → 3′)			
	glsc407	glsc413	glsc651	glsc1129
Glaucoma scintillans	**CAGACCGGGAGACUUAGU**	**GGGAGACUUAGUCUCUAC**	**GCCUUUACUGGUGCGACU**	**GCAUUGUGAACAACGGUG**
AJ511861				
Glaucoma chattoni	==========A===AC==	====A===AC==U=====	===========U=====	U=CC===========G=
S57722, X56533				
Glaucomides bromelicola	**==========A===CU==**	**====A===CU==U=====**	**=====C===========**	**==UC==========AAGC**
AJ810077				
Ciliate sp. WFg2*	====U=====A====C==	====A====C==U=====	=====C===========	==UC==========AAGC
AJ810076				
Bromeliophrya brasiliensis	==============C==	=========C========	=====C=U====U=====	==U=======U===A=GU
AJ810075				
Colpidium campylum	==A=======A===C===	====A===C===U=====	===========U=====	==CC===========GA
X56532				
Tetrahymena empidokyrea	==AG=U====A===A===	====A===A===U=====	=====C======U=====	UGUC==========A=AU
U36222				
Tetrahymena corlissi	==AG=U====A=======	====A=======U=====	=====C======U=====	UGC==========A=GC
U17356				
***Tetrahymena* sp.**	**==AG=U====A==C====**	**====A==C====U=====**	**=====C======U=====**	**UGC==========A=GU**
AJ511862				
Lambornella sp.	==AG=U====A===A===	====A===A===U=====	=====C======U=====	UGUC==========A=AU
AF364043				

Only nucleotides different to the target sequence (mismatches) of *G. scintillans* are shown.
*Sequence derived from an isolate of a ciliate named ''New genus 2 (Ecuador)'' in Foissner et al. (2003).

Probe evaluation. The probes glsc407, glsc413, glsc651, and glsc1129 re-evaluated with the probe match tool of the ARB software package showed that they are all still specific to *G. scintillans* (Table 2). The probes glbr407, glbr413, glbr651, and glbr1129 were constructed to bind to *G. bromelicola* at the same (homologous) binding sites as the probes specific for detection of *G. scintillans*. Thus, they served as a negative control if hybridized with *G. scintillans* and as a positive control if hybridized with *G. bromelicola*. Among these probes, glbr407 and glbr413 are specific for detection of *G. bromelicola*. Probe glbr413 has a single mismatch, that is a non-binding nucleotide site, to *Colpidium campylum* (X56532) (Table 2). The probe glbr651 should bind to the other bromeliad tank ciliate ''New species 2 (Ecuador)'' (Foissner et al. 2003; AJ810076, ''ciliate sp. WFg2'') and has a single mismatch to the homologous target site of species belonging to the genus *Tetrahymena* and to *Lambornella* sp. (AF364043) (Table 2). Probe glbr1129 should also be specific for the detection of the bromeliad tank ciliate (AJ810076) and shows more than one mismatch to the homologous binding sites of other ciliates (for comparison of the probe target sites see Table 2).

FISH comparison. Fluorescence in situ hybridization with the probes EUK1195, glsc407, glsc651, and glsc1129 resulted in distinct signals of all target cells. The intensities of all signals could clearly be differentiated from the weak autofluorescence of the targeted cells (i.e. from signals of cells detected after the control hybridization without any probe). *Glaucoma scintillans* always showed a perceivable increased autofluorescence if exposed to blue light (green emission) compared with *G. bromelicola*, as illustrated in Fig. 10 and in Fig. 13 for *G. scintillans*. Exposed to green light, there was no difference visible (red emission which would turn yellow if superimposed with the green emission). The fluorescence signals of the non-target cells, that is *G. bromelicola* did not show stronger signals than its overall autofluorescence

