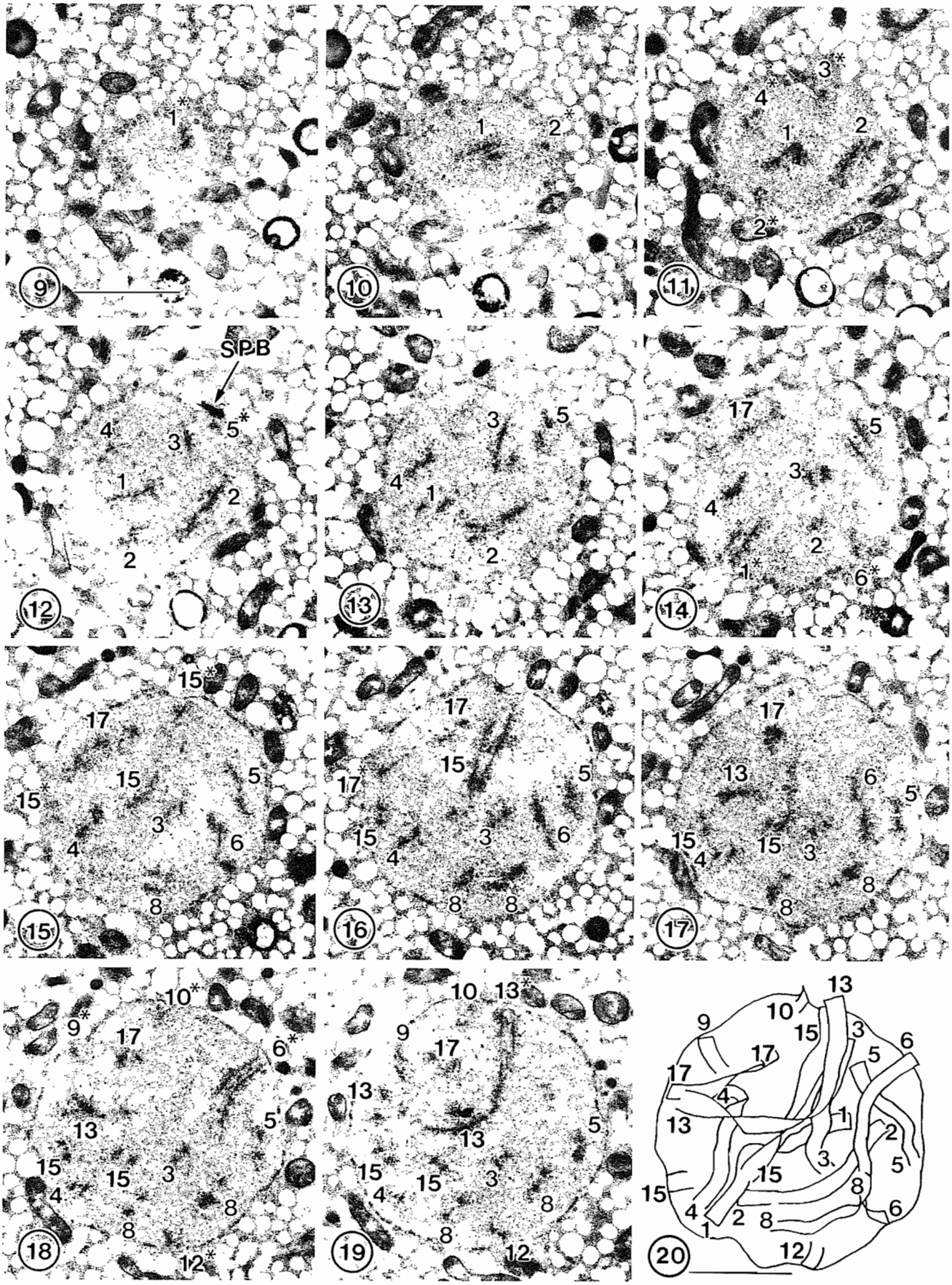
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SPB

