Relative Nuclear DNA Content of Rust Fungi Estimated by Flow Cytometry of Propidium Iodide-Stained Pycniospores

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ABSTRACT

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Flow cytometry was used to estimate the relative nuclear DNA content of pycniospores from 85 collections of 13 species of rust fungi. For a given sample, 10,240 fluorescent events were measured. Sufficient pycniospores could be obtained from as little as one well-developed pycnial cluster. Numbers of fluorescent events plotted against intensity of fluorescence usually exhibited a single well-defined peak with coefficients of variation of 10% or less. Results confirmed and extended data for small numbers of basidiospores obtained earlier with the microscope photometer. DNA content relative to that of a *Puccinia hordei* standard was estimated to be as follows (in order of increasing DNA content): *P. lagenophorae*, 53%; *P. graminis*, 56%; *P. coronata*, 64%; *P. sorghi*, 84%; *P. hordei*, 101%; *P. recondita*, 105%; *Uromyces hippomarathricola*, 107%; *U. reichertii*, 120%; *Tranzschelia pruni-spinosae*, 150%; *P. allii*, 164%; *P. helianthi*, 185%; *U. vignae*, 336%; and *U. appendiculatus*, 346%. Within *P. hordei*, collections from *Hordeum bulbosum* had higher DNA content than collections from other telial host species; within *P. recondita*, collections from each of four telial host species differed, suggesting that these fungi are diverging genetically on some telial host species.

The size of the nuclear genome in rust fungi can provide clues to evolutionary relationships and help in the selection of species for genetic and molecular genetic investigations. Eilam et al (13) estimated the relative DNA content of nine species of rust fungi by measuring the fluorescence intensity of propidium iodide (PI)-stained basidiospore nuclei. The results indicated that DNA content spanned a 10-fold range among the nine species examined and that collections within some species varied with telial host.

Eilam et al (13) took measurements on basidiospores because they had little background fluorescence, although pycniospores also gave satisfactory results. PI was used as the DNA fluorochrome because it intercalates into the DNA backbone independently of base-pair ratio (11,17,26), in contrast to DNA fluorochromes such as 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst 33342, which bind preferentially to AT-rich regions (23), which in turn, can differ among species. Although results were satisfactory, the microscope photometer method was slow and cumbersome to use, and samples were limited to about 100 spores per fungal collection.

Subsequently, we have evaluated the flow cytometer as an alternative method for measuring intensity of nuclear fluorescence in rust fungus spores. Flow cytometry has been used to measure relative nuclear DNA content in cells of diverse organisms by measuring the intensity of fluorescence of nuclei stained with PI or other DNA fluorochromes (8,18) and has recently been used with spores of several species of basidiomycetes and myxomycetes (1). The flow cytometer can be used to measure thousands of fluorescent "events" in a few seconds, and data on forward scatter can indicate relative size of particles. Because PI-stained basidiospores and pycniospores have little or no background fluorescence, both are suitable candidates for flow cytometry. However, large quantities of pycniospores are obtained readily from pycnial clusters, whereas basidiospores must be collected over a period of several days during teliospore germination.

We have used pycniospores to measure relative nuclear DNA content by flow cytometry with results reported here. Our objectives were: 1) to develop a practical, rapid, and accurate method to measure relative nuclear DNA content of rust fungi, including an evaluation of the DNA fluorochromes PI and Hoechst 33342; 2) to confirm and extend the results of Eilam et al (13) by comparing DNA content of a variety of rust fungus species, largely in *Puccinia* and *Uromyces*; and 3) to evaluate variation among collections from different telial hosts for *P. coronata, P. hordei,* and *P. recondita* in which differences in DNA content were indicated in a preliminary way by Eilam et al (13).

MATERIALS AND METHODS

Collections of pycniospores were obtained from pycnial hosts (Table 1). Some of the collections were of rust species used in our earlier investigation of nuclear DNA content in which we used a microscope photometer (13). Other rust species were added to extend the range of species and number of collections investi-

TABLE 1. Species of *Puccinia*, *Tranzschelia*, and *Uromyces*, geographic origin, and hosts of rust collections used as sources of pycniospores for flow cytometry of nuclear DNA

