Portrait of a Species: *Chlamydomonas reinhardtii*

Thomas Pröschold*,¹ Elizabeth H. Harris[†] and Annette W. Coleman*,²

*Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912 and [†]Department of Biology, Duke University, Durham, North Carolina 27708-0338

Manuscript received April 14, 2005
Accepted for publication May 17, 2005

ABSTRACT

Chlamydomonas reinhardtii, the first alga subject to a genome project, has been the object of numerous morphological, physiological, and genetic studies. The organism has two genetically determined mating types (plus and minus) and all stages of the simple life cycle can be evoked in culture. In the nearly 60 years since the first standard laboratory strains were isolated, numerous crosses and exchanges among laboratories have led to some confusion concerning strain genealogy. Here we use analyses of the nuclear internal transcribed spacer regions and other genetic traits to resolve these issues, correctly identify strains currently available, and analyze phylogenetic relationships with all other available similar chlamydomonad types. The presence of a 10-bp indel in ITS2 in some but not all copies of the nuclear ribosomal cistrons of an individual organism, and the changing ratios of these in crosses, provide a tool to investigate mechanisms of concerted evolution. The standard *C. reinhardtii* strains, plus *C. smithii* +, plus the new eastern North American *C. reinhardtii* isolates, comprise one morphological species, one biological species of high sexual intercompatibility, and essentially identical ITS sequences (except the tip of helix I of ITS2). However, variant RFLP patterns characterize strains from each geographic site.

ALTHOUGH several plant species are subjects of genome projects, only one green alga has so far served as a model organism and subject of a genome project, *Chlamydomonas reinhardtii* (HARRIS 2001). Several recent articles discuss the results of its genome sequencing, compare its genome with that of *Arabidopsis*, and provide protocols for its manipulation and transformation (e.g., GROSSMAN *et al.* 2003). An entire book is devoted to its investigative history, cultivation, and manipulation (HARRIS 1989).

C. reinhardtii is a biflagellate photosynthetic unicell, with an easily cultivated haploid vegetative stage. This species occurs as two genetically determined mating types, + and – alleles at a single complex mating-type locus. Sexuality is readily evoked upon nutrient step-down. When mixed, + and – gametes rapidly pair, fuse, and form a diploid cell that becomes a heavy-walled zygospore. Meiosis occurs at zygospore germination, producing four haploid cells in an unordered tetrad; two are of the + and two of the – mating type.

Here we concentrate on three points:

1. The origin and genealogy of the current “standard” *C. reinhardtii* strains, which presumably all are derived from

a single field-isolated zygote in Massachusetts in 1945. This subject has a long and complicated history, thoroughly described up to 1989 by HARRIS (1989).

2. The unique variation among the many nuclear ribosomal RNA cistrons of *C. reinhardtii*. This is a subject ignored by genome sequencing projects of eukaryote organisms because the total length of the set of tandem repeats is too long to be cloned in its entirety.
3. The distribution of *C. reinhardtii* in nature and its relationship to other similar green algae.

C. reinhardtii is certainly the most studied of all algae, for many, many aspects. We consider it worthwhile to bring together both new and related work to fill out the picture of this algal species as an exemplar. Furthermore, the existence of the *Chlamydomonas* genome project makes accurate identification of the currently used strains much more critical. The final clues for this identification have now been revealed by analysis of the internal transcribed spacer (ITS) subregion of the nuclear rDNA cistrons. This information removes prior uncertainties concerning the genetic heritage of the standard strains and provides a snapshot of both its closest and more distant relatives. Furthermore, for the second internal transcribed spacer subregion (ITS2), probably more of the many repeats found within an individual organism have been sequenced in *C. reinhardtii* than for any other eukaryote, and the results are germane to future experiments seeking to resolve how rapidly and by what process(es) ribosomal cistrons homogenize.

¹Present address: CCAP, SAMS, Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland, PA371QA, United Kingdom.

²Corresponding author: Division of Biology and Medicine, Brown University, Providence, RI 02912.
E-mail: annette_coleman@brown.edu

	>-----X-----<
<i>C. reinhardtii</i> "short"	TCGCCCTACTCCAACAT.....GTTTGGAGCAAGAGCGG
<i>C. reinhardtii</i> "long"	TCGCCCTACTCCAACATACACTTGTGTGTTTGGAGCAAGAGCGG
<i>C. smithii</i> +	TCGCCCTACTCCAACAC.....GTTTGGAGCAAGAGCGG
1952 MN	TCGCCCTACTCCAACAC.....GTTTGGAGCAAGAGCGG
1952 MN "long"	TCGCCCTACTCCAACACAC.....TTGTGTGTTTGGAGCAAGAGCGG
2342 PA	TCGCCCTACTCCAACAC.....GTTTGGAGCAAGAGCGG
2342 PA "long"	TCGCCCTACTCCAACACACTTGTGTGTTTGGAGCAAGAGCGG
2931 N.C.	TCGCCCTACTCCAACAC.....GTTTGGAGCAAGAGCGG
2935 Qu	TCGCCCTACTCCAACAC.....GTTTGGAGCAAGAGCGG
2935 QU	TCGCCCTACTCCAACAC.....TTGTGTGTTTGGAGCAAGAGCGG
<i>C. sp.</i> Kenya	TCGCTCTACTCACACCT.....ATGTGTGTGAGTTAAAGCGG
<i>C. incerta</i>	TCGCACACAACTCCTC.....TTCTGGAGTGTGGCGCGCGG
J short	CGCCCTACTCCAACATGTTTGGAG
rein-long	CGCCCTACTCCAACATACACTTG
17-T	TCGCCCTACTCCAACAT
17-C	TCGCCCTACTCCAACAC
smith-long	TCGCCCTACTCCAACACACTTG

FIGURE 1.—Alignment of ITS2, hairpin loop I, sequences of standard *C. reinhardtii* "short" and "long," plus that of *C. smithii* + and of the closely related *Chlamydomonas* strains. Paired nucleotide positions in standard *C. reinhardtii* are overlain with a line. An X marks the nucleotide diagnostic for standard *C. reinhardtii* vs. *C. smithii* +. The strains CC-1952, CC-2342, CC-2931, and CC-2935 are newly collected (sources given) and interfertile with standard *C. reinhardtii*. No long variant was obtained among the few subclones of CC-2931 sequenced. At the bottom are listed the five special primers used (paired with Grev) for analysis of the ITS2 helix I indel.

