The Use of DNA Probes for Taxonomic Study of Dictyostelium Wild Isolates

William B. Evans, Joanne E. Hughes and Dennis L. Welker

Molecular Biology/Biochemistry Program, Department of Biology, Utah State University, Logan, Utah 84322-5500

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ABSTRACT

The classification of 27 wild isolates assigned to Dictyostelium discoideum on the basis of morphological criteria was reexamined using probes specific for DNA sequences cloned from the type strain NC4. These probes included ones specific for ribosomal spacer DNA regions and for a ribosomal RNA coding sequence, as well as probes for two chromosomal gene families (actin and discoind) and for the DIRS-1 transposable element. Four isolates (AC4, WS526, WS584 and ZA3A) which had previously been shown to have unusual mating characteristics were distinctly different from other isolates. We interpret these differences as indicating that the four atypical isolates represent species other than D. discoideum. Probes for the ribosomal spacer DNA either did not hybridize to the DNA of these four isolates or had decreased levels of hybridization to EcoRI restriction fragments of different lengths to that observed with the type strain. With the discoind probe, all isolates had DNA fragments that hybridized but AC4, WS526, WS584 and ZA3A lacked a pair of fragments that were conserved in NC4 and other isolates. With the actin probe, AC4, WS526, WS584 and ZA3A lacked numerous fragments that the other isolates shared with NC4. The DIRS-1 probes showed strong hybridization with ZA3A and weak hybridization to the other three isolates; however, the major EcoRI fragment in WS526 and WS584 was smaller than that in NC4 while ZA3A and AC4 had fragments of similar size to that in NC4. The asexual isolates WS526 and WS584 were closely related to each other and appear to be isolates of the same species although there were some differences in the overall pattern of EcoRI restriction fragments from high copy number sequences and of restriction fragments hybridizing to the actin and discoind gene probes. The homothallic isolates AC4 and ZA3A appear to represent two additional species; ZA3A, and possibly AC4, are more closely related to D. discoideum than the species represented by WS526 and WS584. Overall it appears that the four atypical isolates (AC4, ZA3A, WS526 and WS584) are less distantly related to D. discoideum than are isolates of D. purpureum and D. mucoroides.

The cellular slime mold Dictyostelium discoideum has been studied for many years by developmental biologists. This organism differentiates during asexual fruiting body formation into only two major cell types, spores and stalk cells (LOOMIS 1982; SPUDICH 1987). Molecular genetic analyses involving transformation, insertional mutagenesis, antisense RNA, and amplified gene expression are now possible (NELLEN, SILAN and FIRT EL 1984; KNECHT and LOOMIS 1987; DELOZANNE and SPUDICH 1987; REYMOND, NELLEN and FIRT EL 1985; REYMOND et al. 1986). Molecular approaches are also being employed to expand the genetic linkage map of D. discoideum (GRANT, WELKER and WILLIAMS, 1985; WITKE et al. 1986; WELKER et al. 1986; DINGERMANN et al. 1987) and to study nuclear plasmid DNAs (METZ et al. 1983; NOEGEL et al. 1985; NOEGEL, METZ and WILLIAMS 1985). Many of the recent genetic studies use either protein polymorphisms or restriction fragment length polymorphisms (RFLPs). Most of the polymorphisms utilized have been identified by comparison of different wild isolates. For linkage analyses, the wild isolate must be crossed to a genetically marked haploid tester strain to produce a diploid from which segregant haploids are then obtained (WELKER, HIRTH and WILLIAMS 1985; GRANT, WELKER and WILLIAMS 1985; WELKER et al. 1986). In order to facilitate such studies wild isolates must be properly assigned to their respective species. Likewise, it is important to know that plasmid-bearing isolates are properly identified; particularly in the construction and use of transformation vectors based on endogenous plasmids, so that the host range of the plasmids can be defined and problems associated with it understood.