Species	Collection no.[a]	Origin	Telial host	Pycnial host
P. allii F. Rudolphi	TA-8658	Isr., Golan Heights	*Allium ampeloprasum* L.	*Allium ampeloprasum*
P. allii	TA-8659	Isr. N. Negev	*A. ampeloprasum*	*A. ampeloprasum*
P. allii	TA-8716	Isr., Cent. Coastal Plain	*A. ampeloprasum*	*A. ampeloprasum*
P. coronata Corda	91-CRBN	U.S., Minn.	*Avena sativa* L.	*Rhamnus cathartica* L.
P. coronata	TA-2705	Poland	*A. sativa*	*Rhamnus palaestinus* Boiss.[b]
P. coronata	TA-2706	Poland	*A. sativa*	*R. palaestinus*
P. coronata	TA-8640, TA-8641, TA-8642	Isr., Mount Carmel	*Avena sterilis* L.	*R. palaestinus*
P. coronata	TA-8662	Isr., W. Galilee	*A. sterilis*	*R. palaestinus*
P. coronata	TA-8671	Isr., Judean Foothills	*A. sterilis*	*R. palaestinus*
P. coronata	TA-8681	Isr., Golan Heights	*A. sterilis*	*R. palaestinus*
P. graminis Pers.:Pers. f. sp. *secalis* Eriks. & E. Henn.	T90-21-1-AR	U.S., Minn.	*Agropyron repens* (L.) P. Beauv.	*Berberis vulgaris* L.
P. graminis Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn.	SZA 4A-1	U.S., Minn.	*Triticum aestivum* L.	*B. vulgaris*
	SZA 1C-1	U.S., Wash.		
P. helianthi Schwein.	TA-8619	Isr., Cent. Coastal Plain	*Helianthus annuus* L.	*Helianthus annuus*
P. helianthi	TA-8674, TA-8676	Irs., N. Negev		*H. annuus*
P. hordei G. Otth	TA-5307	Isr., Mount Carmel	*Hordeum bulbosum* L.	*Ornithogalum* spp.
P. hordei	TA-5308, TA-5311	Isr., Judean Foothills	*H. bulbosum*	*Ornithogalum* spp.
P. hordei	TA-5316	Isr., E. Upper Galilee	*H. bulbosum*	*Ornithogalum* spp.
P. hordei	TA-4133, TA-4136	Isr., S. Coastal Plain	*Hordeum glaucum* (=*H. murinum* L.)	Unknown[c]
P. hordei	TA-4139	Isr., W. Galilee	*H. glaucum*	Unknown[c]
P. hordei	TA-4140	Isr., Judean Foothills	*H. glaucum*	Unknown[c]
P. hordei	TA-1687, TA-1690, TA-1691, TA-1697, TA-1698, TA-1699, TA-1701, TA-1706, TA-1709, TA-1710, TA-1711, TA-1712, TA-1732, TA-1740	Isr., Judean Foothills	*Hordeum spontaneum* C. Koch	*Ornithogalum* spp.
P. hordei	TA-1692	Isr., Mount Carmel	*H. spontaneum*	*Ornithogalum* spp.
P. hordei	TA-1696	Isr., N. Negev	*H. spontaneum*	*Ornithogalum* spp.
P. hordei	TA-1701, TA-1702, TA-1703	Isr., Golan Heights	*H. spontaneum*	*Ornithogalum* spp.
P. hordei	TA-879	Isr., Judean Foothills	*Hordeum vulgare* L.	*Ornithogalum* spp.
P. hordei	TA-880	Isr., E. Lower Galilee	*H. vulgare*	*Ornithogalum* spp.
P. lagenophorae Cooke	TA-8631	Isr., Cent. Coastal Plain	*Senecio vernalis* L.	*Senecio vernalis*
	TA-8672			
P. recondita Roberge ex Desmaz.	TA-9308, TA-9382	Isr., N. Negev	*Aegilops longissima* Schw. & Muschl.	*Anchusa* spp.
P. recondita	TA-9351	Isr., Cent. Coastal Plain	*A. longissima*	*Anchusa* spp.
P. recondita	TA-9383, TA-9384 TA-9385	Isr., Judean Foothills	*A. longissima*	*Anchusa* spp.
P. recondita	TA-9285	Isr., Mount Carmel	*Aegilops ovata* L.	*Echium glomeratum* L.
P. recondita	TA-9295 TA-9371	Isr., E. Upper Galilee	*A. ovata*	*E. glomeratum*
P. recondita	TA-9370	Isr., Upper Galilee	*A. ovata*	*E. glomeratum*
P. recondita	TA-9381	Isr., Judean Foothills	*A. ovata*	*E. glomeratum*
P. recondita	TA-2694, TA-2695	Poland	*Triticum aestivum*	*Thalictrum speciosissimum* L.
P. recondita	TA-9300, TA-9306, TA-9356, TA-9365, TA-9395	Isr., S. Coastal Plain	*T. aestivum*	*T. speciosissimum*[b]
P. recondita	TA-9358	Isr., Valley of Esdraelon	*T. aestivum*	*T. speciosissimum*[b]
P. recondita	TA-9367, TA-9372, TA-9394, TA-9411	Isr., Cent. Coastal Plain	*T. aestivum*	*T. speciosissimum*[b]
P. recondita	TA-9386	Isr., Golan Heights	*Triticum dicoccoides* (Kornh. ex Aschers. & Graebn.) Aarons.	*T. speciosissimum*[b]
P. recondita	TA-9399	Isr., Mount Hermon	*T. dicoccoides*	*T. speciosissimum*[b]
P. recondita	TA-2664, TA-2684, TA-2685	Ethiopia	*Triticum durum* Desf.	*T. speciosissimum*[b]
P. recondita	TA-2688	Chile	*T. durum*	*T. speciosissimum*[b]
P. recondita	TA-9291	Isr., Lower Galilee	*T. durum*	*T. speciosissimum*[b]
P. sorghi Schwein.	TA-8709	Isr., Lower Jordan Valley	*Zea mays* L.	*Oxalis corniculata* L.
T. pruni-spinosae (Pers.:Pers.) Dietel	TA-8904	Isr., N. Negev	*Prunus amygdalus* Batsch.	*Anemone coronaria* L.
U. appendiculatus (Pers.:Pers.) Unger	53	U.S., Fla.	*Phaseolus vulgaris* L.	*Phaseolus vulgaris*
U. hippomarathricola S. da Cam	TA-8905	Isr., Cent. Coastal Plain	*Bilacunaria boissieri* Pimenov et Tichomirrov	*Bilacunaria boissieri*
U. reichertii Anikst. & Wahl.	TA-6385	Isr., Valley of Esdraelon	Hordeum bulbosum	*Scilla hyacinthoides* L.
U. vignae Barclay	CPR-1	U.S., Ga.	Vigna unguiculata L.	*Vigna unguiculata*

[a]Collections are designated by isolate or race number.
[b]Shown with greenhouse inoculations only.
[c]Unknown in nature; *Ornithogalum* spp. are hosts when artificially inoculated.

gated. A total of 33 collections of *P. hordei* were included because of their availability and to determine if collections from different telial hosts differed in nuclear DNA content. Collections were mostly from Israel and North America (Table 1).

