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Comparison of three techniques for identifying isomorphic phases of *Chondrus crispus* (Gigartinaceae)

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Abstract

Chondrus crispus, a member of the economically important family Gigartinaceae, alternates between two free-living isomorphic life history phases. The carrageenan composition of the gametophyte and tetrasporophyte phases has been used to identify non-reproductive material, and thus provide a better understanding of the ecology of the species. The aim of this study was to compare three methods for identifying the isomorphic phases. The results obtained by the widely used resorcinol test and an iridescence method are compared with those obtained by a technique that involves the acquisition of mid-infrared absorbance spectra from dried samples by Fourier transform infrared spectroscopy (FT-IR). Both the resorcinol test and FT-IR produced similar results with a calculated gametophyte to tetrasporophyte ratio of 2.1 and 2.3:1, respectively. In contrast the iridescence method was less reliable, with a calculated gametophyte to tetrasporophyte ratio of 1.3:1 and a low predictive value (0.70) for selecting tetrasporophytes. The FT-IR technique provides a rapid assessment, does not involve the use of hazardous chemicals, has the potential to be applied to members of the Gigartinaceae for which the resorcinol and iridescence tests are less applicable and it has greater flexibility in that it can identify additional carrageenan fractions.

Species of the Gigartinaceae, an economically important family of red algae from which carrageenan is extracted, have a triphasic life cycle comprising free living isomorphic gametophytes and tetrasporophytes. While easily identifiable when reproductive structures are present, they are much more difficult to distinguish visually when material is non-reproductive. Two methods are commonly used to separate gametophyte and tetrasporophyte phases of *Chondrus crispus* Stackhouse. With the discovery that κ carrageenan is the predominant form of carrageenan in gametophytes and λ carrageenan in tetrasporophytes, the acetal-resorcinol reagent, originally developed to determine the quantity of 3,6 –anhydrogalactose and fructose in polysaccharides (Yaphe & Arsenault, 1965), is used to distinguish the two phases. The usefulness of the technique was enhanced when Dyck et al., 1985 showed it could be applied directly to fronds, without the need for extracting the carrageenan; gametophytes turn the reagent red, while the reagent remains virtually clear with tetrasporophytes. As a consequence 'the resorcinol test' has been a useful method for investigating population structure and distribution patterns of the different phases of *C. crispus* and other related species (e.g. Dyck & De Wreede, 1995; Lazo et al., 1989; Lindgren & Aberg, 1996). The second method for distinguishing the two phases was first reported by Fournet et al. (1993); there is a correlation between life history phase and iridescence of fronds such that blue iridescence is associated with only gametophytes but not tetrasporophytes. Iridescence in *C. crispus* is thought to be a consequence of the thickness, number and spacing of the cuticular lamellae (Craigie et al., 1992). While some other members of the Gigartinaceae are iridescent, e.g. *Iridaea,* the method has not been employed in the same way as it has for *C. crispus*.

FT-IR spectroscopy is used extensively in carbohydrate chemistry including the characterisation of agar and carrageenan-type polysaccharides from seaweeds (Matsuhiro, 1996). It measures vibrations of functional groups and highly polar bonds providing information on the structures of molecules. By determining the frequencies at which a sample absorbs IR radiation and the intensities of the absorptions the chemical composition can be determined (Griffiths & de Haseth, 1986). Chopin and Whalen (1993) showed that rather than undertaking the laborious task of extracting the carrageenan from tissue samples, it was possible to identify different forms of carrageenan directly on dried, ground material. With this modification the procedure now lends itself to rapid identification and therefore to the discrimination of different life history phases.

There has apparently been no study that compares systematically the three identification methods on the same individuals in order to evaluate their reliability in distinguishing gametophytes from tetrasporophytes. Here we report the results of such a study using individuals sampled from an intertidal population of *C. crispus* that includes material with and without reproductive structures.

Individual fronds of *C. crispus* were randomly sampled from an intertidal population at Sugary Cove, near Dartmouth, South Devon, England. Material was cleaned of epiphytes, visually inspected for the presence of reproductive structures and separated into three groups: female gametophytes (presence of cystocarps); tetrasporophytes (presence of tetrasporangia) and vegetative fronds (no reproductive structures visible, but possibly including male gametophytes). Of the 150 fronds collected, approximately 75% had no visible sign of reproductive structures. Each individual frond was subjected to all three identification methods and then classified as either a gametophyte or tetrasporophyte. The number of gametophytes and tetrasporophytes in the population were scored and the G/T ratio was obtained for each of the methods used.