In the past, morphological features of the amoebae, asexual fruiting bodies and sexual structures have been used for classification in the Dictyostelids (RAPER 1984). However, increased knowledge of the wild isolates has made it apparent that there are limitations associated with classification based solely on morphology, since it is known that morphological characteristics are dependent to some extent on various aspects of culture conditions (RAPER 1984). Nucleic
acid and protein homologies have been useful for species identification and phylogenetic studies in other organisms. Phylogenetic work based on DNA includes determinations of sequences, hybridization studies, and analyses of RFLPs. DNA sequences present in multiple copies per cell have often been utilized. These include mitochondrial DNA, kinetoplast DNA, repetitive DNA, and, in particular, the DNA encoding ribosomal RNA (Dutta 1986; Avise, Lansman and Shade 1979; Morel et al. 1980; Borst et al. 1980; Jeffreys, Wilson and Thein 1985; Appels and Dvorak 1982; Matthews and Debonte 1985; Woese 1987). Genes encoding ribosomal RNAs are highly conserved because of the ubiquitous requirement for these nucleic acids, hence their sequences can be used to determine the phylogenetic relationships of groups that have been separate for long periods of time. DNA sequence analysis of a ribosomal RNA gene indicates that D. discoideum diverged early from other eukaryotes (McCarroll et al. 1983). Characterization of the less highly conserved spacer DNA surrounding the ribosomal RNA genes by sequencing, hybridization or RFLP analysis provides information on more recently separated species.

The DNA encoding the ribosomal genes in D. discoideum has been studied extensively. This rDNA is present in extrachromosomal palindromic dimers of about 88 kb (Cockburn, Taylor and Firtel 1978). Each cell contains about 90 dimers allowing easy visualization of restriction fragments from the rDNA on agarose gels stained with ethidium bromide. Cloned DNA is available for much of the coding and spacer DNA on the rDNA palindromes (Ness et al. 1983; Emery and Weiner 1981). RFLPs affecting this DNA have been identified (Welker, Hirth and Williams 1985). Hence we chose to investigate whether or not wild isolates designated as D. discoideum on the basis of morphological criteria were closely related based on (1) the ability of their DNA to hybridize with probes derived from the rDNA of the D. discoideum type strain NC4 and (2) where hybridization to the rDNA probes occurred, on the presence of RFLPs. We also utilized probes for two protein-coding gene families (actin and discoidin) and the DIRS-1 transposable element. Our findings indicate that 4 of 27 isolates previously designated D. discoideum are not closely related to the others. These results are consistent with those obtained using assays based on protein polymorphisms (Briscoe et al. 1987).

MATERIALS AND METHODS

Strains and growth conditions: Most of the D. discoideum wild isolates (Table 1) have been described previously (Erdoes, Raper and Vogel 1973; Robson and Williams 1979; Robson and Williams 1980; Poole and Firtel 1984; Welker et al. 1986; Dingermann et al. 1987). D. discoideum isolates DD185, DD285 and DD385 were obtained from D. Francis (University of Delaware). Isolates of the other species were obtained from K. B. Raper (University of Wisconsin), D. Francis (University of Delaware), D. Waddell (Bergische Universitat, Wuppertal), J. C. Cavender (Ohio University), G. W. Erdoes (University of Florida), D. H. O'Day (University of Toronto) and the American Type Culture Collection. Strains were grown at 21°C in association with Escherichia coli Br t on DM medium (Poncet and Deering 1980) or with Klebsiella aerogenes on SM medium (Welker 1986; Sussman 1966). The geographical origins and sexual mating characteristics of the D. discoideum isolates are given as part of Table 1.

DNA isolation: DNAs were isolated using a modification of previously published techniques (Welker et al. 1986). DNA isolated from bacterially grown cells was extracted with phenol and chloroform, ethanol precipitated and then further purified using cesium chloride density gradients. After recovery from the gradients the DNA was spool-precipitated with ethanol, rinsed, and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 7.5).

Slot blots, electrophoresis and hybridization: The Schleicher and Schuell Minifold II slot blot apparatus was used for slot blots; genomic DNA (1 µg/slot) was pretreated and loaded according to the manufacturer's instructions. Following application of the DNA, the nitrocellulose membrane was rinsed in 5 × SSC, baked for 2 hr, then prehybridized, hybridized, and hybridization detected according to standard techniques. DNA samples for gels were digested with restriction enzymes and separated on 0.8% agarose gels using a Tris phosphate running buffer (36 mM Tris, 30 mM NaH2PO4, 1 mM EDTA). The separated DNA fragments were blotted to nylon membranes (Zetabind, Bio-Rad) using the alkali transfer protocol (Reed and Mann 1985) or to nitrocellulose (Nitroplus 2000, Micron Separations) by the Southern (1975) protocol. For use as probe DNA, E. coli plasmids with inserts of D. discoideum DNA were nick translated using either 32P-dATP or biotinylated dUTP. Biotin-labeled probe DNA was detected using kits and protocols supplied by Bethesda Research Laboratories. The probes were: pCT1 (Emery and Weiner 1981), pEcoIV and pEcoVII (Ness et al. 1983), pDd812 (Devine, Tsang and Williams 1982), ACTIN-8 (Romans and Firtel 1985), GM45A (Chung, Zuker and Lodish 1983), pB41-6 (Zuker and Lodish 1981). Probes pCT1 and pEcoIV are of ribosomal spacer DNA, pEcoVII is of ribosomal gene-coding DNA; pDd812 is a cDNA clone of a discoidin 1 gene; ACTIN-8 contains a genomic sequence encoding an actin gene; GM45A and pB41-6 contain the DIRS-1 internal EcoRI fragment and terminal repeat, respectively.