Pycnial clusters were obtained either directly from field collections of naturally infected plants, usually all within a square-meter sampling area (collections TA-8904 and TA-8905, Table 1), or from pycnia produced in the greenhouse by inoculating plants with basidiospores from germinating teliospores, following the procedures of Anikster (3). Pycniospores were usually harvested 10–20 days after plants were inoculated. They were collected and stained in a buffer-dye solution of Tris-HCl buffer (0.18 M, pH 7.2) containing PI at 4 μg/ml, RNase at 50 μg/ml, and Triton X-100 at 4 μg/ml. All chemicals were from Sigma Chemical Co. (St. Louis). The RNase stock solution (10 mg/ml) was boiled 10 min to inactivate DNase.

One end of a glass haematocrit capillary tube (1 mm, inside diameter) was placed in the buffer-dye solution until 5–10 mm of the tube was filled by capillary action. The tip of the tube then was touched lightly to the surface of a pycnial cluster, allowing pycnial nectar and pycniospores to enter the tube. The tube was moved lightly over the pycnial surface, with care not to break off fragments of the leaf surface, until spore masses were evident inside the tip of the tube. The tube usually was touched to more than one pycnial cluster, although enough spores for flow cytometry could be collected from one well-developed pycnial cluster when necessary.

The spore suspension was blown from the haematocrit tube into 200 μl of buffer-dye solution in a 13- × 100-mm glass test tube. The tube was shaken on a vortex shaker for 1–2 s. The spore suspension was incubated in darkness at room temperature for 2–3 h to allow PI to stain the nuclei and then was either introduced into the flow cytometer immediately or frozen and stored at −20 C until used. For use as a biological reference in the flow cytometer, a sample of *P. hordei* (collection TA-1699) was harvested and processed in the same manner as the pycniospores to be measured.

Pycniospores were stained with Hoechst 33342 instead of PI in some experiments. The spores were collected in 50% ethanol in the capillary tube and expelled into 200 μl of 50% ethanol in 1-ml Eppendorf microtubes and stored at 4 C for 24–48 h. The spore suspension was centrifuged for 4 min in an Eppendorf microfuge. The spores then were resuspended in Tris-HCl buffer (0.1 M, pH 7.4), spun down a second time, and suspended in the Tris buffer containing Hoechst 33342 at 1.5 μg/ml and 0.1 M NaCl. After 3 h, the spore suspension was introduced into the flow cytometer.

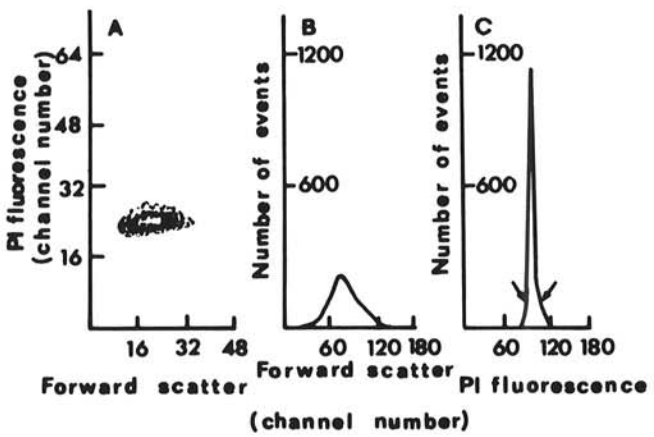

Fig. 1. Example of output data for 10,240 fluorescent events from flow cytometer for propidium iodide-stained pycniospores of *Puccinia hordei* (collection TA-1699). **A,** Contour map of fluorescent events plotted as forward scatter (a function of particle size) on the x-axis and fluorescence intensity (channel number) on the y-axis. Contour lines delimit the percentage of events within each contour as listed. The outermost contour contained 95% of events, the innermost contour, 5%. **B,** Histogram of amount of forward scatter (channel number) on the x-axis and number of fluorescent events on the y-axis. **C,** The data from **A** plotted as a histogram of fluorescence intensity (channel number) on the x-axis and number of fluorescent events on the y-axis. Arrows indicate limits set 2 coefficient of variation units on each side of the mean.

A Becton Dickinson (Mountain View, CA) FACS IV flow cytometer, equipped with an argon laser, was used to measure fluorescence intensity of stained pycniospores. The flow cytometer was calibrated daily with chicken red blood cells as a standard, bringing the output with 650 mW to channel 100. For PI, excitation was at 488 nm, and a 570 nm emission filter was used. For Hoechst 33342, excitation was at 351.1 and 363.8 nm, and a 400 nm emission filter was used. Usually, data for 10,240 particles (events) were collected, which normally required only a small part of the 200-μl sample. Partly because the chicken red blood cells had a much larger DNA content than our samples had, for a standard we used, instead, pycniospores of *P. hordei* (collection TA-1699) harvested and processed in parallel with each group of isolates stained at a given time. Using the standard, the output of the cytometer was set at channel 100 (except for *Uromyces appendiculatus* and *U. vignae*, for which the cytometer was set at 25 and the resulting values were multiplied by four). Fluorescent intensity of samples is expressed as channel number.

Data from the flow cytometer were analyzed with Consort 40 (Mountain View, CA) software. Contour maps of fluorescence intensity versus forward scatter (Fig. 1A) and histograms of forward scatter (Fig. 1B) were examined for gross departures from a well-defined cluster. The amount of forward scatter of light is proportional to particle size. Departure from a well-defined peak may indicate the presence of mixtures or contaminants. The data were viewed as a histogram of nuclei with a given fluorescence intensity (Fig. 1C). Edges of peaks were first trimmed away by a rough estimate of 2 coefficients of variation (C.V.) units on each side of the peaks. After preliminary trimming, the mean and C.V. for the peak were determined. The edges of the peaks then were reset to include all data within 2 C.V. units on each side of the mean (Fig. 1C). The mean value and C.V. are reported for individual samples, a conventional practice in flow cytometry. Variation among samples of a given type is expressed by the standard deviation of the mean. Duncan's multiple range test was used to test for differences among collections from different telial hosts within a rust species.

