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Intraspecific trait variation affecting astaxanthin productivity in two *Haematococcus* (Chlorophyceae) species

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ABSTRACT

Microalgae are increasingly used as commercial sources of high-value compounds. However, the nature and genetic basis of variation in commercially relevant traits remain understudied. This study focuses on the green alga *Haematococcus pluvialis*, well-known for accumulating the carotenoid astaxanthin. We examined intra- and interspecific variation and correlations between six traits related to astaxanthin productivity among 30 natural isolates and cultivated strains of two *Haematococcus* species. Significant intraspecific genotypic variation was found for all traits assessed in both species (broad sense heritability estimates $H^2 = 0.48–0.89$), resulting in a fifteenfold variation in astaxanthin productivity between the poorest and the best-performing strain. The two species differed in five of the six traits. Cultivated strains had a lower astaxanthin productivity compared to natural isolates of *H. pluvialis*, possibly reflecting loss of photoprotective capacity during long-term cultivation. In general, trait correlations were weak yet stronger in *H. rubicundus* than in *H. pluvialis*. Most of the variation in overall astaxanthin productivity could be explained by the differences in post-stress traits. Our results reveal extensive trait variation among isolates of a commercially interesting microalga. We recommend natural strain panels as a valuable tool for cost-efficient trait mapping and to select targets for genetic engineering, marker-assisted strain selection or breeding aiming at the optimization of astaxanthin productivity.

1. Introduction

Modern evolutionary research, particularly population genomic studies, aims to explore the genetic basis of phenotypic variation resulting in a better understanding of the mechanisms governing trait variation [1]. In an era of omics, technological advances have led to increasing amounts of genomic data, from whole genome sequencing-to extensive transcriptomic, methylomic and metabolomics data. Complementing these – omics data with phenotypic data is an essential, yet time consuming and labor intensive step of many biological experiments [2,3,4,5]. As natural sources of value added commodities such as chlorophylls, carotenoids, antioxidants, enzymes, polymers, toxins, sterols and fatty acids [6], microalgae represent a promising and sustainable feedstock supplying food and nonfood markets [7]. From a commercial perspective, however, current yields and processes are often insufficient to sustainably produce bioproducts. In the face of growing competition and significantly larger market opportunities, one major goal in microalgae biotechnology is the screening, selection and development of strains with increased productivity and resilience [8,9]. Although still rare, some studies on model microalgae with economical potential have screened natural populations with the aim to identify better performing strains either for enhanced pigment productivity [10,11] or fatty acid production [3,12].

The green microalga *Haematococcus pluvialis* Flotow (1844) [13] is exploited as the richest biological source of the high value red carotenoid, astaxanthin. The predicted market value for astaxanthin is expected to be over $1.5 billion by 2020 [14]. Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) accumulates in the cytoplasmic oil globules of the aplanospores under various stress conditions [15] where it acts as a protective pigment [16] and reaches up to 1–5% of cell dry weight [17,18]. Synthetic astaxanthin is widely used in the aquaculture sector as salmon colorant [19], while natural astaxanthin from *H. pluvialis* is principally commercialized as a biological nutraceutical with attractive properties [20,21]. To successfully compete with synthetically prepared astaxanthin, *Haematococcus* production processes faces some major challenges [22], among which the most important biological issues are the slow cell growth rate, changes in cell morphology under various conditions and vulnerability to contamination. So far, research has prioritized the production conditions, including the engineering of culture systems and the manipulation of culture conditions (nutrient composition, light intensity, photoperiod, temperature etc.) using single strains from culture collections [23]. However, the use of genetically different strains, as well as the differences in experimental designs, hamper accurate comparisons. Consequently, it is not known to which extend the variation in astaxanthin productivity between different studies is due to strain differences. In fact, little is known about the phenotypic

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variation spectrum in *H. pluvialis*. Few assessments among strains of *Haematococcus* have been made [23,24,25,26,27], which revealed a great deal of physiological diversity between *H. pluvialis* isolates. Yet a better understanding of the nature of this variation is essential for the selection of promising isolates and for the setup of any future selection program.

One prerequisite for assessing the genetic basis of phenotypic variation patterns (at intra and interspecific level), for future breeding programs, is knowledge on species boundaries. Until recently, all *Haematococcus* isolates were assigned to a single species, *Haematococcus pluvialis*, although internal transcribed spacer (ITS) rDNA sequences showed the existence of different lineages [28]. In a recent multi-locus study of the phylogenetic diversity of European isolates of *Haematococcus*, six species-level lineages were found, and two of these formally described [29]. Although the newly described species are also astaxanthin producers, it is not known to what extent their astaxanthin productivity capacities differ from each other and from *H. pluvialis*.

To quantify the genetically based component of phenotypic variation, common-garden experiments are used in which typically comparisons of phenotypic traits of genetically distinct strains, families or populations are made under strict identical environmental conditions [30]. Common garden approaches are becoming increasingly popular over recent years, particularly since the gap between available phenotypic data versus genotypic data is increasing. Currently, these experiments are employed in several disciplines, in applied contexts e.g., in invasion research [31], climate change research [32] and in biotechnology for the selection of promising candidate strains for biodiesel production [33]. In this study, we used a common-garden approach using a total of 30 strains belonging to two *Haematococcus* species, including six strains from culture collections, to study genetically based intra and interspecific (co-)variation in traits considered to be related to astaxanthin productivity in *Haematococcus*, and to identify which of these traits determine overall astaxanthin productivity.