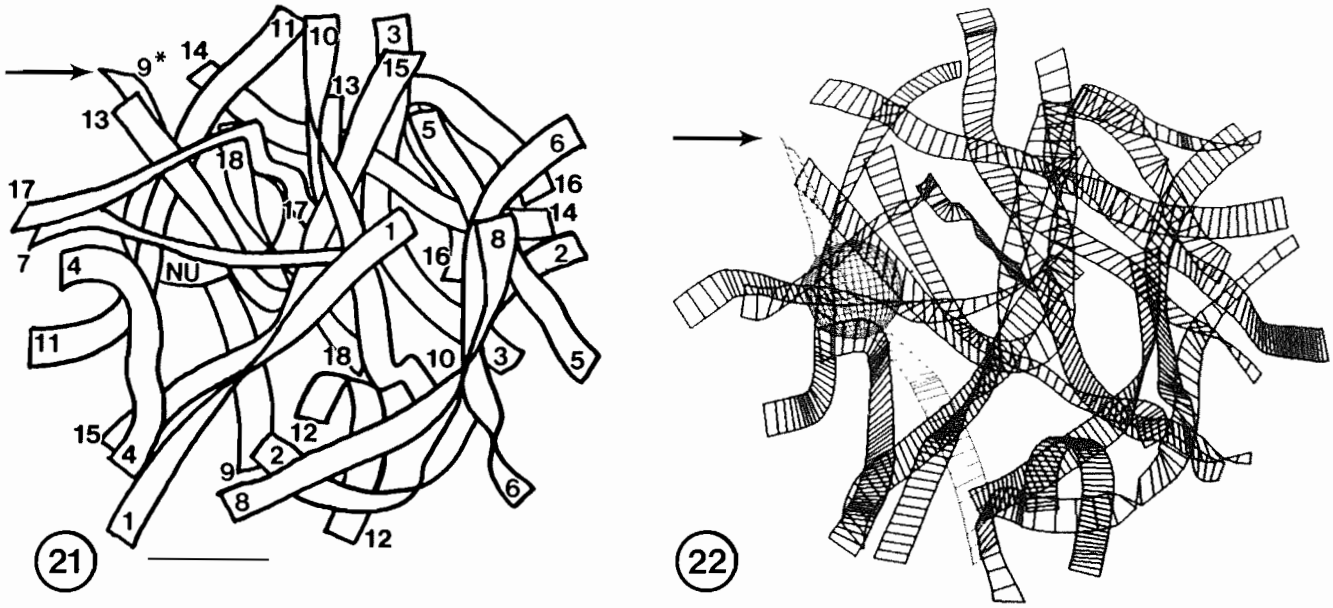



FIG. 21. A manual reconstruction of the same nucleus depicted in Figs. 9–20 of *Puccinia graminis* f.sp. *tritici* (Washington state isolate SZA 2, nucleus A). The NOR bivalent, No. 9 (arrow and *), is the only bivalent that could be identified in the six nuclei owing to its association with the nucleolus (NU). The nuclear envelope is not illustrated. Each of the 18 bivalents are numbered at both ends. Bivalent numbering corresponds to those in Figs. 9–20 and 23. Bar = 1.25 μm. FIG. 22. An orthographic computer-generated, three-dimensional wireframe rendering of the 18 bivalents of *P. graminis* f.sp. *tritici* (Washington state isolate SZA 2, nucleus A). The same nucleus is illustrated in Figs. 9–21 and 23–26. The orientation of the nucleus in Fig. 22 is the same as that in Figs. 21 and 23. The NOR bivalent (arrow) is laterally associated with the nucleolus (sphere).