Stained pycniospores were examined with a Zeiss Universal microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence microscopy. Filter sets were number 14 (green excitation) for PI and number 05 (blueviolet excitation) for Hoechst 33342. To illustrate the nucleus and spore together (Fig. 2),

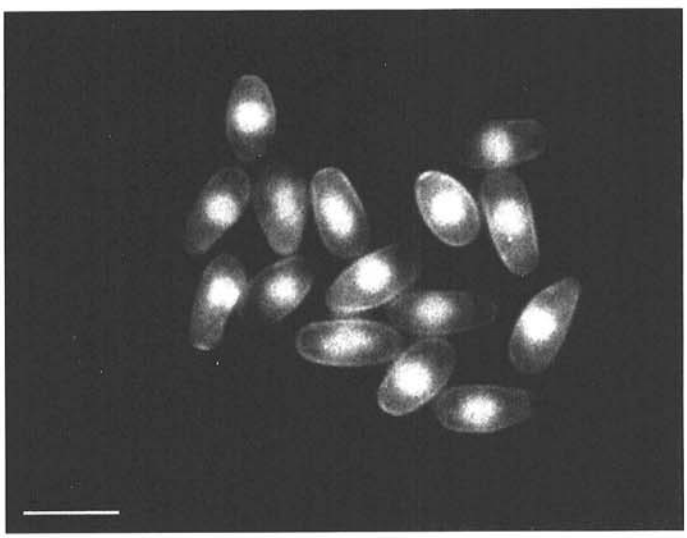

Fig. 2. Pycniospores of *Puccinia recondita* (collection TA-9383) stained with propidium iodide (for nuclei) and with Calcofluor White (for spore walls) as viewed by epifluorescence microscopy. Bar = 10 μm.

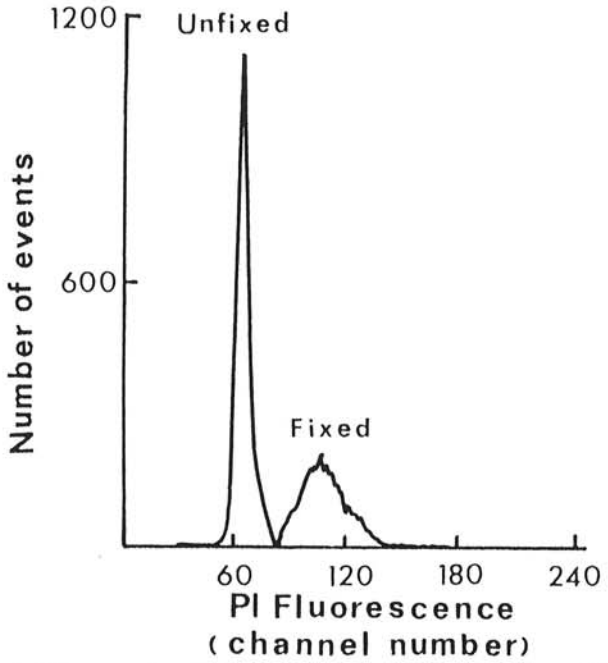

Fig. 3. Histogram data for fluorescence of unfixed and fixed propidium iodide-stained pycniospores of *Puccinia coronata* (collection TA-8641). Coefficient of variation values were much lower with unfixed than with fixed spores.

pycniospores were double stained, first with 0.2% aqueous Calcofluor White (American Cyanamid Co., Chemicals Group, Wayne, NJ), second with PI, and viewed by epifluorescence with filter set number 09 (blue excitation).

RESULTS

Evaluation of methodology. The single nucleus of the pycniospore fluoresced intensely, as viewed by epifluorescence microscopy, whether stained with PI (Fig. 2) or Hoechst 33342 (data not shown). Background fluorescence outside the nucleus was virtually nonexistent. Stained pycniospores (whether with PI or Hoechst 33342) flowed readily through the flow cytometer. Contour mapping of fluorescence intensity versus forward scatter (Fig. 1A) or histograms of forward scatter (Fig. 1B) showed a tight cluster of events. The distribution of forward scatter (an indication of particle size) indicated that few, if any, pycniospores were clumped (Fig. 1A and B). Histograms of numbers of fluorescent events versus amounts of fluorescence (channel number) revealed well-defined peaks (Figs. 1C, 3, and 4). Eighty percent or more of events usually were included when peaks were delimited within 2 C.V. units on each side of the mean (Fig. 1C).

Results with Hoechst 33342 as a nuclear fluorochrome were approximately the same as with PI in that well-defined peaks with low C.V. were obtained. For six species tested, fluorescence intensity relative to the biological reference standard (pycniospores of collection TA-1699, *P. hordei*) was approximately the same with Hoechst 33342 (data not shown). Nevertheless, we