RESULTS

Slot blot analysis of Dictyostelium isolates using rDNA probes: As with other groups of species, the spacer DNA surrounding the ribosomal RNA genes of Dictyostelium species can be used in taxonomic studies of this genus. Spacer DNA probes (pEcoIV, and pCT1) cloned from NC4-derived strains of D. discoideum did not hybridize to DNA purified from D. mucoroides (12 isolates) and D. purpureum (10 isolates). These probes did not hybridize with DNAs from a more limited number of isolates of the following species: D. caveatum, D. giganteum, D. lacteum, D. rosarium and D. sphaerocephalum. A probe for the
TABLE 1

Hybridization of rDNA probes to isolates identified as *D. discoideum* by morphological criteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>pEcoVII</th>
<th>pCt1</th>
<th>pEcoIV</th>
<th>Mating characteristics</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC4</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>Homothallic</td>
<td>Mexico</td>
</tr>
<tr>
<td>DD61</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
<td>Kansas</td>
</tr>
<tr>
<td>DD185</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Not tested</td>
<td>Delaware</td>
</tr>
<tr>
<td>DD285</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Not tested</td>
<td>Delaware</td>
</tr>
<tr>
<td>DD385</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>HU182</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>HU188</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>K10</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Not tested</td>
<td>Ohio</td>
</tr>
<tr>
<td>MFD</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Not tested</td>
<td>Delaware</td>
</tr>
<tr>
<td>NC4</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
<td>North Carolina</td>
</tr>
<tr>
<td>OHIO</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
<td>Ohio</td>
</tr>
<tr>
<td>V12</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>WS7</td>
<td>+++</td>
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</tr>
<tr>
<td>WS51</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
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</tr>
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<td>+</td>
<td>+++</td>
<td>Sexual</td>
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</tr>
<tr>
<td>WS269</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
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</tr>
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<tr>
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</tr>
<tr>
<td>WS526</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>Asexual</td>
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</tr>
<tr>
<td>WS576</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
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<td>Wisconsin</td>
</tr>
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<td>WS582</td>
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<td>+</td>
<td>+++</td>
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</tr>
<tr>
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<td>+</td>
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<tr>
<td>WS584</td>
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<td>-</td>
<td>-</td>
<td>Asexual</td>
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</tr>
<tr>
<td>WS656</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
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<td>Wisconsin</td>
</tr>
<tr>
<td>WS1956</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Sexual</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>WS2162</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Bisexual</td>
<td>Wisconsin</td>
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<tr>
<td>ZA3A</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Homothallic</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Hybridization of probes for ribosomal spacer DNA (pCt1 and pEcoIV) and ribosomal RNA coding DNA (pEcoVII) was scored using slot blots as follows: ++++, strong hybridization; +, weak hybridization; −, no detected hybridization. Mating characteristics and origins are adapted from BRISCOE and coworkers (1987) based on the work of ERDOS, RAPER and VOCEN (1973), of RAPER (1984), and of ROBSON and WILLIAMS (1979, 1980). Particularly relevant to the assignment of WS380B, WS576 and WS269A to the "sexual" isolates are their ability to exchange genetic material by parasexual diploid formation with *D. discoideum* matA tester strains (ROBSON and WILLIAMS 1980).