TABLE 1. Total bivalent length and nuclear volumes for six reconstructed pachytene nuclei (A–F) of *Puccinia graminis* f.sp. *tritici* isolates from Washington (SZA 2), Texas (SZA 12, SZA 14), and Virginia (SZA 17)

| | SZA 2 | | SZA 12 | SZA 14 | | SZA 17 |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| Total length (μm)* | 107.2 | 103.6 | 101.6 | 98.5 | 102.5 | 100.9 |
| Volume ($\mu m^3$)† | 83 | 107 | 92 | 132 | 112 | 104 |

NOTE: Race designation of each isolate is described in Materials and methods.

*Total length of the 18 bivalents for a given nucleus involved summation of individual synaptonemal complex profiles in each section using the Pythagorean Theorem and combining the totals.

†Nuclear volume was derived from $4/3\Pi r^3$, using the average radius obtained from the medial section of each pachytene nucleus.

The 18 bivalents ranged from about 3.0 to 8.9 μm, each comprising 3.0 to 8.8% of the total cytological length of the genome (Fig. 27). Total bivalent lengths were similar among the nuclei, ranging from 98.5 to 107.2 μm, as were nuclear volumes (Table 1). Small variations in section thickness may introduce some variation in calculated bivalent length measurements. Owing to such small differences in calculated length and the similarity in length among bivalents in adjacent ranks, bivalents ranked by length (Fig. 27) may not necessarily be the same within a rank among replicates and isolates.

Computer-derived total bivalent length measurements for nucleus A (not presented) averaged about 14% less than the

FIGS. 9–19. Eleven serial sections through a fusion nucleus in pachynema of *Puccinia graminis* f.sp. *tritici* (Washington state isolate SZA 2, nucleus A) illustrating the continuity of synaptonemal complex profiles. Numbers denote fragments of synaptonemal complexes in various orientations, and asterisks (Figs. 9–12, 14–16, 18, 19) signify initiation and termination sites on the nuclear envelope. Only half of the nucleus is presented and a total of 13 bivalents are visible. Bivalents Nos. 1, 2, and 6 are complete, beginning and terminating within Figs. 9–19, while the rest of the bivalents begin but do not terminate within the 11 sections. The exception is bivalent No. 15, which begins and terminates within the 11 sections but is still incomplete. Bivalent No. 5 terminates near the spindle pole body (SPB) in Fig. 12. Bar = 2.5 μm. FIG. 20. A manual reconstruction, representing half the nucleus, of the previous 11 serial sections with numbered bivalents. Bivalents that begin but do not terminate within the 11 sections are represented by open ends. Bivalent numbering corresponds to that in Figs. 9–19 and 21. Bar = 2.5 μm.

manual length calculations for the same nucleus. Total length may have been underestimated by the use of the cardinal spline curve, which conformed to a series of points and may not have accurately represented true synaptonemal complex length, despite the adjustments that were made to fit the elements to departures from the curves. Alternatively, total length may have been overestimated by manual calculations because portions of the central element may have been measured twice in consecutive grazing sections.

Synaptonemal complexes existed as tripartite ribbonlike structures similar to those previously reported for other heterobasidiomycetes (Boehm and McLaughlin 1991). Centromeres were not observed. In some bivalents, distinct regions of attached condensed chromatin associated with the lateral elements were observed; however, not all bivalents possessed such regions and often several were observed along the length of a single bivalent. The lack of clearly resolved centromeres precluded the use of the centromeric index for cross-correlating individual bivalents as has been done in other hetero- and homo-basidiate fungi (Boehm and McLaughlin 1991; Carmi *et al.* 1978; Holm *et al.* 1981).

*NOR bivalent*

The only bivalent that could be morphologically identified among the six reconstructed pachytene nuclei was that associated with the nucleolus, presumably carrying the NOR. The NOR bivalent (bivalent No. 9, Figs. 21 and 23; bivalent with arrow in Fig. 22) appeared to pass through the nucleolus, although it could not be followed within it. The calculated length of the portions on either side of the nucleolus were nearly identical within a given nucleus, ranging from 2.4 to 3.7 μm among the six nuclei, suggesting a medial association on this particular bivalent. The calculated length of the NOR bivalent did not include the distance between attachment sites on the nucleolus.