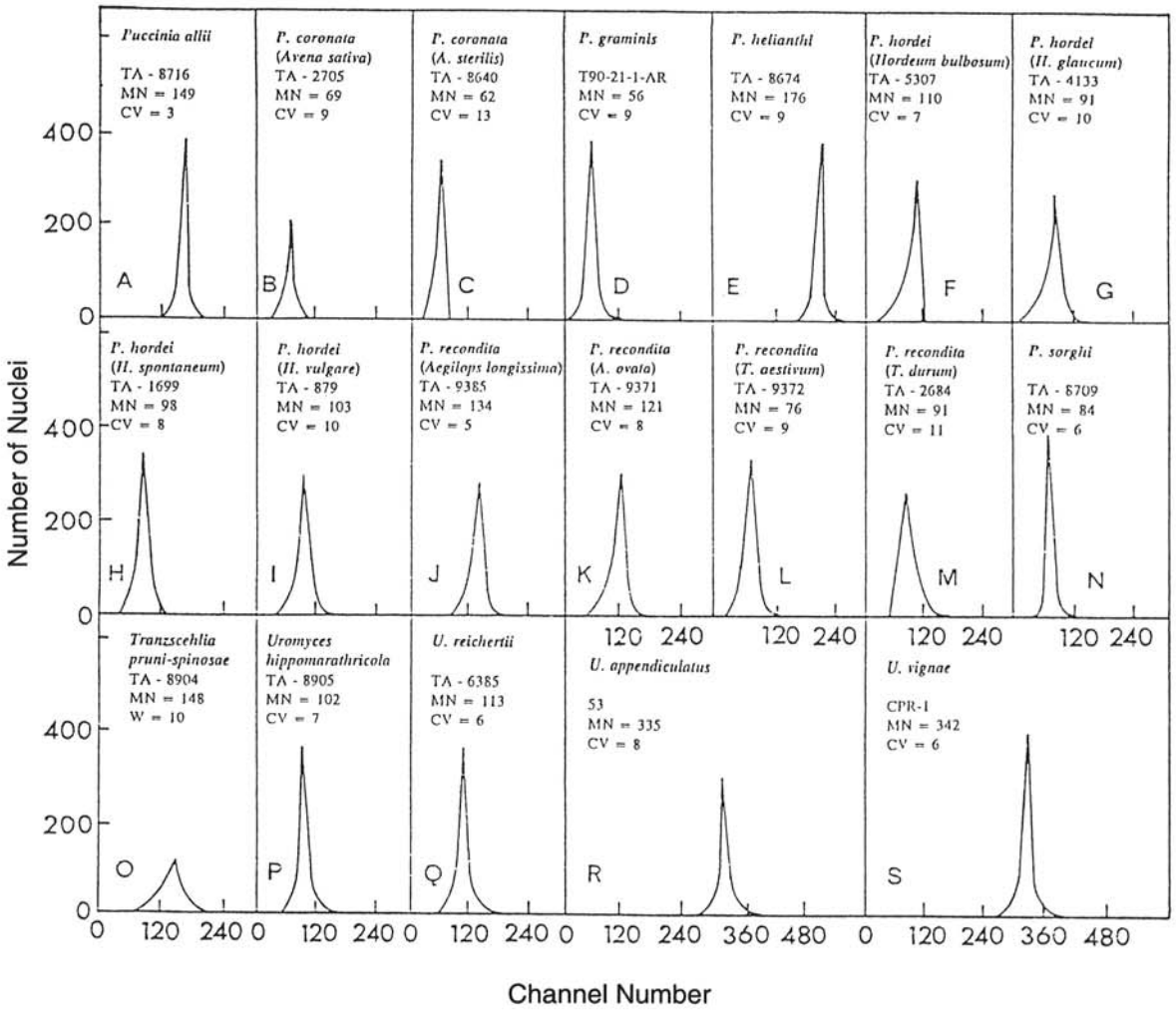

Fig. 4. Histograms showing numbers of nuclei of given fluorescence intensities obtained by flow cytometry for propidium iodide-stained pycniospores of collections representative of 12 species of rust fungi (includes all species used except *Puccinia lagenophorae*). For *P. coronata, P. hordei,* and *P. recondita*, data are shown for representative collections from different telial hosts (indicated in parentheses). Collections are described in Table 1. The mean and coefficient of variation values are listed for the peak for each collection.

elected to use PI for most measurements to avoid possible differences in fluorochrome binding related to differences among species in amount of AT-rich regions in DNA.

Results with PI relative to the *P. hordei* standard were the same whether stained pycniospores were used immediately or frozen and stored before use (data not shown). Results were also the same when spores were frozen before staining (data not shown), except that C.V. values tended to be larger when fresh spores were stained. Fixation of spores in 70% ethanol before staining led to 1.1- to 1.6-fold increases in fluorescence intensity, but unfixed pycniospores yielded narrower and better defined histogram peaks than did fixed spores (Fig. 3). Consequently, we used unfixed instead of fixed spores for the experiments presented here.

If RNase were omitted from the PI solutions used to stain spores, fluorescent intensities were highly variable and usually increased 1.5- to 1.9-fold, indicating that the RNase, when used, was effective in removing large amounts of RNA. The effect of RNase was about the same whether spores were unfixed, frozen, or fixed.

Relative nuclear fluorescence of rust collections. Mean fluorescent intensity expressed as channel number for the 13 species measured spanned a 6.5-fold range (Fig. 4; Table 2). At the low end were *P. coronata, P. graminis,* and *P. lagenophorae* with mean channel numbers of 53–65; at the high end were *U. vignae* and *U. appendiculatus* with mean channel numbers of 336–346.

Within *P. hordei* and *P. recondita*, significant differences were found among collections from different telial hosts. For *P. hordei*, four collections from *Hordeum bulbosum* averaged channel number 113, significantly higher than the values for collections from *H. glaucum, H. spontaneum,* and *H. vulgare* (Table 2; Fig. 4). Likewise for *P. recondita*, collections from each of four telial hosts differed from each other (Fig. 4; Table 2). Collections from *Triticum aestivum* had only 55% of the fluorescence intensity of collections from *Aegilops longissima*; collections from *A. ovata* and *T. durum* were intermediate in fluorescence intensity at 69 and 89% of those from *A. longissima*. Within *P. coronata*, on the other hand, no difference was found among collections from *Avena sativa* and *Avena sterilis* (Fig. 4; Table 2).

Forward scatter values among the 13 species measured tended to increase with increasing intensity of nuclear fluorescence (Table 2). *U. vignae* and *U. appendiculatus* were in a class by themselves with extremely high values for both forward scatter and fluorescence. For the 11 remaining species, correlation analysis showed that forward scatter correlated with fluorescence intensity with an R^2 value of 0.57 ($P < 0.01$). Within a species, however, forward scatter values did not differ significantly among collections, regardless of the telial host, indicating that pycniospore dimensions were similar for all collections within a species.