coding region (pEcoVII) hybridized to DNA purified from all wild isolates, including those of the other Dictyostelid species. Results with representative *D. purpureum* and *D. mucoroides* isolates are shown in Figure 1. These results establish that sequence differences have accumulated in the spacer DNAs in *Dictyostelium* species but that, as expected, the coding regions are more conserved. Hybridization patterns with DNA from 22 of 26 isolates identified as *D. discoideum* on the basis of morphological criteria were similar to that observed for the NC4 wild isolate itself (or its derivative AX3K). This was true whether the probe DNA was obtained from spacer or coding sequences (Figure 1, Table 1). Of the remaining four (AC4, WS526, WS584 and ZA3A), hybridization patterns of WS526 and WS584 were similar to those observed with the isolates known to be from species other than *D. discoideum*; there was no hybridization to pEcoIV and pCt1. More complicated patterns were observed with AC4 and ZA3A. The pCt1 probe did not hybridize with DNA from AC4 but limited hybridization was seen to this probe with DNA from ZA3A; the pEcoIV probe hybridized only slightly to AC4 but appeared to hybridize extensively to ZA3A (Figure 1, and data not shown). All four hybridized to the pEcoVII probe. These four isolates were among isolates previously reported to have unusual sexual mating characteristics. WS526 and WS584 were classed as asexual while AC4 and ZA3A were homothallic (ERDOS, RAPER and VOCEN 1973; ROBSON and WILLIAMS 1979, 1980). Together these results suggest that the four isolates are not closely related to the other isolates presently identified as *D. discoideum*.

**Restriction fragment length analysis of Dictyostelium isolates using rDNA probes:** In previous work using rDNA probes to identify restriction fragment length polymorphisms in 14 *D. discoideum* isolates, several polymorphisms affecting spacer DNA were identified (WELKER, HIRTH and WILLIAMS 1985). That work did not include the four atypical isolates (AC4, WS526, WS584 and ZA3A) identified by the slot blot analysis. To further study the relatedness of these isolates to the other isolates, we analyzed *EcoR*I restriction fragment patterns using ethidium bromide staining and blots of 0.8% agarose gels. This analysis included NC4 and five additional isolates whose
Isolates of *D. mucoroides* and *D. purpureum* analyzed as controls in these experiments gave the expected results for strains of species other than *D. discoideum* (Figure 2). Banding patterns with ethidium bromide were distinctly different from those of the *D. discoideum* isolates. Each of these six isolates had an EcoRI restriction fragment that hybridized to the rRNA gene probe (pEcoVII) but did not contain fragments that hybridized to either of the spacer DNA probes (pCT1 and pEcoIV). However, isolates assigned to *D. purpureum* appeared to represent separate groups; DP2 and DP7, which can mate, were similar to each other but distinct from DP3645. All three isolates assigned to *D. mucoroides* had a different pattern of bands.

Restriction fragment length analysis using probes for chromosomal gene families: Additional information on the status of AC4, WS526, WS584 and ZA3A was obtained from analysis of the actin and discoidin gene families. Both gene families have been characterized in previous work, so cloned probes were available (Romans and Firtel 1985; Devine, Tsang and Williams 1982). Restriction fragment length polymorphisms among known *D. discoideum* isolates also have been identified for these gene families (Welker et al. 1986; Poole and Firtel 1984; D. L. Welker, unpublished results). Analysis of these genes was expected to reveal differences both in the presence or absence of hybridizing sequences and in the length of restriction fragments carrying these sequences.

The copy numbers of actin genes in the four atypical isolates were similar to that in NC4 but the pattern of HindIII fragments hybridizing with the ACTIN-8 probe were markedly different from that observed with NC4 (Figure 3). AC4 and ZA3A had unique patterns while the patterns in WS526 and WS584 shared numerous bands. While the other *D. discoideum* isolates, as expected, differed somewhat from NC4, most restriction fragments in these isolates were indistinguishable in size from those of NC4 (Figure 3).

Isolates of *D. purpureum* and *D. mucoroides* had similar actin gene numbers to that found in *D. discoideum* but, as expected, the pattern of restriction fragments was distinctly different from that in NC4 (Figure 3). As on the ethidium bromide stained gel, fragment patterns of *D. purpureum* isolates DP2 and DP7 were related; DP3645 had a pattern distinct from that of DP2 and DP7. The pattern of each of the three *D. mucoroides* isolates differed from each other.

