Hydrobiologia **512:** 141–144, 2004.
P.O. Ang, Jr. (ed.), Asian Pacific Phycology in the 21st Century: Prospects and Challenges.

An improved chromosome preparation from male gametophyte of *Laminaria japonica* (Heterokontophyta)

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Key words: China, chromosome, male gametophyte, *Laminaria japonica*

Abstract

An improved method for the preparation of chromosomes from the male gametophyte of the alga *Laminaria japonica* Aresch. was described. The male gametophyte was pretreated with pDB (p-dichlorobenzene) and 8-hydroxyquinoline in order to clear cell wall and soften cytoplasm. The samples were treated by mordant iron alum [$FeNH_4$ $(SO_4)_2 \cdot 6H_2O$] followed by staining with haemotoxylin. Well-spread and highly stained chromosomes were observed without precipitation. The chromosome number of male gametophyte of *L. japonica* was estimated to be 31.

Introduction

Parthenogenesis was observed in the gametophyte of *Laminaria japonica* Aresch. when gametophytes were cultured individually and grown vegetatively into sporophytes (Fang et al., 1978; Fang & Dai, 1980; Dai et al., 1993). Cytological observation on natural parthenogenesis of this species indicated that the doubling of chromosomes only occurred in female parthenogenetic sporophyte, while this phenomenon was absent in male parthenosporophyte (Fang & Dai, 1980; Dai et al., 1993). Therefore, unlike female parthenosporophytes, the males were sterile, for no active sperms were produced (Fang & Dai, 1980; Dai et al., 1993). It has been reported by Jiang & Tang (1979) that reproductive haploid sporophytes could be obtained from male gametophytes, however, these plants were abnormal in shape and repeated attempts to produce haploid sporophytes have failed. All thalli derived from male gametophytes were found to be haploid, the shapes of which were mostly abnormal and no spores could be released from these thalli (Dai et al., 1997). It was postulated by Dai et al. (1997) that a single set of chromosomes cannot lead to successful meiosis and no spores could thus be produced from haploid male parthenosporophytes. Theoretically, the chromosome-doubling chemical, colchicine, could be used as a strategy to improve the propagation of the male pathenosporophyte. Furthermore, hybridization of diploid male and female parthenosporophytes, both of which possess good growing properties, would greatly promote the field output of this kelp (Dai et al., 1997). A chromosome number of $n = 22$ for *L. japonica* has been widely accepted for decades (Abe, 1939; Yabu, 1973; Tai & Fang, 1977; Lewis et al., 1993), but the latest observation revealed a new number of $n = 31$ (Yabu & Yasui, 1991). It must be emphasized that the chromosome number should be confirmed before any chromosome doubling experiment can be carried out.

The classic paraffin method was popular in cytological and chromosomal studies of algae before the appearance of the squashing method (Abe, 1939; Nishibayashi & Inoh, 1956; Yabu, 1964). After the squashing technique of aceto-iron-haematoxylin-chloral hydrate has been published (Wittmann, 1962, 1965), it was rapidly applied to algal chromosome preparation (Yabu & Tokida, 1966; Lewis, 1993). The chromosome numbers of *Laminaria japonica* (Yabu, 1973); *L. yendoana* Miyabe (Yasui, 1992); *Kjellmaniella crassifolia* Miyabe (Yabu et al., 1985) and *Ecklonia kurome* Okamura (Yabu & Taniguchi, 1990)

were preliminarily identified with Wittman's method where chloral hydrate had a clearing function. However, chloral hydrate is highly toxic, and is particularly harmful when tests were performed on a large scale. Therefore, an improved chromosome preparation with equal efficiency but a higher safety factor is urgently needed.

The aims of this study were to improve the method of chromosome preparation from male gametophyte of *L. japonica* and to determine its chromosome number.

Material and methods

Male gametophyte of *L. japonica* used in this study was the strain lj 4-2.1982, isolated in 1982 from a sporophyte and preserved at 4 °C in the Laboratory of Genetics, Ocean University of Qingdao, China.

The male gametophyte was digested with gentle stirring in a multi-enzyme solution (5% cellulase: 4% pectinase = 1:1) for 3 h, and centrifuged at 1000 × ***g*** for 5 min. The supernatant was discarded and the pellet was re-suspended in sterile seawater and filtered three times through a 40 μm mesh. Fragments that passed through the mesh were allowed to attach to the glass slides. The gametophyte fragments were cultured in enriched sterile seawater ([N] 3×10^{-4} mol l^{-1}, [P] 1×10^{-5} mol l^{-1}) in an illuminating incubator at 18 °C under cool-white fluorescent light at 2000 lx with a 12h:12h LD photoperiod. Two weeks later, when the fragments grew into numerous filaments, they were ready for pretreatment.

Three or four hours into the dark period, filaments were successively treated with 0.1% colchicine, saturated pDB (p-dichlorobenzene), and saturated 8-hydroxyquinoline for 3 h, 1 h and 15 min, respectively. After each treatment, samples were washed to remove chemical residues.

The filaments were fixed in Carnoy's fixative (100% alcohol: 100% acetic acid = 3:1) for at least 24 h. Prior to squashing, samples were mordant in 2% iron alum for 15 min to allow binding of iron on the chromosomes. The preparation was washed in tap water, and then stained in 5% haematoxylin for 5 min. At this time, the filaments were stained dark blue and it was impossible to differentiate the chromosomes from the cytoplasm. Thus, it is necessary to remove excess stain from the sample with a drop of 45% acetic acid. The color of the filaments then turned into pale blue, a cover slip was placed in position and a flat instrument was used for squashing. Squashing required more pressure than with other materials because of solid nature of the filament cell wall.