Occasionally, histograms of fluorescence from the flow cytometer revealed a small second peak at about twice the channel number of the main peak (Fig. 5A and C). Second peaks were seen more frequently with *P. coronata* than with other species and more frequently with the use of Hoechst 33342 than with PI, but they occurred in several species, sometimes with PI. Second peaks could be seen more distinctly if event numbers per sample were increased to 30,000 (instead of the usual 10,240). The proportion of the spore population in the second peak was 5–10%. Histograms of forward scatter showed no sign of a second peak (Fig. 5B and D), indicating that the DNA peak was not associated with pairs of spores or spores of doubled size. Populations showing the second peak were examined by epifluorescence microscopy for number of nuclei, but pycniospores with more than one nucleus were not found. We also checked for possible contamination by aeciospores but rarely found them and also found that nuclei of PI-stained aeciospores did not fluoresce. Anticipating that DNA might replicate as pycnial clusters age, we measured fluorescence intensity in pycniospores from old pycnial clusters (30 days after pycnial clusters were first seen) but found second peaks no more consistently than in young pycnial clusters.

DISCUSSION

The results for relative fluorescence of PI-stained nuclei obtained here by flow cytometry generally confirmed and extended results obtained by Eilam et al (13) with microscope photometry. Data taken by both methods for eight collections (Table 3) gave highly correlated results when expressed as fluorescence intensity

TABLE 2. Relative fluorescence intensity and forward scatter of propidium iodide-stained pycniospore nuclei from 13 species of *Puccinia*, *Tranzschelia*, and *Uromyces* as measured by flow cytometry[a]

Species	Telial host[b]	No. of collections tested[c]	Fluorescence intensity (chan. no. ± SD)[d]	Forward scatter, (chan. no. ± SD)[d]
P. allii		3	164.0 ± 11.4	98.9 ± 9.5
P. coronata	*Avena sativa*	3	65.1 ± 1.2	70.3 ± 7.8
	A. sterilis	6	63.8 ± 1.4	66.2 ± 3.4
P. graminis f. sp. *secalis*	*Agropyron repens*	1	56.0	42
P. g. tritici	*Triticum aestivum*	2	55.5 ± 0.5	45 ± 0
P. helianthi		3	185.3 ± 10.3	137.2 ± 9.0
P. hordei	*Hordeum bulbosum*	4	113.2 ± 3.3[e]	87.7 ± 7.0
	H. glaucum	4	92.0 ± 5.8	94.5 ± 6.2
	H. spontaneum	19	97.4 ± 5.0	97.8 ± 12.3
	H. vulgare	2	102.0 ± 3.0	95.0 ± 18.2
P. lagenophorae		2	53.0 ± 3.1	89.3 ± 2.3
P. recondita	*T. aestivum*	11	74.2 ± 4.1[e]	85.5 ± 8.5
	Aegilops longissima	6	133.8 ± 6.5[e]	97.6 ± 3.0
	A. ovata	5	118.7 ± 8.5[e]	90.0 ± 4.6
	T. durum	5	92.1 ± 2.8[e]	93.7 ± 6.0
P. sorghi		1	83.6	121.2
T. pruni-spinosae		1	150.0	120.0
U. appendiculatus		1	346.0	460.0
U. hippomarathricola		1	107.0	105.0
U. reichertii		2	119.7 ± 5.0	115.0 ± 5.0
U. vignae		1	336.0	560.0

[a] Intensities are relative to the fluorescence of a reference standard, collection TA-1699 of *P. hordei*.
[b] Telial host as in Table 1.
[c] Collections are listed in Table 1.
[d] Channel numbers (chan. no.) are relative to that of the reference standard, collection TA-1699 of *P. hordei*, set at channel 100. Standard deviation based on variation of mean values among collections.
[e] Significantly different from values for other hosts within the species by Duncan's multiple range test, $P < 0.01$.

relative to that of *P. hordei* from *H. spontaneum* (the collection used as a standard in the present investigation). Overall, the range and rank in amount of fluorescence of individual species was similar by both methods: *P. graminis* and *P. coronata* were among those with the least fluorescence; *U. appendiculatus* and *U. vignae* had the most fluorescence; and *P. hordei, P. recondita,* and *U. reichertii* were intermediate. This comparison indicates that flow cytometry accurately measures the amount of nuclear fluorescence of PI-stained rust fungus nuclei. As discussed by Eilam et al (13), comparisons between PI- and Feulgen-stained nuclei of diverse animals and plants generally show that PI fluorescence is a good indicator of amount of nuclear DNA. However, an undetermined amount of fluorescence from pycniospores, as measured by flow cytometry, was from mitochondrial DNA. Fluorescing mitochondria were not readily visible in PI-stained pycniospores adjacent to the bright fluorescence of nuclei (Fig. 2) and probably contributed negligible amounts of fluorescence to the total.

Genome size, as measured in megabase pairs (Mbp), can be estimated for a given collection by comparing amount of fluorescence to that of *P. graminis* f. sp. *tritici*. Backlund and Szabo (6) determined from reassociation kinetics that this fungus has 67 Mbp. The relative DNA content of their isolate (74-36-924-A) was not determined by fluorescence from either pycniospores (here) or basidiospores (13). However, a measurement with urediniospores indicated that the isolate was similar in DNA content to four other isolates of the fungus, as measured in basidiospores, which in turn differed little among each other in relative DNA content (13). Therefore, the two isolates of *P. g. tritici* used here, with mean fluorescence intensity 55.5 (Table 2), can be assumed to have about 67 Mbp. Thus, our reference standard (collection TA-1699 of *P. hordei*), with a fluorescence intensity of 100, had 1.8 times the fluorescence of the two isolates of *P. g. tritici* and, therefore, an estimated genome size of 121 Mbp. By similar calculation, *P. lagenophorae* with the smallest genome in our investigation had 64 Mbp; *U. appendiculatus* with the largest genome had 418 Mbp.

