Gametangial Development in *Allomyces macrogynus*
II. Evidence against Mitochondrial Involvement in Sexual Differentiation

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Abstract. The possible role of mitochondria in determining the sex of the gametangia of *Allomyces macrogynus* was investigated. Quantitative studies of mitochondrial distribution in vegetative hyphae confirmed previous reports of apical mitochondrial clustering. However, by the time the male and female gametangia were partitioned off, no significant difference in mitochondrial distribution between the two sexes was present. Possible mechanisms for the redistribution of mitochondria during early differentiation are discussed. In addition, cytochrome oxidase activity was demonstrated in all mitochondria of both male and female gametangia by the use of diaminobenzidine. It is concluded that neither mitochondrial distribution nor differential mitochondrial activity plays a determining role in the differentiation of the sexual cells in *Euallomyces*.

Key words: Phycocyanete - *Allomyces* - Mitochondria - Gametangial differentiation - Sex - Diaminobenzidine - Cytochrome oxidase.

During the gametophytic phase of the life cycle of *Allomyces* subgen. *Euallomyces* (Emerson, 1941) morphologically and biochemically distinct male and female gametangia are produced in pairs at the tips of the gametophytic hyphae. The male gametangia are smaller and occupy an apical or subapical position depending upon the species. *Allomyces arbuscula* exhibits hypogyny whereas *A. macrogynus* exhibits epigyny. In *A. macrogynus* the female gametangia may attain lengths two to three times that of the male. Posteriorly uniflagellate, anisogamous gametes are discharged from the gametangia; the male gametes are smaller and pigmented. Among the biochemical markers which distinguish the gametangia are (1) the production of γ-carotene by the male gametangia (Emerson and Fox, 1940) and (2) the production of sirenin by the mature, undischarged female gametangia and female gametes (Machlis, 1958; Machlis et al., 1966). Furthermore the male gametes respond chemotactically to sirenin.

The control mechanism which directs the formation of these two morphologically and biochemically distinct gametangia, born on a thallus whose nuclei are all derived mitotically from the single, haploid meiospore nucleus, has been a subject of conjecture and investigation for over 40 years (Hatch, 1935; Turian, 1957, 1958, 1960a, b, c; Turian and Viswanathan, 1966; Turian et al., 1969; Fähnrich, 1974; Stumm and Croes, 1975). As a consequence of studies by Hatch (1935) and Turian (1958, 1969), there is correlative evidence which implicates mitochondria in the determination of the sex of gametangia. Two proposals which involve mitochondria have been presented for the mechanism of sexual differentiation in *Allomyces*. The first is (Hatch, 1935) that there is a differential distribution of mitochondria in developing gametangia; more mitochondria lead to femaleness, fewer mitochondria cause maleness. Although a differential distribution of mitochondria was reported for vegetative hyphae of *Allomyces* (Hatch, 1935; Turian, 1957), no quantitative data have been presented on the distribution of mitochondria either in hyphae or in developing gametangia. The second proposal is that maleness results from a lower proportion of oxidatively functional mitochondria in that region of the hypha destined to become a male gametangium (Turian, 1958, 1969). If indeed mitochondria do play a role in sexual differentiation of *Allomyces*, however, convincing evidence has not been presented. The present study shows that the mitochondria of both male and female gametangia contain cytochrome.

Abbreviations. % M = percent area occupied by mitochondria;
DAB = diaminobenzidine
oxidation and provides evidence against a differential distribution of mitochondria in developing gametangia of *A. macrognus*.

**MATERIALS AND METHODS**

*Organism and Culture Conditions. Allomyces macrognus* (strain Burma 3-35) was used in this study as before and all details of culture and handling are provided in the preceding paper, Part I of this series (Morrison, 1977a). Gametangial development is induced at will by transferring growing vegetative hyphae to non-nutrient medium at time zero.

**Determination of Mitochondrial Distribution.** The distribution of mitochondria was determined in hyphal tips from 0, 30, and 90 min hyphal balls (Morrison, 1977a). Comparisons were made between % M, the percent area occupied by mitochondria, in male versus % M in female protoplasm. Since the hyphal tips from 0 and 30 min hyphal balls are not yet partitioned into male and female regions as are the hyphal tips from 90 min hyphal balls (Morrison, 1977a), the terms presumptive male ("♂") and presumptive female ("♀") were employed. Since the male (♂) gametangium is terminal and its measured length is ca. 20–32 μm (author's measurements), portions of the hypha ca. 0–25 μm from the hyphal apex were designated "♂". Since the measured length of the female (♀) gametangium is ca. 50–74 μm (author's measurements), portions of the hypha ca. 35–95 μm from the hyphal apex were designated "♀". Thus, the "♂" closely corresponded to that portion of the hypha which would develop into a ♀ if differentiation were allowed to proceed to completion, and the "♀" corresponded to that portion of the hypha which would develop into a ♀.