Heterogeneity was observed for the cytological length of the NOR bivalent among isolates (Fig. 27). In the Washington state isolate (SZA 2, nuclei A and B) and the Texas isolate (SZA 12, nucleus C), the NOR bivalent was intermediate in length, consisting of 5.1 and 5.3% of the total bivalent length, respectively, whereas in the other Texas isolate (SZA 14, nuclei D and E) and the Virginia isolate (SZA 17, Nucleus F), the NOR bivalent ranged from 6.7 to 7.8% of the total bivalent length. Among replicates of a given isolate, however, differences in NOR bivalent length were small and probably insignificant (Fig. 27).

## Discussion

*Pachytene karyotype of* P. graminis *f.sp.* tritici

Ultrastructural analysis of six pachytene teliospore nuclei provided a definitive karyotype of $n = 18$ for four isolates of *P. graminis* f.sp. *tritici*, representative of both sexual and asexual North American populations. The 18 bivalents in *P. graminis* f.sp. *tritici* formed a finely graded series of chromosome pairs, possessing similar total lengths among the isolates examined (Fig. 27). The total length of the 18 bivalents averaged 102.3 μm over the six nuclei (Table 1); this is larger than those reported for other fungal karyotypes determined by the pachytene reconstruction method (Boehm and McLaughlin 1991; Braselton 1984; Carmi *et al.* 1978; Gillies 1972; Holm *et al.* 1981; Slezec 1984; Tanaka *et al.* 1982; Zickler 1977). Recently, reassociation kinetics has been used to determine the genome size of *P. graminis* f.sp. *tritici* to be $5.8 \times 10^7$ bp (Backlund and Szabo 1991). This genome size is rather larger than other fungal genomes analyzed to date, in line with our cytological data.