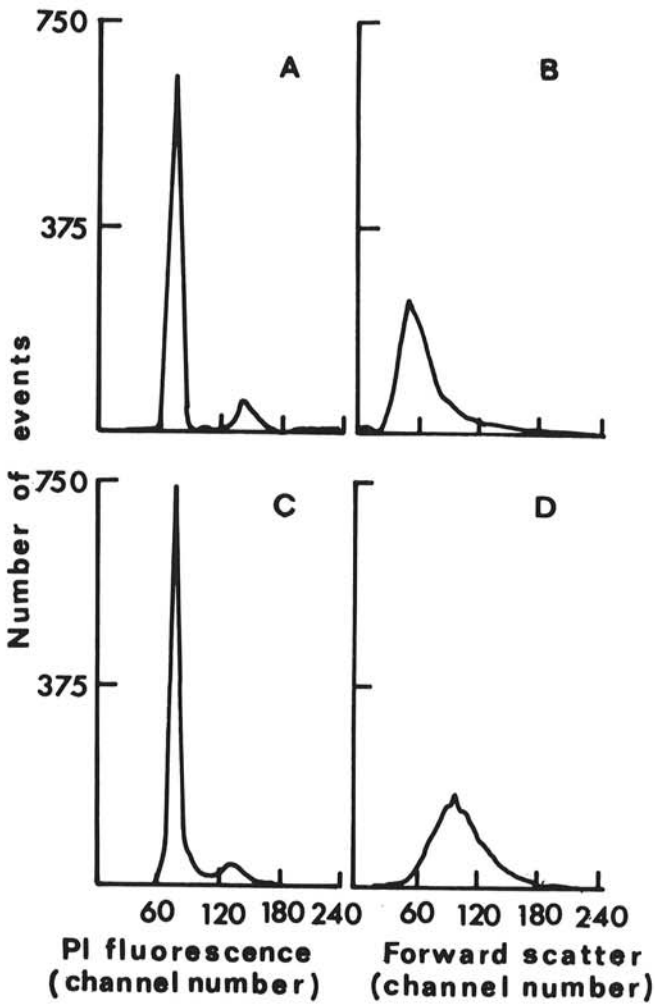

Fig. 5. A and C, Histograms of fluorescence and **B and D,** forward scatter for pycniospore collections exhibiting two peaks of fluorescence. The mean channel number of the small peak (right peak) was approximately twice that of the main peak (**A and C**). No second peak of forward scatter was evident (**B and D**), indicating that the second small peak in fluorescence was not associated with spore couplets or spores of doubled size. **A and B,** *Puccinia coronata*, collection TA-8640; **C and D,** *P. recondita*, collection TA-2784.

In contrast to other species, *P. sorghi* gave a different result in the present study compared to that of Eilam et al (13). The nuclear fluorescence value for *P. sorghi* was 84% of the value for *P. hordei* in the present study but only 48% in the study of Eilam et al (13). We used a collection from Israel (lower Jordan Valley, Table 1), whereas Eilam et al used a collection from North America (Minnesota). Because results with other species were consistent between the two investigations, we tentatively conclude that the two collections of *P. sorghi* differed in DNA content. *P. sorghi* is of special interest because the genome size of an Australian collection has been estimated by reassociation kinetics to contain 47 Mbp (2). This is 68% of the estimated value of 67 Mbp for *P. graminis* (6). A similar percent was obtained for relative DNA content of *P. sorghi* and *P. graminis* as obtained by Eilam et al (13) with the North American isolate of *P. sorghi*, whereas the Israeli isolate had 144% of the DNA content of *P. graminis* in the present study. A further survey of collections is needed to clarify the degree of diversity in DNA content within *P. sorghi*.

The results here confirmed our earlier report (13) that *P. hordei* from *H. bulbosum* has about 1.2 times (1.11–1.23) more nuclear DNA than do collections from other telial host species. The earlier study included only one collection from *H. bulbosum*; here we used four collections that were all equal in the intensity of nuclear fluorescence. Collections from *H. bulbosum* "differ from others in telial host range, in alternate host, and in ability to hybridize in nature with collections from other hosts" (13), suggesting that collections from *H. bulbosum* may be a distinct forma specialis (3,12).

Collections of *P. recondita* from four telial host species differed from each other in nuclear fluorescence (Table 2). Collections from *T. aestivum* had the least nuclear fluorescence, whereas

TABLE 3. Comparison of results by flow cytometry (FC) (pycniospores, present investigation) to results by microscope photometry (MP) (basidiospores, Eilam et al [13]) for relative fluorescence intensity of propidium iodide-stained nuclei of *Puccinia* and *Uromyces*

Species Collection no.	Telial host	Fluorescent intensity[a] FC	MP
P. coronata			
91-CRBN	*Avena sativa*	65	54
TA-8640	*A. sterilis*	64	74
P. graminis			
T90-21-1-AR		56	55
P. hordei			
TA-5311	*Hordeum bulbosum*	111	124
TA-1690	*H. spontaneum*	102	85
P. recondita			
TA-9295		122	126
U. appendiculatus			
53		346	415
U. vignae			
CPR-1		336	262

[a]For flow cytometry, the values are relative to 100, collection TA-1699 of *P. hordei* from *H. spontaneum*, used as the reference standard; for microscope photometry, the values are relative to the average (55.7 AU) of seven collections of *P. hordei* from *H. spontaneum* (Eilam et al [13]). Correlation analysis indicated that results from the two methods correlated with an R^2 value of 0.90 ($P < 0.001$).

collections from *T. durum*, *A. ovata*, and *A. longissima* had 1.2, 1.6, and 1.8 times the amount, respectively, in collections from *T. aestivum* (Table 2). In line with this, Eilam et al (13) reported that a collection originating from *T. durum* had about two-thirds the nuclear DNA content of isolates from four species of *Aegilops*. These results are in accord with studies of the host range of *P. recondita*, which showed two different types of *P. recondita* from *Triticum*, one on *T. durum* and a second on *T. aestivum* (T. Eilam and Y. Anikster, *unpublished data*). Likewise, two distinct types were found in *A. longissima* and *A. ovata*. To determine if the collections from different telial hosts represent different formae speciales, subspecies, or species, evidence is needed on genetic compatibility among the groups.