MATERIALS AND METHODS

The algal cultures utilized, and their sources, are listed in supplementary Tables S1 and S2 at <http://www.genetics.org/supplemental/>. All the algal strains were cultured in SoilWater medium (STARR and ZEIKUS 1993) at 24° in 60 µE/m²/sec constant light. Pairings of strains to test their mating potential were done in the same medium and under the same growth conditions, as well as by the standard mating protocols for *C. reinhardtii* (HARRIS 1989).

Polymerase chain reactions and sequencing: DNA to serve as template in PCR reactions was extracted from ~0.1 mg wet weight of cells, using InstaGene Matrix (Bio-Rad, Hercules, CA). The standard protocol used to obtain PCR products encompassing the entire ITS1, 5.8S, and ITS2 regions was 95° for 5 min; five repetitions of 90° for 1 min, 50° for 2 min, and 72° for 1 min; and then 30 cycles of 90° for 1 min, 60° for 1 min, 72° for 1 min, ending with a final 72° for 10 min. Taq polymerase was added after the reaction reached 95°.

Initial studies involved purifying the PCR products from agarose gels (QIAquick gel extraction kit; QIAGEN, Valencia, CA), subcloning them into pT7 Blue T-vector (Novagen, Madison, WI), infecting *Escherichia coli*, and preparing DNA by miniprep (Wizard Plus miniprep kit; Promega, Madison, WI). Later studies utilized direct sequencing of the gel-purified mixture of PCR products. Primers included the standard pair (derived from WHITE *et al.* 1990, ITS5 and ITS4) that we call here Gfor and Grev, priming, respectively, in the 3' end of the small subunit (SSU) rDNA and the 5' end of the large subunit (LSU) rDNA and producing the full ITS1-5.8S-ITS2 as the PCR product. Additional special-purpose forward primers, each paired with Grev, are shown in Figure 1. Sequencing in both directions was done earlier manually [United States Biochemicals (Cleveland) 2.0 kit] and later using an automatic sequencing system (ABI dye systems and ABI Prism 377 automated sequencer; Applied Biosystems, Foster City, CA). Primers for sequencing were those used for the initial PCR plus a pair in the 5.8S (ITS3 and ITS2 of WHITE *et al.* 1990).

Alignment of sequences utilized MacVector and AssemblyLIGN software (Kodak, International Biotechnologies, New

Haven, CT) and took into account the known secondary structure of the ITS1 and ITS2 RNA transcripts (Figure 2). Phylogenetic comparison utilized PAUP* version 4.0b10 (SWOFFORD 2002). The evolutionary model for the data sets was calculated by Modeltest 3.06 (POSADA and CRANDALL 1998). The ITS sequences have been deposited in GenBank as listed in supplementary Table S2 (<http://www.genetics.org/supplemental/>).

Genomic DNA analyses: Genomic DNA was isolated from a washed pellet of algal cells grown in 500 ml of HSM (HARRIS 1989). These were lysed in 4% SDS, 0.2 M NaCl, 0.05 M Tris pH 8, 0.1 M EDTA containing 0.1 mg/ml proteinase K. The extract was phenol extracted, then phenol:chloroform extracted, and the aqueous solution brought to 200 mM NaCl and put on ice. Two and one-half volumes of 100% ethanol was added and allowed to stand on ice overnight. The precipitated nucleic acids were collected by centrifugation, washed in 70% ethanol, and air dried. The pellet, resuspended in TE, was treated with 0.1 mg/ml RNase A at 37° for 2 hr, extracted once with phenol:chloroform, and precipitated and resuspended again in TE.

For endonuclease reactions (*Afl*III and *Bam*HI; New England BioLabs, Beverly, MA), multiple aliquots of genomic DNA were digested, under conditions described by the manufacturer, for differing periods of time to ensure a complete reaction. Comparable quantities of these aliquots were run on 1% agarose gels and stained in ethidium bromide, and then the DNA was transferred to a nylon membrane (Boehringer Mannheim, Indianapolis) by standard methods (SAMBROOK *et al.* 1989). A miniprep of a cloned *C. reinhardtii* ITS sequence was radiolabeled and used as probe. Probing and rinsing followed the standard protocol of SAMBROOK *et al.* (1989).

For mapping, we worked from the *C. reinhardtii* nuclear ribosomal cistron map of MARCO and ROCHAIX (1980) and checked *C. reinhardtii* GenBank sequences M32703 (SSU) and AF183463 (LSU partial).

RESULTS AND DISCUSSION

The fundamental problem, the genealogical history of *C. reinhardtii*, is treated first, including the critical information derived from the analyses of ITS2 that is described subsequently.

Background information: What we refer to here as the "standard *C. reinhardtii*" strains are those in use widely, all allegedly derived from the meiotic products of a single zygote isolated in 1945 by G. M. Smith from a Massachusetts site (HARRIS 1989). A laboratory history of the standard strains, modified from HARRIS (1989) and from KUBO *et al.* (2002), is provided in Figure 3. Three basic sublines, I, II, and III, are reconstructed from the literature and culture collection records.

The genetic constitution characterizing the three major sublines of the standard *C. reinhardtii* strains for five unlinked loci is shown in the Figure 3 diagram. One locus is mating type, found on linkage group VI (HARRIS 1989). Two, *nit1* and *nit2* (respectively, linkage group IX and III), singly or together, prevent growth on nitrate; organisms utilize ammonia or urea as a nitrogen source instead, and Smith used a medium containing ammonia, so would have been unaware of any genetic variation in this trait. Essentially all subline I (the only exception is UTEX 2247, which we believe to be a late exchange