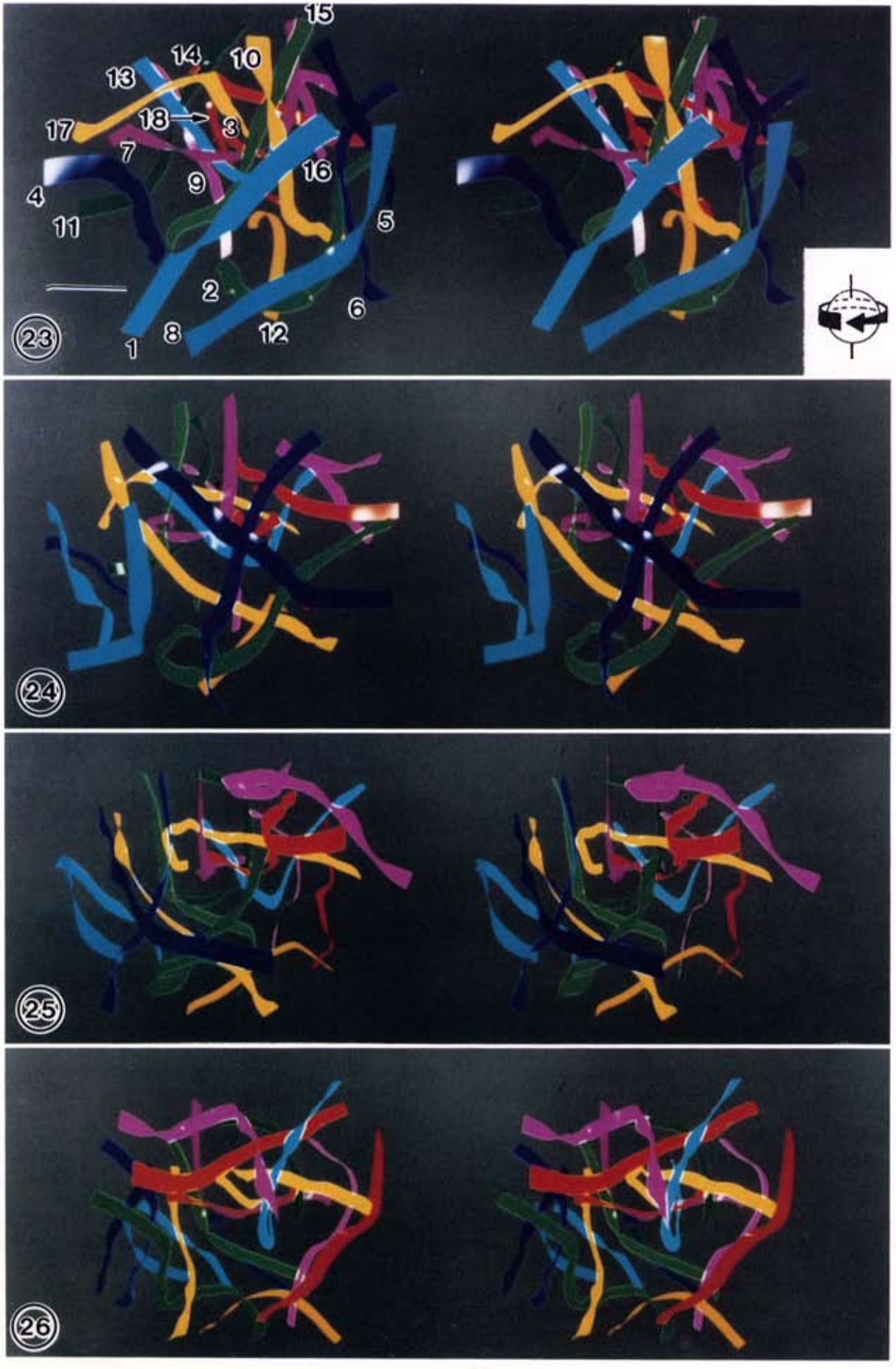
Manual reconstructions of pachytene nuclei provided the basic information needed for the karyotype of *P. graminis* f.sp. *tritici*, i.e., bivalent number and calculated lengths. However, in manually derived, two-dimensional composite line drawings the spatial relationships among bivalents were not clearly evident because most were oriented obliquely to the plane of sectioning and therefore were foreshortened to varying degrees. With computer assistance, bivalents could be represented in either orthographic or perspective orientations and were capable of being rendered as rotatable stereo pairs. Although bivalents in pachynema have been reconstructed with computer assistance in other studies (Byers and Goetsch 1975; Moens and Ashton 1985; Moens and Moens 1981; Peeples and Goldstein 1989; Tanaka *et al.* 1982), the stereo pairs presented here in perspective orientation provide a less diagramatic representation.

*Earlier rust fungus karyotypes*

Previous determinations by light microscopy of the karyotype for *P. graminis* are clearly at odds with our findings. A karyotype of $n = 6$ was presented by McGinnis (1953) for an isolate of *P. graminis* obtained from *Agropyron trachycaulum*. McGinnis (1953) used mitotic divisional phases of aceto–orcein-stained basidiospores that were analyzed prior to and coincident with germination. The chromosomes were of similar size and existed as three pairs, which led McGinnis (1956) to suggest a polyploid series within the genus. Others, working with axenic cultures of *P. graminis* f.sp. *tritici* and using light microscopy of nonpachytene nuclei, have seemingly confirmed this karyotype (Maclean *et al.* 1974; Williams and Hartley 1971). Karyotypic studies in other rust fungi have also relied on light microscopic analysis of nonpachytene nuclei and have yielded haploid chromosome numbers similar to those reported by McGinnis (1953) for *P. graminis* (Allen 1933; McGinnis 1954; Olive 1949; Singh 1972; Valkoun and Bartos 1974; Wright and Leonard 1978). Sansome (1959) was the first to derive a karyotype for a rust fungus by counting pachytene bivalents; a haploid chromosome number of $n = 20–22$ was determined for *Puccinia kraussiana* by light microscopy of pachytene bivalents released from crushed teliospores and stained with aceto–carmine.