The discoidin probe (pDd812) detected more extensive polymorphism among the *D. discoideum* isolates. However, each of the five isolates (WS112B, WS269A, WS380B, WS576, and WS2162) we tested apparently shared a pair of fragments with NC4 (Figure 4). This pair of fragments was missing from...
AC4, WS526, WS584, ZA3A and isolates of *D. purpureum* and *D. mucoroides* (Figure 4). In other work, the 1.8 kb fragment was shown to vary in size in some *D. discoideum* isolates but no polymorphisms affecting the 1.4-kb fragment have been observed (D. L. Welker, unpublished results). *D. purpureum* and *D. mucoroides* isolates contained fragments that hybridized with the discoidin gene probe but the weaker hybridization to these fragments indicated that there were differences in the DNA sequence. WS526 and WS584 had related patterns of fragments as did the *D. purpureum* isolates DP2 and DP7. AC4, ZA3A and the remaining *D. purpureum* and *D. mucoroides* isolates had unique banding patterns.

Restriction fragment length analysis using probes for the DIRS-1 transposable element: Information obtained using probes for the internal DIRS-1 coding sequence and for the DIRS-1 terminal repeats was consistent with the data obtained with the other probes (Figure 5). However, results suggest that the four atypical isolates (AC4, ZA3A, WS526 and WS584) are more closely related to *D. discoideum* than the isolates of *D. purpureum* and *D. mucoroides* are to *D. discoideum*. There was little or no hybridization with *D. purpureum* and *D. mucoroides* isolates (data not shown) but there was some hybridization with each of the four atypical isolates. ZA3A hybridized strongly with both probes and the major hybridizing band was indistinguishable in size from that observed with *D. discoideum* isolates. AC4 hybridized weakly to both probes but the major hybridizing band observed with these probes was again similar in size to that seen in NC4. WS526 and WS584 also hybridized weakly to both probes. However, the size of the major hybridizing band in each case was smaller than in NC4. The sizes of these bands in WS526 and WS584 were similar.

**DISCUSSION**

Our results indicate that 4 of 27 wild isolates classified as *D. discoideum* on the basis of morphological criteria (AC4, WS526, WS584, ZA3A) differ extensively from the *D. discoideum* type strain NC4.
and other *D. discoideum* isolates. These findings are consistent with data based on a set of allozyme and cell surface proteins (Briscoe et al. 1987). Our interpretation is that these four isolates are of species other than *D. discoideum*. The concept of a biological species being “the members in aggregate of a group of populations that interbreed or potentially interbreed with each other under natural conditions” (Futuyma 1986) is applicable to *D. discoideum*. *D.
Genetic exchange between strains of a species modulates the effects of genetic changes, for example by facilitating their spread throughout the species. The presence of a set of similar restriction fragments in two strains is a direct indication of shared genetic material. The absence of similar fragments in strains presumed to have a common ancestor is an unequivocal indication of the accumulation of genetic change. The evidence for the accumulation of extensive genetic change uncovered in our work and that of Briscoe and coworkers (1987) coupled with the inability of AC4, ZA3A, WS526 and WS584 to exchange genetic material with known D. discoideum strains provides a compelling argument for the removal of these four isolates from the species D. discoideum.

Conversely, our data also support the findings of Briscoe and coworkers (1987) that the four atypical isolates are more related to D. discoideum than are isolates identified as D. purpureum and D. mucoroides. All four isolates retained sequences sufficiently similar to hybridize with some or all of the probes used, while with isolates of D. purpureum and D. mucoroides hybridization to fewer of the probes was found. The extent of hybridization and the sizes of the homologous fragments observed with the rDNA and DIRS-1 probes suggest that ZA3A is most closely related to D. discoideum, AC4 is somewhat less related, and WS526 and WS584 are least related to D. discoideum. This sequence similarity is consistent with the prior classification of these isolates as D. discoideum; these isolates are expected to have some affinity to D. discoideum unless their similar morphological characteristics arose independently. We interpret these results as implying descent of D. discoideum and the four atypical isolates from a common ancestor not shared by the species D. purpureum or D. mucoroides.