FIGURE 2.—Secondary-structure diagram of *Chlamydomonas reinhardtii* ITS2 RNA transcripts. In the ITS2 diagram, the relatively conserved positions (MAI and COLEMAN 1997) are presented in boldface type. All nucleotide variants of the standard *C. reinhardtii* and of *C. smithii* + are indicated by arrows. (A) ITS1. Four nucleotide variants were each found in only one subcloned sequence of the standard *C. reinhardtii* strains (in order, CC-278, CC124J, CC-620, and UTEX 2247); the nucleotide in parentheses was a variant in one subclone of *C. smithii* +. (B) ITS2 had no variant nucleotide positions among all the standard *C. reinhardtii* subclones sequenced; as indicated by the arrows, in *C. smithii* +, one subclone had a variant position, compensatory for pairing, in helix II, while all *C. smithii* + differed from all standard *C. reinhardtii* at the circled position in helix I. Shown in addition to the “long” form of helix I is the alternative “short” form of helix I, found in differing proportions in the standard *C. reinhardtii* strains. In the short form, the *Af*III site is highlighted in boldface type. Only the short form, with the C substitution, has been recovered so far from *C. smithii* +, which is otherwise identical to *C. reinhardtii* in both ITS1 and ITS2.

between the Duke and Sager laboratories) and subline II strains can grow on nitrate, while subline III strains cannot (HARRIS 1989; SAITO *et al.* 1998). Where analyzed, they bear both the *nit1* and *nit2* alleles. The next locus is the nucleolar organizer repeats, as yet unmapped. The final locus contains genes for “autolysins,” concerned with wall digestion, metalloproteases surveyed by Matsuda’s laboratory (KUBO *et al.* 2001, 2002). Not shown in Figure 3 are other known genetic differences that have led to some confusion among different laboratories in the past. These are documented in HARRIS (1998) and in SAITO *et al.* (1998) and include green *vs.* yellow colony color when growing in the dark and the requirement for light to evoke and maintain gamete activity.

As made obvious from Figures 3 and 4, the current strains of standard *C. reinhardtii* cannot all be the immediate products of a single zygote as previously assumed (see HARRIS 1989), a point made clearly by Matsuda’s laboratory (KUBO *et al.* 2002). Two other possible sources of participants exist. At the time Smith was dispersing samples, at least by February of 1950, he had in his laboratory literally hundreds of *C. reinhardtii* F₁ clones, generated by the doctoral research of Regnery (SMITH and REGNERY 1950). In addition, a single strain designated *C. smithii* (HOSHAW and ETTL 1966) that is mating-type plus, also collected from Massachusetts in 1945, was present in Smith’s laboratory and is fully interfertile with the standard strains (HARRIS 1989). *C. smithii* + shares with the standard *C. reinhardtii* most of the alleles

shown in Figure 3. It can use nitrate and has the B allele of the *mmp1-mmp2* locus, but a variant allele of the *mmp3* locus (KUBO *et al.* 2002).

The ITS2 data (below) rule out participation of *C. smithii* + in the standard *C. reinhardtii* lineages. *C. smithii* + has only “short” versions of the ITS2, whereas all standard *C. reinhardtii* strains are mixed for this character. More importantly, *C. smithii* + is uniform for a C residue where all standard *C. reinhardtii* have a T in the ITS2 sequences (Figure 1).

This leaves us with the single zygote germinated by Smith. Is it possible to derive all the strains listed in Figure 3 from a single zygote? No it is not (not directly), even if all four zygote products were isolated. All the strains could, however, be derived from three of the four products of a single zygote, plus one or more F₁ products of their intercross with one another (possibilities are illustrated in Figure 4). Perhaps the earliest pair of strains Smith gave out was the pair in lines I and II that might be two of the original zygote tetrad. Line III appears later with strains brought by Ebersold to Levine’s laboratory; these are two new genotypes, perhaps an additional one of the originals and one F₁.

Heterogeneity among nuclear ribosomal repeats in standard *C. reinhardtii*: Almost all eukaryotes have multiple copies of their nuclear ribosomal RNA cistrons, arranged in a long tandem array. Each cistron contains one gene for SSU, one for 5.8S, and one for LSU ribosomal RNA. These are transcribed as one long RNA; “RNA processing” then removes the transcribed spacers

FIGURE 3.—Genealogy of standard *C. reinhardtii* laboratory strains, modified from HARRIS (1989). Note that CC-1690 (thick box) was used for creation of EST libraries for cDNA sequencing in the Chlamydomonas Genome Project. The three major sublines each are uniform for the genetic loci cited, with certain striking exceptions. In subline 1, two strains that Sager had by 1980 (UTEX 2246 and 2247) are unlike the remainder of subline 1 and probably came from the Gillham laboratory in an exchange. In subline 3, the CC-124J in Matsuda's laboratory, although obtained from Harris in 1983, is almost pure for the "long" ITS2 form, in contrast with the other subline 3 strains. Likewise, all the strains derived from the cell-wall-less mutants of DAVIES and PLASKITT (1971) are almost pure "shorts," while the remainder of subline 3 are clearly mixed for ITS2 type. Data for *mmp1-mmp2* are from KUBO *et al.* (2002). In sublines I and II, the + mating type has the RFLP allele called A for the *mmp1-mmp2* region of linkage group XIX, while the - mating type has the B allele. In subline III, both mating types carry the A allele. The final metalloprotease gene (*mmp3*) is not yet mapped but segregates independently of all the other loci and also has two alleles by RFLP analysis. Again, in lines I and II, the + mating types have the A allele and the - mating type the B, while in subline III both mating types carry the A allele for this gene.

(ITS1 and ITS2—see supplementary Figure S1 at <http://www.genetics.org/supplemental/>) lying between the genic regions, salvaging only the rRNAs. The genic sequences are relatively stable, evolutionarily. By contrast the ITS regions combine stable and less stable regions and have gained wide usage in phylogenetics at lower taxonomic levels (COLEMAN 2003). Within a single organism (with the exception of hybrids), the nuclear ribosomal ITS sequences are typically essentially identical among all the repeats.

C. reinhardtii is known to have at least 200 copies of the nuclear ribosomal repeats (HOWELL and WALKER 1976). We have sequenced the internal transcribed spacer regions flanking the 5.8S gene and concentrated on the second of these, the ITS2. Our initial sequencing by subcloning of the ITS2 region revealed a 10-nucleotide INDEL, found in some cistrons but not others, of the same clonal strain of cells. The position of the INDEL in ITS2 is shown in Figures 1 and 2 in its two alternative

forms. Thirty-three standard *C. reinhardtii* strains tested all have both short and long forms of this region, but in varying proportions, as assessed by direct sequencing of the PCR product or sequencing of multiple subclones. Where subcloning frequency had suggested a near equal proportion of "longs" and "shorts" in a genome, direct sequencing of the mixture of PCR products gave an unreadable series starting at just the INDEL position. Where subcloning suggested a predominance of, for example, the short version, direct sequencing of the PCR product gave a clear read, but close examination revealed minor peaks starting at the INDEL position. Only CC-124J, UTEX 2246, and the strains associated with the cell-wall-less subgroup appeared to lack two forms. Likewise, the isolate known as *C. smithii* + appeared to have only the short form, and its sequence was identical in all three extent samples of the strain (UTEX 1062, SAG 54.72, and CC-1373).