Reconstructions of serial sections through metaphase spindles have also been used to derive chromosome numbers in the rust fungi. Such studies have yielded $n = 14$ for both *Uromyces*

FIGS. 23–26. A computer-generated, three-dimensional perspective view of the 18 bivalents of *Puccinia graminis* f.sp. *tritici* (Washington isolate SZA 2, nucleus A; the same nucleus as in Figs. 9–22) shown as colorized stereo pairs rotating in the *y*-axis in increments of 45°. The nucleus illustrated in Fig. 23 is in the same orientation as Figs. 20–22 (i.e., 0°), whereas Figs. 24–26 are 45°, 90°, and 135°, respectively, in relation to Figs. 20–23. Bivalents closest to the viewer appear larger than those in the background and are highlighted for depth enhancement. The nucleolus and the nuclear envelope are not illustrated to allow for the visibility of all 18 bivalents. The numbering of the 18 bivalents in Fig. 23 corresponds to those in Figs. 9–21. Bar = 1.25 μm.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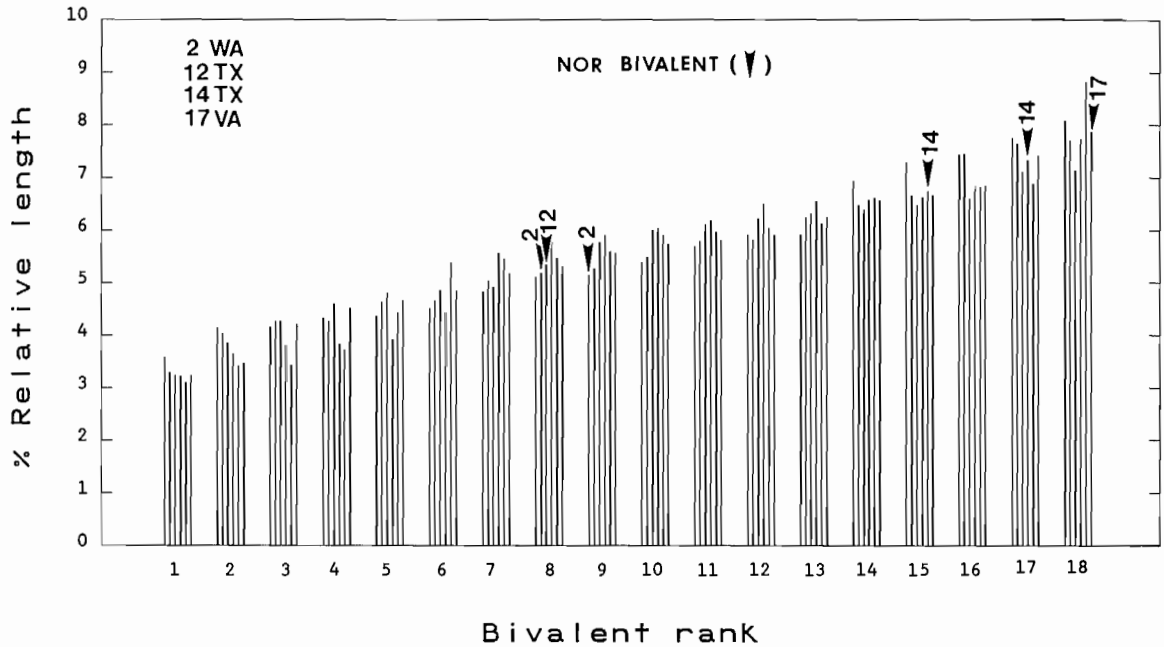


FIG. 27. The 18 bivalents of *Puccinia graminis* f.sp. *tritici* ranked by relative length (i.e., the percentage of the total cytologic genomic length). Measurements from six reconstructed pachytene nuclei are presented for each ranked bivalent in the order A–F as given in Table 1. The 18 bivalents measure only between 3.0 and 8.8% of the total length and form a graded series of similar lengths. The bivalents within a rank are not necessarily the same since lengths may overlap among adjacent ranks. The NOR bivalent for each replicate nucleus is denoted by arrowheads corresponding to the four isolates analyzed (SZA 2, 12, 14, and 17; Materials and methods and Table 1). Note that the NOR bivalent length differs among, but not within, isolates.