Tetrasporophytes and gametophytes were identified on the basis of the resorcinol reaction following the protocol outlined by Garbary and De Wreede (1988). A disc (5 mm^2) of material, lacking reproductive structures, was sampled from the basal portion of all fronds. Each individual disc was placed in a boiling tube, 2 mL of resorcinol-acetal reagent added and then heated to 80–90 °C in a water bath for 60 s. The colour reaction was recorded immediately, dark opaque red for gametophytes or clear pink for tetrasporophytes. For iridescence, fronds were immersed in seawater and visually inspected under background laboratory lighting. Each of the three authors independently assessed the fronds; identical results were obtained by all.

Basal portions (approximately 5 g fresh biomass) of each frond, avoiding reproductive tissue, were oven dried at 60 °C overnight and then ground (10 min) to a fine powder using a pestle and mortar. Standards of pure κ and λ carrageenan (Sigma Chemical Co.) and dried samples of *C. crispus* were further ground with KBr powder in an approximate ratio of 1:10 (sample: KBr) to ensure consistent reproducibility of spectra, as recommended by Chopin and Whalen (1993). This mixture filled to capacity a 13 mm wide $\times$ 2mm deep sampling cup mounted on a SPECAC "praying mantis" diffuse reflectance accessory (DRIFT) with a KBr background. Spectra (32 scans) were recorded with a Bruker IFS 66 FT-IR spectrometer at 4.0 cm^{-1} resolution and then investigated for the presence or absence of peaks corresponding to the absorption bands of the structural elements of κ carrageenan (wave number of 930 and 845 cm^{-1}) and λ carrageenan (wave number of 830 and 820 cm^{-1}).

The numbers of gametophytes and tetrasporophytes obtained by the different identification methods were compared using McNemar's test ($p < 0.05$); this is the equivalent of the chi-squared test for paired data (Zar, 1996). The results from the resorcinol and iridescence tests were evaluated further using a diagnostic test procedure (Campbell & Machin, 1999) that provides information on the proportion of gametophytes and tetrasporophytes correctly identified by the two techniques when compared with the results obtained by FT-IR which were *a priori* considered to be 100% accurate (i.e. considered to be controls). The predictive values, i.e. the proportion of gametophytes/tetrasporophytes that according to the tests are gametophytes/tetrasporophytes were also derived.

Reproductive structures were present on 28 (approximately 25%) of the 150 individuals sampled. Of this subset 68% were visually identified as gametophytes and 32% as tetrasporophytes, giving a G/T ratio of 2.1:1. The results obtained by FT-IR perfectly matched those of the visual observations and justifies their use as controls in the statistical analysis. Except for one gametophyte that was identified as a tetrasporophyte, the results obtained by the resorcinol test were in agreement ($p > 0.05$) with FT-IR and visual observations, and the G/T ratio obtained was 1.8:1. From the iridescence test, three individuals with cystocarps and one individual with tetrasporangia would have been

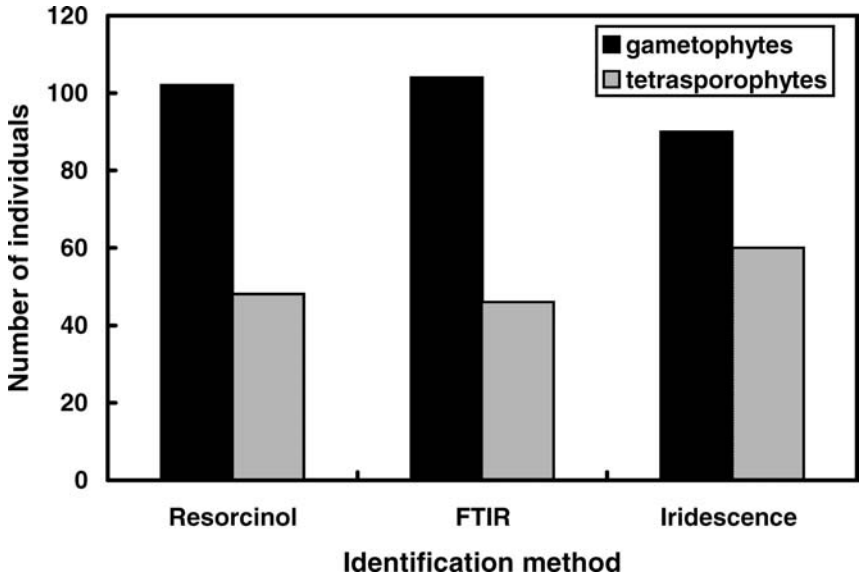

Figure 1. The number of fronds of *Chondrus crispus*, including those with ($n = 28$) and without reproductive structures, identified as gametophytes and tetrasporophytes by FT-IR, the resorcinol test and iridescence ($n = 150$).