Our investigation was set up to be a qualitative study of genetic relatedness among these isolates. For this, probes that identify restriction fragments bearing members of multigene families are excellent. The differences observed with the actin probe and the discoidin probe are striking. However, it is difficult to quantitate the data for comparison to work with other species, primarily because one is unsure that fragments of similar size come from homologous regions of the genome rather than having a similar size due to chance. The data presented in Table 2 is consequently an underestimate of the genetic change. The approach of Briscoe and coworkers (1987) provides a better basis for comparison of polymorphisms in Dictyostelium to those in other species; we refer the reader to their discussion for such a comparison. For quantitation it would be better to use a collection of unique sequence probes so that no ambiguity exists in the identification of homologous fragments. Of the probes we used the rDNA spacer and rDNA gene coding probes as well as the DIRS-1 central fragment probe best fit this criterion. We wish to point out that the different probes pick out different degrees of polymorphism. The sequences surrounding different gene families in D. discoideum have diverged to different degrees. Restriction fragments bearing actin genes are more conserved among D. discoideum isolates than are fragments bearing discoidin genes (this work, Welker et al. 1986; D. L. Welker, unpublished data). Restriction fragments bearing tRNA genes or copies of the Tdd-2 element are even less conserved (Dingermann et al. 1987; D. L. Welker unpublished results). These highly polymorphic fragments may be useful for studying intraspecific relationships. Preliminary results with unique gene probes such as those for myosin or calmodulin indicate that sequences surrounding essential single copy genes, not unexpectedly, have a much higher degree of conservation (D. L. Welker, unpublished results). These differences in conservation of fragment length reflect the nonessential nature of specific members of multigene families versus the requirement to maintain a functional single copy gene. Differences in the apparent rate of change observed with probes for different gene families are not unexpected considering that characters in other species have also been shown to evolve at different rates.

Of the four atypical isolates, WS526 and WS584 were closely related to each other. Except for a few
differences in the sizes of high copy number EcoRI restriction fragments and of fragments hybridizing to the actin and discoidin probes, patterns were identical for this pair of isolates. Using probes and monoclonal antibodies to study α-actinin, WITKE and coworkers (1986) observed that these two isolates were similar to each other but distinct from a set of *D. discoideum* isolates. Likewise BRISCOE and coworkers (1987) found no differences affecting the mobility of enzymes and no differences in the reaction with monoclonal antibodies to cell surface proteins between WS526 and WS584. On the basis of the evidence of close genetic relatedness, we conclude that WS526 and WS584 are isolates of a single species. Indeed WS526 and WS584 are closer genetically to each other than most of the sexual *D. discoideum* isolates are to NC4.

AC4 and ZA3A were different from all other isolates examined and appear to represent single isolates of two additional species. This pair of isolates had distinctly different patterns of high copy number DNA fragments from other isolates, and the sizes of EcoRI fragments that hybridized to specific probes for regions of the rDNA palindrome were unique. In particular, sizes of fragments hybridizing with pEcoVII, which carries portions of the NC4 rRNA genes, differed from sizes observed in all other isolates. Unlike WS526 and WS584, AC4 and ZA3A had sequences that hybridized with the rDNA spacer probes from NC4. Both AC4 and ZA3A hybridized to pEcoIV; ZA3A also hybridized to pCT1. But again the sizes of the hybridizing fragments differed from those in all other isolates. ZA3A and AC4 also had unique patterns of fragments that hybridized to the actin and discoidin probes. The data obtained with the DIRS-1 probes are consistent with the hypothesis that ZA3A and AC4 represent separate species. With these probes the amount of hybridization to ZA3A was similar to that of *D. discoideum* isolates, as was the size of the major hybridizing band. While AC4 retained hybridization to DIRS-1 probes and the size of the major hybridizing band was similar to that in NC4, the amount of hybridization was decreased. The differences observed with the DIRS-1 probe are in keeping with previously characterized sequence divergence of DIRS-1 elements isolated from a single *D. discoideum* strain; these included both single base pair changes as well as deletions (ZUKER et al. 1984; CAPPELLO, COHEN and LODISH 1984). The variation in DIRS-1 copy number is also consistent with variations in copy numbers of transposable elements seen with other species.

Isolates presently designated *D. purpureum* and *D. mucoroides* had no detectable hybridization to the DIRS-1 probes or the rDNA spacer probes but as expected did hybridize to the rRNA gene probe. However, numerous differences were detected by ethidium bromide staining and the gene family probes in the small set of *D. purpureum* and *D. mucoroides* isolates that we analyzed in more detail. These findings and supporting data of BRISCOE and coworkers (1987) suggest that isolates presently designated *D. purpureum* and *D. mucoroides* each may also represent several species. This is not surprising considering the almost worldwide distribution of isolates and the range of sexual mating characteristics attributed to these species (RAPER 1984). We are presently studying these species in more detail and our results to date are consistent with this conjecture.

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