Results and discussion

Chromosome preparations from male gametophyte were clear and countable (Figs 1 and 2). Chromosomes dispersed well without dye precipitation, and cytoplasm was stained light (Fig. 1). The chromosome numbers counted in male gametophyte cells ranged from 29 to 32, most of which were 31 (Fig. 2).

This study showed that the chemical pDB has a cytoplasm-decomposing effect on gametophyte cells. Previously, pDB was seldom applied to chromosome preparation of higher plants as pretreatment reagent

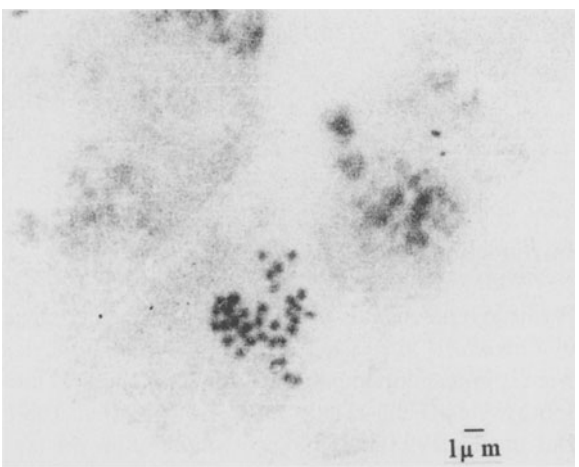

Figure 1. Chromosomes of the male gametophyte of *L. japonica* (partial view). Scale bar = 1 μm.

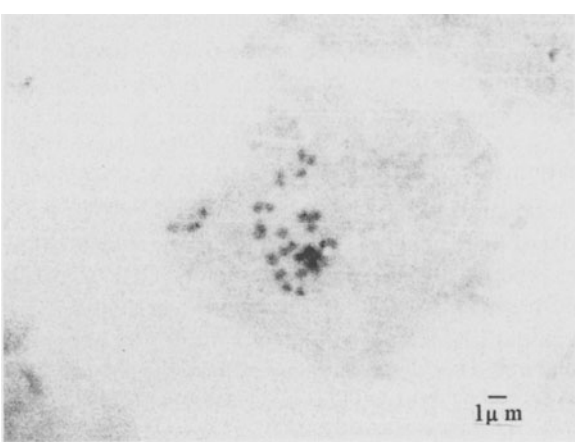

Figure 2. Chromosomes in a cell of the male gametophyte of *L. japonica* (n = 31). Scale bar = 1 μm.

because its strong scavenging effect may lead to chromosome fracture (Li & Zhang, 1991). In this study, however, it was demonstrated that the clearing property of pDB was effective in male gametophyte of lower plant *L. japonica* and cytoplasm staining has been reduced, leading to a light-colored background. Furthermore, using pDB not in working stain but as pretreatment has protected operators from contacting the harmful dye during squashing. 8-hydroxyquinoline is generally applied as a condensing reagent for chromosome preparation in higher plants (Li & Zhang, 1991). This study has shown that after 8-hydroxyquinoline treatment, the cell wall of male gametophyte became crisp and could be easily crushed but the mechanism of action of 8-hydroxyquinoline is still unclear.

The stain haematoxylin, combined with iron-mordant, yielded intense and selective chromosome staining in plant materials. Previous work involved mixing of iron alum and haematoxylin just before staining. Stain precipitation would rapidly occur from the binding of mordant and dye, the resulting light scatter would cause difficulty in chromosome counting. In this study, however, the mordant and stain were stored separately and used sequentially in order to avoid stain precipitation. Otherwise, complete washing of the mordant from samples could also help reduce possible precipitation. The present method was easy to perform and highly effective in producing chromosome preparation from the male gametophyte of *L. japonica*. The technique could equally be applied to chromosome preparation of other algal species.

A chromosome number of $n = 22$ was first observed in the sporangium of *L. japonica* (Abe, 1939), and this conclusion was supported by numerous reports (Yabu, 1973; Tai & Fang, 1977; Lewis et al., 1993). The evidence that nuclei in gametophytes of *Laminaria japonica* are haploid and those in sporophytes are diploid was put foward by Yabu (1973). Yabu (1973) suggested that it was hard to count the chromosome number in gametophyte and the number ranged from 16 to 22 occasionally in female gametophytes, rarely in males. Recently, Yabu & Yasui (1991) reported that the chromosome number of *L. japonica* was normally $n = 32$, which is similar to that of *L. angustata* Kjellm., *L. ochotensis* Miyabe and *L. religiosa* Miyabe. The chromosome number of male gametophytes examined in most samples in the present study was 31, which was different from those reported by Yabu (1973) ($n = 22$), Lewis et al. (1993) ($n = 22$), and by Nakahara (1984) (n = 28-35), but similar to the latest report of $n = 32$ by Yabu & Yasui (1991). Results presented here mostly supported Yabu's conclusion with minor differences. Most of the former results were obtained from sporophytes of *L. japonica*, while this study used male gametophytes. It is reasonable to believe that the data obtained from the haploid is more reliable, because a smaller chromosome number can reduce the counting error. The reason for the number discrepancy is unknown, although it seems that differences in methodology may be responsible. Future effort will be directed at improving the squash method.

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