To check these exceptional standard *C. reinhardtii*

FIGURE 4.—Possible origin of standard *Chlamydomonas reinhardtii* strains. The distribution of alleles in sublines I, II, and III cannot be accommodated in a single tetrad, but could be generated if at least one F₁ is included.

strains, we turned to a further method of assessment. The short version of the ITS has an *Af*/III site (ACPu-PyGT) in the terminus of the first hairpin loop in the ITS2 transcript secondary structure; this cut site is absent from the long version. The *Af*/III site in the short version of ITS2 is shown in Figure 2, and it and the additional *Af*/III site in the adjacent LSU are mapped in supplementary Figure S1 (<http://www.genetics.org/supplemental/>). After *Bam*HI/*Af*/III endonuclease digestion of genomic DNA, short cistrons should have two bands, of 870 and 1650 nucleotides in length, where long cistrons should retain a single band of 2500 nucleotides.

To ascertain directly what the genomic DNA contained, we digested total DNA with the diagnostic restriction endonucleases, separated the products on an agarose gel, and prepared a Southern blot. This was probed with a radioactively labeled plasmid containing a complete *C. reinhardtii* ITS1-5.8S-ITS2 sequence (supplementary Figure S2 at <http://www.genetics.org/supplemental/>).

Of 13 different standard *C. reinhardtii* strains examined this way, most showed autoradiography patterns on Southern blots indicating the presence of both long and short versions of the ITS within a genome, and the proportions of each roughly corresponded with what had been observed either by direct PCR sequencing or by frequency among multiple subclones (supplementary Table S1 at <http://www.genetics.org/supplemental/>). There were still, however, strains that appeared to be

pure types, from the diagnostic bands on the autoradiogram. CC-124J, the strain from Matsuda in Japan, appeared to be lacking any short version; this was not true of the CC-124 we obtained directly from the CC collection. Several strains collectively associated with the cell-wall-less mutation work of DAVIES and PLASKITT (1971) appeared to be lacking longs. The same is true of two other strains, UTEX 2246 shown in Figure 3, subline I, a strain deposited by Sager in UTEX in 1980, and *C. smithii* +.

To discern finally whether these exceptional strains were indeed homogeneous for only one version of the ITS2, forward primers designed to discriminate between shorts and longs were used, with Grev, for PCR. Using the J short primer, a band resulted from the Japanese sample CC-124J mating-type minus, and its sequence was the expected short. Likewise, use of the rein-long and smith-long special primers on the cell-wall-less strains and on UTEX 2246 produced a band that, when sequenced, was the expected long version. Thus specific primers were capable of detecting a minority cistron type not detectable by genomic digestion and probing, and all standard *C. reinhardtii* organisms contain both long and short versions of ITS2, albeit in very different proportions in different strains.

However, no long band was obtained with *C. smithii* + DNA, using either of the special long primers. *C. smithii* + seems to be uniform for the short version, in agreement with the absence of any indication of variation in this region on direct sequencing of mixtures of PCR products of the Gfor-Grev primer pair, and from analysis of genomic DNA. More important to the genealogy study, *C. smithii* + appeared to differ from all standard *C. reinhardtii* at one nucleotide site, marked X in Figure 1. For a more stringent examination of this nucleotide position we designed two relatively short forward primers differing only by C/T at the 3' end (Figure 1, 17-T and 17-C), and paired each with Grev for PCR to test whether this nucleotide position was indeed homogenized completely to T in *C. reinhardtii* and to C in *C. smithii* +. With the use of higher annealing temperatures, as seen in supplementary Figure S3 (<http://www.genetics.org/supplemental/>), 17-T succeeded in priming a PCR product only with standard *C. reinhardtii* DNA, while 17-C succeeded only with *C. smithii* + DNA, indicating that this nucleotide position is uniformly T in standard *C. reinhardtii* and C in *C. smithii* +.

It then seems most likely that there was no contribution of *C. smithii* + to the standard strains of *C. reinhardtii* unless there were absolutely no crossover events in the entire set of ribosomal repeats (8.5 kb $\times$ 200 = 1700 kb) in a hypothetical *reinhardtii* $\times$ *smithii* zygote germination. In a final effort to examine the possibility that a cross between *C. reinhardtii* and *C. smithii* + had been made, but only *C. reinhardtii* cistrons were present in the product because there had been no crossover in the region of the ribosomal repeats, we examined known products of such a cross. We obtained 10 randomly

selected F_1 products of a cross of standard *C. reinhardtii* (CC-29) $\times$ *C. smithii* + (RANUM *et al.* 1988) and determined the sequence of their Gfor-Grev PCR products as well as whether DNA from each could be primed successfully by 17-T and by 17-C. The CC-29 parent was examined as well and proved to contain predominantly shorts; as expected, these primed with 17-T but failed to prime with 17-C. Two of the F_1 products had the same phenotype and might well be uninterrupted standard *C. reinhardtii* parental cistrons. The other eight F_1 clones, however, displayed a wide variety of sequence disturbances starting at the position equivalent to the end of helix I in ITS2 and primed successfully with both 17-T and 17-C at discriminatory reannealing temperatures. We conclude then that in the region of ribosomal repeats the average frequency of crossover per zygote is about two; that is, the vast majority of zygote products would have a mixture of *C. reinhardtii* and *C. smithii* + repeats. With the additional information that *C. smithii* + has a plastid DNA restriction pattern with a number of differences from that of standard *C. reinhardtii*, but no *C. reinhardtii* examined has the *C. smithii* + plastid DNA pattern (HARRIS *et al.* 1991), we conclude that *C. smithii* + has not contributed genetically to the standard *C. reinhardtii* strains.