wrongly identified using this technique if reproductive structures had not been present. On the basis of this test, 17 of the 28 individuals were identified as gametophytes and 11 as tetrasporophytes, giving a G/T ratio of 1.5:1. However, because of the small sample size ($n = 28$) no significant difference was found between the results from this method and the other three. Extending the analyses to include individuals with no observable reproductive structures confirmed the general correspondence between FT-IR and the resorcinol test and the differences between these methods and that of the iridescence test. Figure 1 shows the number of individuals identified as gametophytes and tetrasporophytes by the three discrimination methods. Of the 150 individuals included in the study, 22 were misidentified by the iridescence test, 18 of which were wrongly assigned as tetrasporophytes and 4 as gametophytes. These values differ significantly ($p < 0.05$) from those of the resorcinol test and FT-IR, with only 60% of the population identified as gametophytes compared with 68% by the resorcinol test and 70% by FT-IR, and hence G/T ratios of 1.5, 2.1:1 and 2.3:1, respectively. From the diagnostic test, the probability that iridescence will positively identify a gametophyte (termed the sensitivity of the test) and the probability that the test result will be negative if applied to tetrasporophytes (termed the specificity of the test) were 83% and 91%, respectively. This compares with a sensitivity of 95% and specificity of 100% for the resorcinol test.

The results clearly demonstrate the accuracy of FT-IR spectroscopy for identifying the isomorphic reproductive phases of *Chondrus crispus* when applied to dried, ground algal samples, confirming the results obtained by Chopin and Whalen (1993). Furthermore, the suitability of the method to discriminate between gametophytes and tetrasporophytes in the absence of reproductive material is also shown. The results further illustrate that FT-IR is largely matched by the resorcinol test; of the 150 individuals sampled only 2 were wrongly assigned as tetrasporophytes on the basis of a negative resorcinol reaction. This contrasts with the results of the iridescent test with 22 misidentifications, of which 18 were wrongly identified as tetrasporophytes and 4 as gametophytes. These results contrast with those of Fournet et al (1993) who considered iridescence to be a rapid and reliable technique that could be equally employed in the field or in the laboratory on both immature and mature fronds. The reason for the disparity between their results and ours may relate to sources of variation associated with varying ecological properties of the habitat that have not been previously accounted for and should be investigated further. Alternatively, it may be related to the eye of the observer. The technique is subjective and so, as well as ensuring adequate replication it is important to consider verification of the results by including several independent observations of the same samples, as has been done here. Neither the number of replicates nor number of

observers was provided by Fournet et al (1993). On the basis of our results the differences in the sensitivity and specificity values derived from the diagnostic test lead to the conclusion that while the predictive capability of resorcinol and iridescence for correctly identifying gametophytes is reasonably similar (predictive values of 1.00 and 0.95, respectively), the absence of iridescence has a much lower predictive value (0.70 versus 0.96 for resorcinol) in selecting tetrasporophytes. Hence, if the iridescence method is to be employed, then all non- iridescent vegetative fronds should be further tested to accurately identify the life history stage.

The resorcinol test is straightforward, requires minimal and cheap equipment and its accuracy almost matches that of FT-IR for use with *Chondrus crispus.* However, the technique is not equally applicable to all members of the Gigartinaceae, as exemplified by the study of Shaughnessy & De Wreede (1991). They found that storage method, weight of material used and time allowed for colour development could all influence the reliability of the technique. A further disadvantage of the method is the hazardous nature of the chemicals used in the test. Acetal (1,1-diethoxyethane) is a highly flammable, volatile liquid the vapours from which may form explosive mixtures with air. Inhalation or contact can irritate or burn the skin and eyes. Resorcinol (1, 3-dihydroxybenzene) is also harmful if inhaled and in contact with the skin and particularly the eyes. Furthermore it is toxic to aquatic organisms, possibly acting as an endocrine disruptor (Colborn et al., 1993).

Of the three techniques tested FT-IR spectroscopy proved the most accurate in discriminating gametophytes from tetrasporophytes. The method requires the minimum of sample preparation and small quantities of material. Individual spectra can be obtained in less than one minute, allowing for large numbers of samples to be analysed relatively quickly. The major drawback is the initial equipment cost although, this is now standard equipment in most university Chemistry Departments. Furthermore, applicability of FT-IR may be less restrictive than either the iridescence or resorcinol tests, although further detailed studies on species of the Gigartinaceae are required before the generality of its utility can be fully assessed. Employing this technique should make it possible to study the population ecology, including dispersal and recruitment, of the two life history phases of other economically important species of the Gigartinaceae and as a consequence, this should permit the development of more informed management strategies for sustainable wild harvesting. Moreover, since other carrageenan fractions can be characterised from minimally treated material, the FT-IR spectra are representative of the natural composition of carrageenophytes and thus can provide valuable information on the quality of harvested or farmed material for industrial purposes. It is predicted that greater use will be made of this technique in ecological and eco-physiological investigations.

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