*vignae* (= *U. phaseoli* var. *vignae* (Cummins 1978)) (Heath and Heath 1976) and *Puccinia malvacearum* (O'Donnell and McLaughlin 1981*b*). However, unlike pachytene bivalents associated with synaptonemal complexes, metaphase chromosomes do not exist as morphologically distinct structures, often are dispersed along the length of the spindle, and possess minute kinetochoric regions with few attached microtubules (Heath 1978), thus precluding ready reconstruction of the karyotype. Results presented here and elsewhere (Braselton 1982; Carmi *et al.* 1978; Harris *et al.* 1980; Maniotis 1980; Tanaka *et al.* 1982), however, emphasize the need to reevaluate earlier light and electron microscopic fungal karyotypes using ultrastructural reconstructions of pachytene nuclei.

*Timing of pachynema*

The progression of meiotic prophase I stages, i.e., leptonema through diplonema, in the cereal rusts is not accompanied by any discernible change in probasidial (teliospore) morphology. This necessitated direct nuclear analysis using epifluorescence microscopy to select nuclei for serial sectioning. Nevertheless, further selection at the ultrastructural level was required to choose nuclei precisely at pachynema, where synaptonemal complexes had minimal lateral chromatin spread, were anchored at both ends of the nuclear envelope, and extended the full length of the bivalent; such nuclei were considered to be in the same stage and were therefore more likely to possess comparable bivalent length measurements.

Fusion nuclei in non-overwintered teliospores that stained homogeneously, with no evidence of condensed bivalents, apparently were either in early meiotic prophase (leptonema and zygonema) or in postpachynema stages (diplonema). The overwintered, homogeneously stained fusion nuclei are interpreted as being in diplonema. Since no pachytene nuclei were detected in young, hyaline, postkaryogamic teliospores and since few were detected in mature non-overwintered teliospores, we conclude that pachynema occurred concomitant with the pigmentation process or shortly thereafter, i.e., shortly after karyogamy. These observations suggest that *P. graminis* f.sp. *tritici* is in diplonema as it enters dormancy, with meiotic recombination occurring prior to overwintering. This agrees with the findings of Mims (1977, 1981), who presented the first ultrastructural evidence for synaptonemal complexes in a rust fungus and reported pachynema occurring shortly after karyogamy in species of *Gymnosporangium*, and of O'Donnell and McLaughlin (1981*a*), who reported that teliosporic fusion nuclei were in late diplonema as they entered developing metabasidia of *P. malvacearum*.

*Centromères*

The lack of clearly resolved centromeric regions at pachynema in *P. graminis* f.sp. *tritici* precluded the use of the centromeric index to cross-correlate individual bivalents among replicate nuclei. In fungi, pachytene centromeres have been resolved at the ultrastructural level amost exclusively among the homobasidiomycetes and have been used to cross-correlate individual bivalents using the centromeric index (Carmi *et al.* 1978; Holm *et al.* 1981; Slezec 1984). Ultrastructurally resolved centromeres have not been reported from the ascomycetes (Byers and Goetsch 1975; Gillies 1972; Zickler 1977), Chytridiomycetes (Borkhardt and Olson 1979), Oomycetes (Tanaka *et al.* 1982), and Plasmodiophoromycetes (Braselton 1982, 1984); however, pachytene centromeres have been reported at the light microscope level in *Neurospora* (Singleton 1953).