Algal strain confusions: Not only is our knowledge of past strain exchanges among laboratories incomplete, but also in the course of the 60 years since the *C. reinhardtii* standard strains were first isolated and dispersed to other laboratories, some laboratories subsequently have deposited strains in culture collections as *C. reinhardtii* from different collection sites. The older strains in supplementary Table S1 (<http://www.genetics.org/supplemental/>) purporting to be *C. reinhardtii* from various different places are all actually standard *C. reinhardtii* strains, since their ITS sequences are identical to those of the standard strains, their plastid DNA shows identical endonuclease restriction fragment patterns (HARRIS 1998), and their distribution of copies of the Gulliver transposon matches those of the standard strains (FERRIS 1989). These include the strains ostensibly from the Caroline Islands (SAG 11-32c), Florida (SAG18.79), France (SAG 77.81), Japan (SAG 73.72), and Pringsheim's strain SAG 11-31. In addition, the short ITS2 sequence of *C. reinhardtii* in GenBank (AF156601), sequenced in China, is actually from a strain obtained from a Denmark laboratory, one of the standard *C. reinhardtii* lines.

The *C. smithii* story is a cautionary tale for control reactions in mating experiments. Culture collections actually contain two cultures labeled *C. smithii*, one designated a (+) mating type (UTEX 1062, SAG 54.72, and CC-1373) and the other a (−) mating type (UTEX 1061 and SAG 53.72). This latter strain was isolated in California by Smith. Several laboratories used this mating-type minus strain and its presumed partner *C. smithii* + in pairings with standard *C. reinhardtii*, but HARRIS (1989)

noted that the (−) strain did not seem to mate with *C. reinhardtii* while the (+) strain did. Subsequently, the ITS sequence of this minus strain was found to be very different from that of *C. reinhardtii*, more like *C. culleus*, and it was found to be homothallic, making zygotes in clonal culture (COLEMAN and MAI 1997).

With respect to *C. incerta* (SAG 7.73) from Cuba, as recognized by ITS2 sequence identity two other strains are also identical, SAG 81.72 from Holland (originally submitted as *C. globosa*) and two equivalent strains, NIVA Chl13 = NIVA Chl21, from Norway. The Norway strains were originally labeled *C. reinhardtii*. Their putative SAG duplicate (SAG23.90) is not the same, but is rather a *C. noctigama*. The culture called *C. incerta*, SAG 23.72, from France is morphologically very different, a large cell with four flagella (SCHLÖSSER 1994).

How homogeneous are the ribosomal cistrons of an organism? In the course of this study, some 73 subclones of ITS were sequenced, and an additional 63 sequences were obtained by direct PCR sequencing (supplementary Table S1 at <http://www.genetics.org/supplemental/>). For many of these, the ITS1 was largely ignored, but the ITS2 was completed. Among the ITS1 sequences of all strains of standard *C. reinhardtii*, four examples of a single-nucleotide variant were found. For each of these four cases, either the nucleotide is unpaired in the RNA transcript secondary structure or the substituted nucleotide is compensatory and preserves the pairing at that position (see COLEMAN and MAI 1997). For ITS2, except at the tip of the first helix of ITS2, no nucleotide position variants were encountered among the standard *C. reinhardtii* strains. A single variant nucleotide was found in one subclone of *C. smithii* +, in helix II of ITS2, a compensatory change preserving the pairing, as indicated in Figure 2.

Some additional strains called *C. reinhardtii*, infertile with the standard strains, have subsequently been isolated from other areas (see supplementary Table S2 at <http://www.genetics.org/supplemental/>). At least three of the newly collected *C. reinhardtii* strains (from Minnesota, Pennsylvania, and Quebec) have both long and short versions of ITS2 helix I (Figure 1), as revealed by sequencing subclones of the ITS. Each long version is slightly different in sequence. Except for the INDEL and the C/T position differentiating standard *C. reinhardtii* and *C. smithii* +, there is essentially no other difference in either ITS1 or ITS2 of all these strains capable of interbreeding.

It remains unknown how rapidly ITS is homogenized within a genome. Homogenization of ITS repeats is apparent in all the standard *C. reinhardtii*, even to the crucial nucleotide that distinguishes it from its near relative *C. smithii* +, with the sole exception of the loop at the end of helix I in ITS2. This remains unhomogenized in the standard *C. reinhardtii* and also in the more recently collected interfertile *C. reinhardtii* strains. This is a region that is relatively poorly conserved between

species evolutionarily, but is almost universally homogenized within an organism. Why should this exception exist? We offer no suggestion, except that these *Chlamydomonas* strains would seem to provide ideal material for further study, given the apparent frequency of cross-over events among the ribosomal cistrons.

Phylogenetics: Prompted by the studies of Goodenough and Ferris (FERRIS *et al.* 1997, 2002) on the structure of the *C. reinhardtii* mating-type locus, we obtained and sequenced the ITS region of all available chlamydomonads of grossly similar morphology, as well as the ITS of newly collected strains and others examined in the literature (*e.g.*, NOZAKI *et al.* 2002). At the same time we made pairings of these organisms with standard plus and minus mating types of *C. reinhardtii* to assess mating potential.

These latter pairings include the one *C. smithii* + isolate from Massachusetts and isolates from Eastern Canada (SACK *et al.* 1994), Minnesota (GROSS *et al.* 1988), Pennsylvania and Florida (SPANIER *et al.* 1992), and North Carolina (HARRIS 1998). All show high interfertility, zygote formation, and zygote germination with viable progeny when crossed with the standard *C. reinhardtii*. All the strains found capable of interbreeding with *C. reinhardtii* originate from the east coast of North America, extending as far west as Minnesota.