In *P. coronata*, we did not find differences in nuclear fluorescence between collections from cultivated oats, *Avena sativa*, and wild oats, *Avena sterilis*. This is in contrast to preliminary data by Eilam et al (13) who used only one collection from each host species. The absence of a consistent difference fits the conclusion that the same forma specialis attacks both species (14,15).

Flow cytometry offered several advantages over microscope photometry as used earlier by Eilam et al (13) for comparisons among rust fungus species. The protocols of sample preparation for flow cytometry are simple. Data could be obtained for 10,000 or more spores in a few minutes compared to only 100 spores in several hours by microscope photometry. The large sample size with the flow cytometer increased the accuracy of mean values. Furthermore, as discussed later, the flow cytometer revealed that some samples contained two classes of pycniospores, apparently differing in DNA content.

Pycniospores worked well for comparing fluorescence intensity of PI-stained nuclei among rust fungus species. The small, uninucleate pycniospore flowed through the cytometer without clumping. The spore was virtually colorless and produced no autofluorescence with the excitation used for PI or Hoechst 33342. Enough pycniospores for flow cytometry could be obtained from one pycnial cluster, whether young or old, and DNA content was stable with pycnial age in the spore populations. Furthermore, pycniospores could be frozen after staining and stored for several weeks before use. The pycniospores of *P. hordei* from *H. spontaneum* were an effective reference standard for flow cytometry because abundant pycnial clusters could be produced reliably on *Ornithogalum*, and the intensity of nuclear fluorescence was mid-range among the majority of rust fungus species measured.

Basidiospores also would probably give satisfactory results in flow cytometry because they also have little background fluorescence. They would provide a way to measure DNA content of microcyclic rusts, which do not produce pycniospores. As noted earlier, basidiospores are difficult to obtain in large numbers.

PI-stained urediniospores have not given satisfactory results for estimating nuclear fluorescence by flow cytometry. Relative values among species by flow cytometry correlated poorly with values by microscope photometry (data not shown). This was probably a consequence of the large amount of background fluorescence outside the nucleus in PI-stained urediniospores. Background fluorescence is abundant as viewed by epifluorescence microscopy. Although much of this background could be masked out by microscope photometry, variation in nuclear configuration remained a problem (13). We do not recommend urediniospores for estimating nuclear DNA content regardless of the method to be used.

Unexpectedly, unstained urediniospores autofluoresced when excited by the wavelengths of light used for fluorescein isothiocyanate in the flow cytometer (data not shown). We are evaluating this signal to learn if it will assist in detecting and counting urediniospores alone or in combination with forward or wide-angle scatter for distinguishing urediniospores from other spores or objects in the flow cytometer. Forward scatter combined with DNA content of basidiospores has shown promise for identification of certain species of basidiomycetes (1,22).

PI was chosen as the fluorochrome for nuclear DNA in our investigation because its binding to DNA was not dependent on AT-rich regions (11), it stained unfixed spores, it gave minimal background fluorescence, and it worked well in our earlier investigation with microscope photometry (13). However, similar results were obtained with Hoechst 33342 (Fig. 4), which binds preferentially to AT-rich regions (19,25,27). This suggests that the species of rust fungi tested with Hoechst 33342 are similar to each other in the amount of AT-rich regions in the genome. Hoechst 33342, in contrast to PI, does not stain RNA (5,7), obviating the need for RNase treatment.

Occasionally, histograms of nuclear fluorescence showed a second peak indicating that a small part of the population had twice the DNA content of the main population (Fig. 5A and C). Histograms of forward scatter showed no sign of a corresponding population of spores with increased size (Fig. 5B and D). However, samples with second peaks could not be obtained consistently or predictably whether pycnial clusters were young or old. Consequently, we are uncertain about the significance of the second peak. Nuclei of pycniospores are thought to divide after they migrate into flexuous hyphae (9,10,21). If DNA replicates in some pycniospores while still in the pycnial cluster, it follows that the remainder of the pycniospores are in the G1 phase of the nuclear division cycle.

Individual nuclei of aeciospores, basidiospores, pycniospores, and urediniospores all have the same amount of DNA, at least for certain rust species (13,28). If pycniospores are in G1, as suggested by our results, then the other spore types are also in G1. This conflicts with data by Kwon and Hoch (20) for germinating urediniospores of *U. appendiculatus* and by Hu (16) for basidiospores of *Cronartium quercuum*, indicating that these spores are in G2. Recently, Narisawa et al (24) showed by microscope photometry that nuclei of *P. coronata* in pycniospores (spermatia), aeciospores, and hyphae of axenic cultures derived from pycnial clusters were equal in DNA content. Because the hyphae were unlikely to be uniformly in G2, their results indicate that the spores and hyphae were all in G1. The finding of nuclei with doubled DNA content in some populations of pycniospores lends support to the possibility that nuclei in aeciospores, basidiospores, pycniospores, and urediniospores are predominantly in G1, at least in some species of rust fungi.

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