Hyphal balls were prepared for electron microscopy as previously described (Morrison, 1977a). Ultrathin longitudinal sections of "♂", "♀", ♀ and ♀ were photographed at a magnification of 6500 ×. The smallest intermediate aperture on the Zeiss 9A-EM was used to measure the length of the hyphal section and to determine the position of the "♂" and "♀" regions being analyzed.

Since the mitochondria of *A. macrognus* are approximately cylindrical (Turian, 1957), the configurations of profiles of mitochondria range from circular to elliptical. Thus, profile diameters were not used as a measure of the size of the mitochondria, since calculation of the area of each mitochondrion based on its diameter and shape would have been arduous. Instead tracings were made of hyphal sections and their mitochondrial profiles from each electron micrograph. From these tracings, the areas of hyphal protoplasm and mitochondrial profiles were determined with an optical planimeter (Filotecnia Salmoiragi S.p.A., Model 236/A, Milano, Italy). % M was calculated from the total area occupied by mitochondrion in a given hyphal section and the area of the corresponding hyphal protoplasm. The differences between % M in "♂" and "♀" from 0 and 30 min hyphal balls and between % M in ♀ and ♀ from 90 min hyphal balls were analyzed statistically using a standard *t* test on an Olivetti Underwood Programma 101 computer and a Monroe Calculator, Model 1800.

**Cytochemistry.** Differentiating hyphal balls (105 min) were prefixed for 1 h with ice cold 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2 containing 0.1 M sucrose. After four washes in the same buffer solution over a period of 30 min, the hyphal balls were placed in vials containing 2 ml of reaction mixture. The reaction mixture was composed of 3.3-diaminobenzidine tetrahydrochloride (DAB) (G. Frederick Smith Chemical Co.), 1.0 mg/ml or 2.5 mg/ml, in 0.05 M phosphate buffer, pH 7.2, containing 0.1 M sucrose. The final pH of the reaction mixture was 5.9. In order to minimize photooxidation (Hirai, 1971), DAB was added to the reaction mixture immediately prior to use in a darkroom illuminated with red light. The vials were then covered with aluminum foil and incubated at 27°C for 30 min or for 60 min. Control hyphal balls were (1) incubated in reaction mixture minus DAB, (2) pre-incubated with 1 mM KCN in the buffer solution for 30 min at room temperature then incubated in reaction mixture. All controls were incubated at 27°C for 60 min. The reaction was terminated by washing 4–5 times with ice cold buffer solution for 1 h. The hyphal balls were post-fixed for 1 h with ice cold 1% OsO4 in 0.05 M phosphate buffer, pH 7.2; washed two times with phosphate buffer, pH 7.2, at room temperature; and stained for 2 h with 2% uranyl acetate at room temperature. The hyphal balls were then prepared for electron microscopy (Morrison, 1977a) except that the sections were not post-stained with lead citrate. Mitochondria in male and female gametangia were examined for the presence of electron-dense deposits on the cristal and inner membranes. Oxidized DAB polymerizes spontaneously and then reacts with OsO4 to form an electron-dense reaction product called osmium black (Seligman et al., 1968).

**RESULTS**

**Mitochondrial Distribution.**

The hyphal tips of 0 min hyphal balls are characterized by an apical cluster of mitochondria (Turian and Oulevey, 1971; Morrison, 1977a). This cluster occurs within the "♂". Comparisons were made of % M in "♂" and % M in "♀" within individual presumptive gametangial pairs, and these data were then used to calculate average % M for all "♂" and for all "♀". On an individual hypha basis, all of the "♂" exhibited a greater % M than did their corresponding "♀" (Table 1). In addition, the average % M for all "♂" was 32% more than the average % M for all "♀". This difference is statistically significant at a level of 5%.

At 30 min, when the organization of the apical zone is disappearing (Morrison, 1977a), comparisons of % M in "♂" and % M in "♀" for individual, presumptive gametangial pairs show considerable variation in the pattern of mitochondrial distribution (Table 2). Unlike the hyphae of 0 min hyphal balls, there is a greater % M in the "♂" than in the "♀" only 3 out of 8 of the hyphae. However, when the average % M from all of the "♂" is compared with the average % M from all of the "♀", the % M of the "♂" is still 29% more than the % M of the "♀". This difference is statistically significant at a level of 5%.

Comparisons of % M in the ♀ and % M in the ♀ for individual pairs of gametangia at 90 min again reveal wide variations in mitochondrial distribution. Indeed, half of the pairs show a greater distribution in the ♀, whereas the other half show a greater distribution in the ♀ (Table 3). However, the average % M from all ♀s is 11.60 and from all ♀s is 11.90, a difference which has no significance at the 5% level.