Recently, pachytene centromeres have been resolved in reconstructed nuclei of *Eocronartium muscicola* (Boehm and

McLaughlin 1991), a heterobasidiomycetous moss parasite phylogenetically related to the rusts but differing in its mode of parasitism (Boehm and McLaughlin 1988) and in details of nuclear division (Boehm and McLaughlin 1989). The presence of resolvable pachytene centromeres at the ultrastructural level in the homobasidiomycetes and in one heterobasidiomycete (*E. muscicola*; Boehm and McLaughlin 1991) but not in another (*P. graminis* f.sp. *tritici*; this study) appears to be phylogenetically significant. The presence of centromeres in *E. muscicola* further supports the argument that it is not a member of the Uredinales (Boehm and McLaughlin 1989). The rust fungi share with the ascomycetes certain nuclear features including the intranuclear spindle composed of a central bundle of nonkinetochore microtubules and the extranuclear multilayered discoid spindle pole bodies that become inserted in a close fitting pore in the nuclear envelope during division (Heath and Heath 1976; O'Donnell and McLaughlin 1981*a*, 1981*b*). To this list we tentatively add the ascomycetous feature of a lack of ultrastructurally resolved pachytene centromeres.

*NOR bivalent*

We interpret the two portions of the synaptonemal complex that are associated with the nucleolus in *P. graminis* f.sp. *tritici* to be parts of a single bivalent that traverses the nucleolus. A similar situation as viewed cytologically in *Saccharomyces cerevisiae* (Byers and Goetsch 1975; Dresser and Giroux 1988; Moens and Ashton 1985) was proven by genetic analysis and pulsed-field gel electrophoretic separation of chromosomes to be a single bivalent carrying a medially located NOR (Petes 1979; Schwartz and Cantor 1984). In *E. muscicola*, the fungus most closely related to *P. graminis* f.sp. *tritici* for which an ultrastructural pachytene karyotype is available (Boehm and McLaughlin 1991), the NOR bivalent is confined to a peripheral depression on the side of the nucleolus where it can be traced for its full length; a centromere further confirmed that only a single bivalent was associated with the nucleolus. In several other fungi, however, the nucleolus is terminally associated with a bivalent (Carmi *et al.* 1978; Gillies 1972; Holm *et al.* 1981; Zickler 1977), and in *Pleurotus eryngii*, the NOR apparently can be either terminal or medial, depending on the isolate (Slezec 1984). Since we could not follow the NOR bivalent through the nucleolus in *P. graminis* f.sp. *tritici*, the alternative, that there are two bivalents terminally associated with the nucleolus thus bringing the haploid number of chromosomes to 19, cannot be ruled out.

Discrepancy in length for the NOR bivalent among isolates of *P. graminis* f.sp. *tritici* suggests that other chromosomal length polymorphisms may occur, despite the same total number of chromosomes and a relatively constant genome size. The total length of the two arms of the NOR bivalent varied among isolates, but within a single nucleus each bivalent arm proved similar in length. The length heterogeneity for the NOR bivalent did not relate to whether or not the isolates were from putative sexual or asexual populations. NOR bivalent length heterogeneity among isolates of *P. graminis* f.sp. *tritici* may represent translocations similar to those reported for the NOR bivalent in yeast (Moens and Ashton 1985).

Electrophoretic methods have been developed recently for separating intact fungal chromosomes and deriving electrophoretic karyotypes (reviewed by Mills and McCluskey 1990; Skinner *et al.* 1991). Some fungi have a great deal of intraspecific polymorphism in chromosome number and especially in size, which can result in divergent total genome sizes (Howlett 1989; Kinscherf and Leong 1988; Masel *et al.* 1990; McCluskey and Mills 1990; Ono and Ishino-Arao 1988). The uncertainty of the number of bivalents associated with the nucleolus in *P. graminis* f.sp. *tritici* might be resolved by separating whole chromosomes or chromosome fragments with pulsed-field gel electrophoresis. We are now attempting to electrophoretically separate chromosomes of this fungus and other rusts to compare their cytological karyotypes directly, as revealed from ultrastructural reconstructions of pachytene nuclei, with those obtained from pulsed-field gel electrophoresis.

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