Figure 5 presents the phylogenetic analysis of organisms found to be most similar to *C. reinhardtii* in ITS sequence. Also included are *Volvox carteri*, several *Gonium* isolates, and an array of *Chlamydomonas* species, omitting many groups of even more distant chlamydomonads (*e.g.*, *C. moewusii/eugametos*). Since the group encompasses considerable phylogenetic depth we first utilized the relatively conserved nucleotide positions of ITS2 (Figure 2, boldface type). As indicated by the asterisk in Figure 5, tree A, there is a major evolutionary dichotomy in the backbone. Of the six most conserved pairings in the whole Volvocacean ITS2 (MAI and COLEMAN 1997), one has undergone a compensatory base change (CBC) that separates the top half of the tree from the bottom; U-A has changed to Pu-Py. The only other CBCs involving these conserved pairings are two; the U-A change to G-U supporting the clade of SAG 5.93 with SAG 62.72 and the A-U change to a G-C, a CBC supporting the association of SAG19.90 with SAG 18.90 and *C. asymmetrica*. The second marker of the major dichotomy concerns the most conserved DNA sequence in all of ITS2, a marker found on the 5' side of helix III. The top half of the tree is uniform for GGCCTCTACTGGGTAGGCA at that position, except for one transition in a secondary structure bulge in *V. carteri*, while several variants appear among these sequences in the bottom half of the tree.

We then selected the strains of the top half of the tree and examined their relationships using all positions of ITS1 and ITS2, including gaps as a fifth character. This produces a slightly more subdivided branching,

at least in distance analysis, but did not change the relationships of the *C. reinhardtii* and their closest relatives, as shown in Figure 5B. As expected, all the interbreeding strains of *C. reinhardtii*/*smithii* + group together. Their ITS1 and ITS2 sequences are identical (except the tip of helix I, ITS2). The *C. reinhardtii*/*smithii* + strains are barely separated from *C. incerta* and *Chlamydomonas* sp. from Kenya; all are identical for nucleotide positions in ITS2 found to be relatively conserved (Figure 2).

The next closest chlamydomonads are *Chlamydomonas* sp. (from Kenya) and *C. incerta* (from Cuba). Strains isolated from the same site in Kenya several times, and identical in ITS sequence, fail to interact sexually with each other or with *C. reinhardtii*. *C. incerta* from Cuba is a single strain that does not interact sexually with either *C. reinhardtii* mating type or the Kenya strains. The second *C. incerta* (NIVA Chl13/21) from Norway has an identical ITS sequence to that from Cuba, as does the *C. incerta* (SAG 81.72) from the Netherlands. All are equally intractable for mating. Since we never obtained a sexual pair of the Kenya material, and the *C. incerta* strains have been in culture for years, all might possibly be sterile. *C. incerta* from Cuba is genetically mating-type minus, on the basis of the presence of the MID gene in the mating-type locus (FERRIS *et al.* 1997), and so also is the Netherlands strain, but this latter strain differs by RFLP profile from the Cuba strain (T. PRÖSCHOLD, unpublished results).

The next closest relative, by DNA comparison, is a 16-celled colonial green alga, *Gonium pectorale*. With respect to the set of relatively conserved nucleotide positions in ITS2, *G. pectorale* Alaska differs from the taxa above it in the tree at only two positions (one in a single-stranded region and one transition at the distal-most pairing of helix II), and *G. pectorale* Africa differs at one additional nucleotide, a bulge in helix II. A number of *G. pectorale* mating-type pairs capable of interbreeding are available (FABRY *et al.* 1999); none crosses with *C. reinhardtii*, but the Alaska pair of strains is most similar to *C. reinhardtii* by ITS comparison.

A relatively close evolutionary relationship between colonial greens and the *C. reinhardtii* grouping is supported by other evidence. In a study of their hatching enzymes, autolysins, enzymes that act to release daughter cells from the mother cell wall, both SCHLÖSSER (1976, 1984) and MATSUDA *et al.* (1987) found that chlamydomonads fall into subgroups, on the basis of their autolysin sensitivity. The autolysin enzyme isolated from one member of a subgroup would digest the mother wall of all the members of the same subgroup. The chlamydomonads found to be in the same autolysin subgroup as *C. reinhardtii* included *C. incerta*, *C. smithii* +, and *C. globosa* SAG 81.72 (known now to be *C. incerta* from the Netherlands). *C. reinhardtii*, *C. smithii* +, and *C. incerta* also share recognizably similar *ypt 2* and actin gene exons (LISS *et al.* 1997).

FIGURE 5.—Phylogenetic analyses of *Chlamydomonas reinhardtii* strains and putative relatives. (A) Tree, based on comparisons of the relatively conserved positions of ITS-2 of 36 taxa (116 positions marked in boldface type in Figure 2B), representing a maximum-likelihood analysis using the model according to TAMURA and NEI (1993) with equal base frequencies and Gamma distribution shape parameter ($G = 0.3853$; TrNef + G). The model was calculated as the best model with Modeltest 3.06 (POSADA and CRANDALL, 1998). The upper bootstrap values are neighbor joining (boldface type; 1000 replicates), using the same model criteria; the lower bootstrap values are maximum parsimony (boldface italic type; 1000 replicates). The tree was rooted using the basal clade, marked with a bracket. (B) The smaller tree presents the further analysis of the 23 nearest neighbors of *C. reinhardtii* (boxed in A, a branch marked with an asterisk), using the entire ITS-1 and ITS-2 sequence (851 positions) and strains UTEX 1341 and SAG 73.81 as outgroup. The tree was obtained using maximum-likelihood analysis, the Tamura and Nei model (TrN + I + G), with the proportion of invariable sites ($I = 0.2182$) and Gamma distribution shape parameter ($G = 0.5983$) calculated as best model with Modeltest. The bootstrap values are neighbor joining (boldface type; 1000 replicates), using the same model above and maximum parsimony (boldface italic type; 1000 replicates) below. Only bootstrap values $>50\%$ are presented. Taxon names in boldface type are newly sequenced here. The brace on the tree in B indicates interfertile *Chlamydomonas* strains.

The gametangium autolysin of *C. reinhardtii* can also digest the gametangium stage of six species of *Gonium* (MATSUDA *et al.* 1987), an indication of genetic relatedness further supported here since the next closest relatives by ITS analysis are a pair of *G. pectorale* strains and *V. carteri*—in fact, this is roughly the position of all the Volvocaceae. The next most similar taxon for these relatively conserved nucleotide positions is *G. sacculiferum*, which differs at 5 positions. *C. parastreptata* differs at 18 positions.

The discovery that the colonial alga *G. pectorale* is more similar on a DNA basis to *C. reinhardtii* than are many other chlamydomonads is not entirely unexpected. In

the asexual reproduction of these algae, the mother cell undergoes 2^n mitoses before release of the daughter cells. Morphologically, the colonial forms appear to derive from the delay of completion of cytokinesis of the multiple daughter cells that the chlamydomonad mother cell makes.