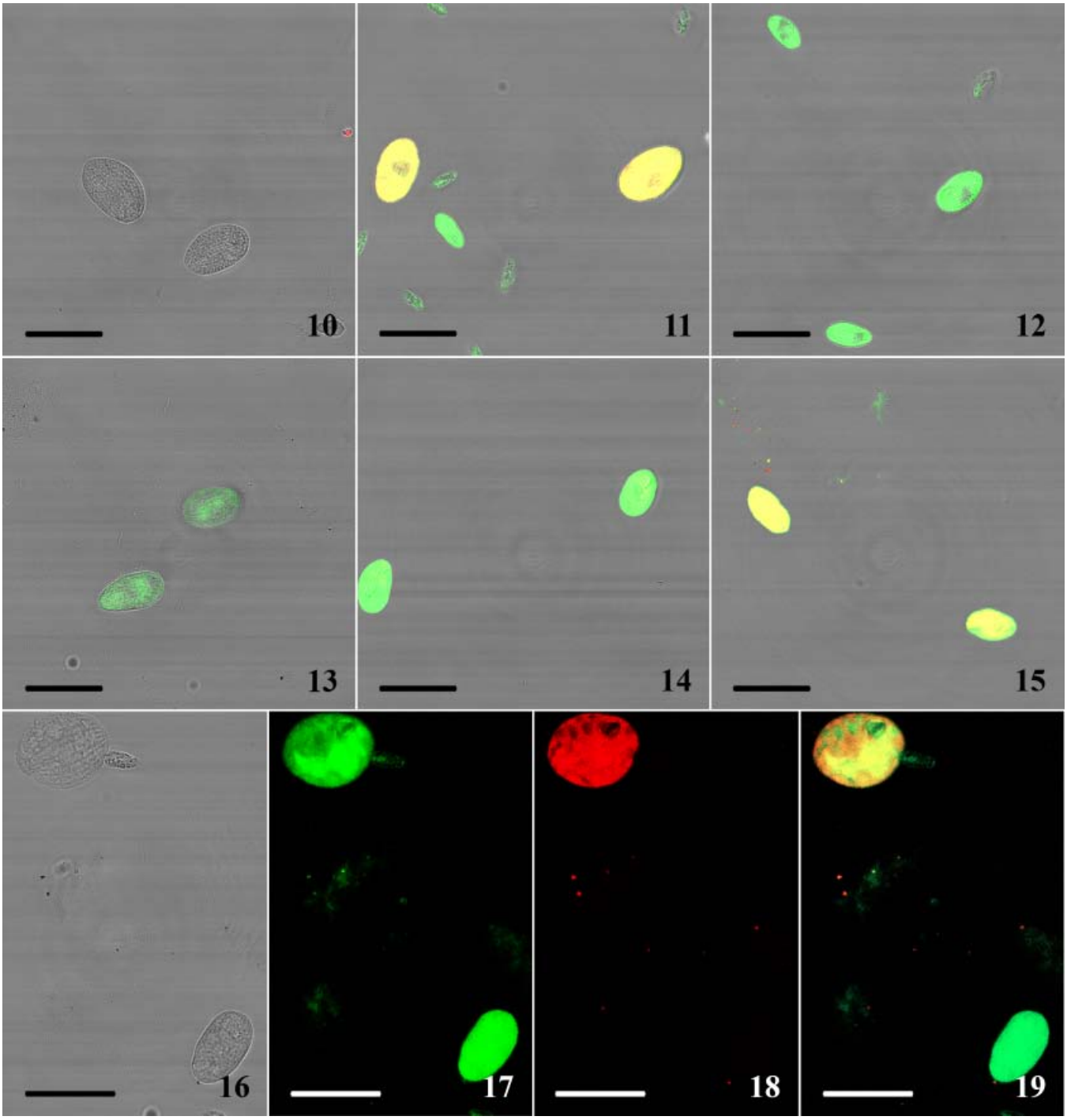

Fig. **10–19.** Whole-cell-based differentiation of *Glaucomides bromelicola* and *Glaucoma scintillans* due to fluorescence in situ hybridization (FISH) with a set of 18S rRNA-directed oligonucleotide probes. **10–12.** *Glaucomides bromelicola*. **13–15.** *Glaucoma scintillans*. **16–19.** A mixture of both species mentioned above. Fig. 10 and Fig. 13 are the resulting images after a control hybridization without probes (autofluorescence). Fig. **11,12,14,** and Fig. **15** show cells after hybridization with the universal Eukarya-specific probe EUK1195, labeled with fluorescein (green and yellow) and the *G. bromelicola*-specific probe glbr651, labeled with Cy3 (red and yellow; Fig. 11, 14) or the *G. scintillans*-specific probe glsc651, labeled with Cy3 (red and yellow, Fig. 12, 15). Fig. **16–19** shows the results after FISH on a sample of both ciliates mixed together. Phase contrast image by laser transmission detection (Fig. 16), hybridization with the Eukarya-specific probe EUK1195, labeled with fluorescein (green; Fig. 17), and the *G. bromelicola*-specific probe glbr407, labeled with Cy3 (red; Fig. 18). Fig. **19** is generated by superimposition of Fig. 17 and Fig. 18. All images were recorded by laser scanning microscopy at 200x magnification. Fig. **10–15** are triple-channel images, Fig. **16,17,18** are single channel records, Fig. **19** is a superimposition of Fig. 17 and 18. All channels were recorded separately by either the light information of phase contrast (laser transmission), fluorescein-derived light emission (green), or Cy3-dye-derived light emission (red). Superimposition of those three channels resulted in the images presented. All scale bars = 50 μm.

after hybridization with the *G. scintillans*-specific probes, except after hybridization with probe glsc651, which caused a slightly increased fluorescence of *G. bromelicola*. But even in this case, signals obtained from *G. scintillans* could clearly be differentiated from the much weaker signals of *G. bromelicola* (compare yellow stain of Fig. 12, 15). All cells observed showed strong signals after hybridization with the probe EUK1195 (Fig. 11, 12, 14, 15, 17, 19, green and yellow). Hybridizations of *G. scintillans* with probe glsc413 resulted in slightly stronger signals compared with the overall autofluorescence of this species (data not shown). By optimizing the settings for signal acquisition, probe glsc413-derived fluorescence signals could be clearly differentiated from the autofluorescence of *G. scintillans*. However, the control hybridization with probe glbr413 did not confirm this result (i.e. bound neither to the target organism *G. bromelicola* nor to the non-target organism *G. scintillans*). All negative control hybridizations with the EUB338 probe mix resulted in cell signals, which were too weak to be detected by the CCD camera or CLSM detector at up to 200X original magnification (data not shown). Within spots of mixed cells, *G. scintillans* could always clearly be differentiated from the non-fluorescent *G. bromelicola* if hybridized with one of the four probes specific for *G. scintillans* (e.g. Fig. 16–19). Even with a single nucleotide mismatch, it was possible to differentiate *G. scintillans* from *G. bromelicola* (glsc651, glbr651; Table 2 and Fig. 11, 12, 14, 15; yellow light emitting cells). Simultaneous hybridizations of two differently labeled probes (e.g. the Cy5-labeled probe EUK1195 and the Cy3-labeled probe glbr651) with their target organisms were possible (double probe detection; e.g. Fig. 11, 15 and Fig. 17–19).

DISCUSSION

Glaucoma scintillans is a cosmopolitan and very frequent ciliate in many types of freshwater habitats (Foissner et al. 1994). Thus, it was reasonable to assume that it occurs also in the tanks of bromeliads. However, all populations found so far belong to another species. Indeed, *G. scintillans*-like ciliates are frequent in bromeliad tanks (Foissner et al. 2003; Foissner, W., unpubl. data), and *G. bromelicola* is as common in tank bromeliads of Central America as is *G. scintillans* in European freshwaters (Foissner, W., unpubl. data).

With exception of the probes glsc413 and glbr413, the oligonucleotide probes designed specifically to identify *G. scintillans* (Fried et al. 2002) and the new probes, developed specifically for *G. bromelicola*, clearly distinguished these two species. This was possible even if there was only a single nucleotide difference (single mismatch; glsc651, glbr651; Table 2 and Fig. 11, 12). The possibility of a single mismatch discrimination by oligonucleotide probes is well known and has been demonstrated previously for bacteria (Amann, Glöckner, and Neef 1997) and for ciliates, too (Schmidt et al. 2006). The weak signals, obtained after hybridization with probes glsc413 and glbr413 to their target organisms, might be explained by inaccessibility of the probes to the target molecule, a phenomenon that is also known from other organisms (Behrens et al. 2003a, b). The probes glsc413 and glbr413 are only target site shifted variants (six nucleotides to the 3′-end of the target region) of the probes glsc407 and glbr407. Thus, the inaccessible part of the molecule is probably located at the 3′-end of the probe target region as probe glsc407 (glbr407) clearly binds to its target (see Table 2).

The species investigated in this study and some other species have a highly similar morphology and are thus easily misidentified (Fig. 1–4). Once more after Schmidt et al. (2006), who differentiated *Stylonychia mytilus* from the morphological scarcely distinguishable *Stylonychia lemnae* with the same FISH technique, this study reconfirms ciliate taxa can be distinguished by only a single nucleotide differentiation. Thus, a specific tool, like FISH with rRNA-targeted probes, will greatly improve identifications and data on geographic distribution. However, it is still necessary to have more 18S rDNA sequences from other closely related species, as the probes tested in this study are based on an updated (since the publication of Schmidt et al. 2006), but still restricted dataset of sequences; *Glaucoma* is represented by only two species, *G. scintillans* and *Glaucoma chattoni*. However, the possibility of misidentifications by using non-specific probes (i.e. the detection of species not yet represented in today's 18S rDNA datasets) will be very unlikely if several probes targeting different sites of the molecule are used to detect a given taxon. This multiple-probe concept (Behr et al. 2000) was considered during the development of the probes used in this study.

ACKNOWLEDGMENTS

Supported by the German Research Foundation (DFG projects LU421/3-1, LU421/3-2, and 421/3-3), the Austrian Science Foundation (FWF project P-15017), the Universität Innsbruck, Austria, and the ''Verein zur Förderung der wissenschaftlichen Ausbildung und Tätigkeit von Südtirolern an der Landesuniversität Innsbruck,'' Innsbruck, Austria. The technical assistance of Andreas Zankl, Dr. Eva Herzog (Universität Salzburg, Austria), and Dorle Begert, and helpful comments on the manuscript by Dr. Wolfgang Ludwig and Prof. Dr. Karl Heinz Schleifer (Technische Universität München, Freising, Germany), are greatly acknowledged.

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Received: 08/04/04, 12/09/04, 05/16/07; accepted: 05/23/07