Biogeography: All the interbreeding strains of *C. reinhardtii* and the one interfertile strain of *C. smithii* occur naturally in eastern North America. We have not found any organisms capable of interbreeding with it, or sharing ITS sequence, anywhere outside this area, despite considerable collecting and also sequencing of all available germane organisms. The studies of unicellular

green soil algae by Bold's laboratory (DEASON and BOLD 1960) in Texas also failed to isolate any organism interfertile with *C. reinhardtii*, although they report finding at least seven different chlamydomonads of similar morphology.

One additional insight from the recent successful rediscoveries of *C. reinhardtii* lends support to the idea of its relatively localized distribution. Four separate laboratories instituted a search for chlamydomonads interfertile with the standard *C. reinhardtii*. None mentions any excessive numbers of natural samples tried before attaining success. GROSS *et al.* (1988) mention sampling 24 sites, obtaining chlamydomonad-like isolates from each, but only one (a minus mating type from Minnesota) that interacted with *C. reinhardtii*. SPANIER *et al.* (1992) found three clones (two from Pennsylvania and one from Florida) interfertile with standard *C. reinhardtii* from ~300 sampled sites in Pennsylvania and Florida. SACK *et al.* (1994) examined 352 samples from 22 localities in Quebec and Ontario, Canada, and the midwestern United States, differing in their soil type. Nineteen of the agricultural soil types, and these only, yielded strains interfertile with *C. reinhardtii*, and all of these came from Quebec. Finally, HARRIS (1998) isolated + and - mating types of a strain interfertile with *C. reinhardtii* from a single sample of garden soil taken in North Carolina. Together with *C. smithii* +, all these strains constitute a clear biological species, since their interfertility and survival rates of intercross zygote progeny are high. They also constitute a single Z clade (COLEMAN 2000). These organisms also share an essentially identical ITS sequence, with the exception of the tip of ITS2 helix I (Figure 1), which lies in a region that is relatively nonconserved evolutionarily and which is known to vary in other algae capable of interbreeding.

Genomic variation at a finer level: There is an abundance of DNA length polymorphisms among the genomes of the interfertile strains of *C. reinhardtii* collected from different sites in the eastern United States, both in nuclear DNA and in plastid DNA. The variants have proven very useful for mapping genes. These interfertile strains are far from genetically identical at this level. Not only are the alleles shown in Figure 3 present in variant form, but also in each case where a probe for organellar DNA, or a known gene, or short repeat DNA or transposon has been used, isolates from different collections can be distinguished easily (DAY *et al.* 1988; GROSS *et al.* 1988; RANUM *et al.* 1988; FERRIS 1989; HARRIS 1989, 1998; SPANIER *et al.* 1992; HAILS *et al.* 1993; SACK *et al.* 1994; LISS *et al.* 1997). Only a mating pair collected at the same time in the same place seems to lack this level of genetic variation, as also found for other green microalgae (COLEMAN and GOFF 1991). It is this geographic site-specific genetic variation that gives us confidence in our presumptions about misidentified strains of *C. reinhardtii* and *C. incerta*, since these

were also examined for RFLPs and displayed the exact pattern expected.

Thus *C. reinhardtii* is a near ideal paradigm of a "species" as recognized among terrestrial plants and animals, with a biogeography localization to match. There is no way to conclude that the biological species *C. reinhardtii* is found only in eastern North America, since exhaustive collecting is impossible. However, considering the long history and notoriety of the standard strains and their ease of access and manipulation for mating tests, the lack of any other geographic source at least suggests that a concentration of this species exists in eastern North America.

We are very grateful to L. Krienitz (Institute of Aquatic Ecology, Neuglobsow) for dedicated collecting in Kenya, to C. Forest (Brooklyn College) for *C. reinhardtii* strains, and to Y. Matsuda (Kobe University) for calling our attention to the unusual ITS repeat structure of his CC-24J strain and providing it to us. We are also grateful for support provided to T.P. by Deutsche Forschungsgemeinschaft grant PR 682/1-1&2.

LITERATURE CITED

- COLEMAN, A. W., 2000 The significance of a coincidence between evolutionary landmarks found in mating affinity and a DNA sequence. *Protist* **151**: 1-9.
- COLEMAN, A. W., 2003 ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends Genet.* **19**: 370-375.
- COLEMAN, A. W., and L. J. GOFF, 1991 DNA analysis of eukaryote algal species. *J. Phycol.* **27**: 463-473.
- COLEMAN, A. W., and J. C. MAI, 1997 Ribosomal DNA ITS-1 and ITS-2 sequence comparisons as a tool for predicting genetic relatedness. *J. Mol. Evol.* **45**: 168-177.
- DAVIES, D. R., and A. PLASKITT, 1971 Genetic and structural analyses of cell-wall formation in *Chlamydomonas reinhardtii*. *Genet. Res.* **17**: 33-43.
- DAY, A., M. SCHIRMER-RAHIRE, M. R. KUCHKA, S. P. MAYFIELD and J.-D. ROCHAIX, 1988 A transposon with an unusual arrangement of long terminal repeats in the green alga *Chlamydomonas reinhardtii*. *EMBO J.* **7**: 1917-1927.
- DEASON, T. R., and H. C. BOLD, 1960 *Phycological Studies I. Exploratory Studies of Texas Soil Algae*. Pub. 6022, University of Texas, Austin, TX.
- FABRY, S., A. KOHLER and A. W. COLEMAN, 1999 Intraspecies analysis: comparison of ITS sequence data and gene intron sequence data with breeding data for a world-wide collection of *Gonium pectorale*. *J. Mol. Evol.* **48**: 94-101.
- FERRIS, P. J., 1989 Characterization of a *Chlamydomonas* transposon, *Gulliver*, resembling those in higher plants. *Genetics* **122**: 363-377.
- FERRIS, P. J., C. PAVLOVIC, S. FABRY and U. W. GOODENOUGH, 1997 Rapid evolution of sex-related genes in *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA* **94**: 8634-8639.
- FERRIS, P. J., E. A. ARMBRUST and U. W. GOODENOUGH, 2002 Genetic structure of the mating type locus of *Chlamydomonas reinhardtii*. *Genetics* **160**: 181-200.
- GROSS, C. H., L. P. W. RANUM and P. A. LEFEBVRE, 1988 Extensive restriction fragment length polymorphisms in a new isolate of *Chlamydomonas reinhardtii*. *Curr. Genet.* **13**: 503-508.
- GROSSMAN, A. R., E. H. HARRIS, C. HAUSER, P. A. LEFEBVRE, D. MARTINEZ *et al.*, 2003 *Chlamydomonas reinhardtii* at the crossroads of genomics. *Eukaryot. Cell* **2**: 1137-1150.
- HAILS, T., M. JOBLING and A. DAY, 1993 Large arrays of tandemly repeated DNA sequences in the green alga *Chlamydomonas reinhardtii*. *Chromosoma* **102**: 500-507.
- HARRIS, E. H., 1989 *The Chlamydomonas Sourcebook*. Academic Press, San Diego.
- HARRIS, E. H., 1998 Introduction to *Chlamydomonas*, pp. 1-11 in *The*

- Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas*, *Advances in Photosynthesis*, Vol. 7, edited by J.-D. ROCHAIX, M. GOLDSCHMIDT-CLERMONT and S. MERCHANT. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- HARRIS, E. H., 2001 *Chlamydomonas* as a model organism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**: 363–406.
- HARRIS, E. H., J. E. BOYNTON, N. W. GILLHAM, B. D. BURKHART and S. M. NEWMAN, 1991 Chloroplast genome organization in *Chlamydomonas*. *Arch. Protistenk.* **139**: 183–192.
- HOSHAW, R. W., and H. ETTL, 1966 *Chlamydomonas smithii* sp. nov.—a chlamydomonad interfertile with *Chlamydomonas reinhardtii*. *J. Phycol.* **2**: 93–96.
- HOWELL, S. H., and L. L. WALKER, 1976 Informational complexity of the nuclear and chloroplast genomes of *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta* **418**: 249–256.
- KUBO, T., T. SAITO, H. FUKUZAWA and Y. MATSUDA, 2001 Two tandemly-located matrix metalloprotease genes with different expression patterns in the *Chlamydomonas* sexual cell cycle. *Curr. Genet.* **40**: 136–143.
- KUBO, T., J. ABE, T. SAITO and Y. MATSUDA, 2002 Genealogical relationships among laboratory strains of *Chlamydomonas reinhardtii* as inferred from matrix metalloprotease genes. *Curr. Genet.* **41**: 115–122.
- LISS, M., D. L. KIRK, K. BEYSER and S. FABRY, 1997 Intron sequences provide a tool for high-resolution phylogenetic analysis of volvocine algae. *Curr. Genet.* **31**: 214–227.
- MAI, J. C., and A. W. COLEMAN, 1997 The internal transcribed spacer 2 exhibits a common secondary structure in green algae and flowering plants. *J. Mol. Evol.* **44**: 258–271.
- MARCO, Y., and J.-D. ROCHAIX, 1980 Organization of the nuclear ribosomal DNA of *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* **177**: 715–723.
- MATSUDA, Y., A. MUSGRAVE, H. VAN DEN ENDE and K. ROBERTS, 1987 Cell walls of algae in the Volvocales: their sensitivity to a cell wall enzyme and labeling with an anti-cell wall glycopeptide of *Chlamydomonas reinhardtii*. *Bot. Mag. Tokyo* **100**: 373–384.
- NOZAKI, H., K. ONICHI and E. MORITA, 2002 Differences in pyrenoid morphology are correlated with differences in the *rbcL* genes of members of the *Chloromonas* lineage (Volvocales, Chlorophyceae). *J. Mol. Evol.* **55**: 414–430.
- POSADA, D., and K. A. CRANDALL, 1998 Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.
- RANUM, L. P. W., M. D. THOMPSON, J. A. SCHLOSS, P. A. LEFEBVRE and C. D. SILFLOW, 1988 Mapping flagellar genes in *Chlamydomonas* using restriction fragment length polymorphisms. *Genetics* **120**: 109–122.
- SACK, L., C. ZEYL, G. BELL, T. SHARBEL, X. REBOUD *et al.*, 1994 Isolation of four new strains of *Chlamydomonas reinhardtii* (Chlorophyta) from soil samples. *J. Phycol.* **30**: 770–773.
- SAITO, T., M. INOUE, M. YAMADA and Y. MATSUDA, 1998 Control of gametic differentiation and activity by light in *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* **39**: 8–15.
- SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- SCHLÖSSER, U. G., 1976 Entwicklungsstadien—und sippenspezifische zellwand—autolysine bei der freisetzung von fortpflanzungszellen in der gattung *Chlamydomonas*. *Ber. Deutsch. Bot. Ges.* **89**: 1–56.
- SCHLÖSSER, U. G., 1984 Species-specific sporangium autolysins (cell-wall-dissolving enzymes) in the genus *Chlamydomonas*, pp. 409–418 in *Systematics of the Green Algae*, edited by D. E. B. IRVINE and D. M. JOHN. Academic Press, New York.
- SCHLÖSSER, U. G., 1994 SAG-sammlung von algenkulturen at the University of Göttingen—catalogue of strains 1994. *Bot. Acta* **107**: 113–186.
- SMITH, G. M., and D. C. REGNER, 1950 Inheritance of sexuality in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **36**: 246–248.
- SPANIER, J. G., J. E. GRAHAM and J. W. JARVIK, 1992 Isolation and preliminary characterization of three *Chlamydomonas* strains interfertile with *Chlamydomonas reinhardtii* (Chlorophyta). *J. Phycol.* **28**: 822–828.
- STARR, R. C., and J. A. ZEIKUS, 1993 UTEX—the culture collection of algae at the University of Texas at Austin. *J. Phycol.* **29** (Suppl): 1–106.
- Swofford, D. L., 2002 *PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods)*, Version 4. Sinauer Associates, Sunderland, MA.
- TAMURA, K., and M. NEI, 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**: 512–526.
- WHITE, T. J., T. BRUNS, S. LEE and J. TAYLOR, 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322 in *PCR Protocols*, edited by M. A. INNIS, D. H. GELFAND, J. H. SNINSKY and T. J. WHITE. Academic Press, New York.

Communicating editor: B. BARTEL