2. Materials and methods

2.1. Cultures and experimental set-up

Most strains used for this study are new natural isolates (obtained via single cell isolation), all of which belong to different genotypes based on intraspecific differences in ITS sequences (Fig. 1) with the exception of the strain pairs BE03_02 and BE03_05, BE02_09 and BE02_05, NL02_08 and NL02_10, CZ01_06 and CZ01_08 as well as NL03_06 and NL03_07. Finally BE08_04, BE08_06 and BE08_12 also had identical ITS sequences. Strains were identified to species based on their ITS rDNA sequence [29] (Fig. 1). Seventeen *H. pluvialis* and thirteen *H. rubicundus* strains were selected from a larger set of strains for the common-garden experiment. Generally, two isolates per sampled location were selected (Table 1). For *H. pluvialis*, six additional strains were included from culture collections (Culture Collection of Algae and Protozoa, UK (CCAP) (34/7, 34/13, 34/14), Sammlung von Algenkulturen at the University of Göttingen (SAG) (192.80, 49.94 = CCAP 34/13), the Scandinavian Culture Center for Algae and Protozoan (SCCAP) (K-0084) and the Culture Collection for the National Institute for Environmental Studies, Japan (NIES) (144). All new isolates are cryopreserved and maintained at the Diatom Collection Ghent of the Belgian Coordinated Collections of Microorganisms BCCM/DCG) (http://www.bccm.belspo.be/catalogues/dcg-catalogue-search).

Four replicates per strain were grown up at 23 °C from stock cultures (preserved at 6 °C) in Bold Basal Medium modified with 3-fold Nitrogen (BBM-3 N, [34]) using a 16:8 h light:dark regime and a light intensity of 20 ± 2 μmol photons m^{-2} s^{-1}. Strains were re-inoculated twice with an interval of three days at the same initial F_0 value of 0.023 (settings 6-1-2, as measured using a PAM fluorometer; see below) before starting the experiment, to ensure that they were in exponential and flagellated stage. Cells were harvested by centrifugation (3000 rpm at 20 °C during 5 min) in 15 ml falcon tubes, and re-suspended in 12 multi-well plates (Greiner Bio-One) with a working volume of 4 ml using BBM-3N at an initial F_0 value of 0.023 (settings 6-1-2, see below). Each of the four replicates per strain was split in two separate wells at the start of the experiment, for each replicate two technical replicates needed to measure all traits (see below). A bi-phasic approach was used for the cultivation of *Haematococcus* strains for astaxanthin synthesis, involving different conditions for biomass and for astaxanthin accumulation (Fig. 2).

For biomass accumulation the above-described growing conditions were used to measure the following pre-stress traits:

2.1.1. Growth rate

Growth rates of the strains under assessment were determined based on daily measurements of Chl *a* fluorescence by Pulse-Amplitude-Modulated (PAM) fluorescence using a Waltz MAXI-Imaging (PAM) with default settings, intensity of 1, gain of 1 and damping of 2. Minimal fluorescence F_0 [35] was used as proxy for Chl *a* content of the algal cultures [36]. Before measurement, the cultures were dark acclimated for 15 min. After each measurement, the well plates were randomly displayed under the experimental conditions. Growth rate was calculated as the slope of the linear regression of the log2-transformed F_0 fluorescence vs time for each culture during the exponential phase (using minimal 6 measurements).

2.1.2. Stationary phase DW

Algal biomass concentration was measured gravimetrically on the second day of the stationary phase (based on F_0 fluorescence) for each strain separately. One of the two technical replicates was harvested by filtering 3 ml of homogenized culture through a pre-weighed and muffled (550 °C for 2 h) glass fiber filter (GF/F, diam. 25 mm, pore size 0.7 μm; Whatman, Kent, UK) and washing twice with de-ionized water (5 ml) to remove remaining salts. Filters were further freeze-dried at −80 °C overnight before determining the dry weight (DW).

2.1.3. Palmelloid cells

Palmelloid cells were distinguished based on [37] as follows: non-motile coccoid green cells of which the spherical protoplast is enveloped within a closely adherent palmella membrane, the primary cell wall. In a preliminary experiment, the evolution of the percentage of palmelloid cells was followed during batch culture growth (as followed using F_0) with increases in F_0 for six strains (Supporting information Fig. S1). All strains produced palmella cells yet at a different rate: for four of the six strains a maximal percentage palmelloid cells was reached when cultures where in stationary phase for two consecutive days (F_0-based), while for the two remaining strains at this same time point, the percentage palmelloid cells kept increasing gradually. Based on these preliminary data, the proportion of palmelloid cells in the cultures was quantified on the second consecutive day in stationary phase (time at which palmelloid cell formation reached a maximum) as follows. Subsamples of 50 μl of each homogenized culture were taken and fixed with formaldehyde borax (final concentration 1%). After settlement, pictures of each replicate were taken using a digital camera (Powershot G3, Canon) and the relative abundance of flagellated cells, dead cells and palmelloid cells ($n \geq 200$, per replicate) determined using Image J software (version 1.48; National Institutes of Health, USA). The final palmelloid cells (expressed as % of the total live cells) was used as a proxy for the rate at which cells transformed from flagellated stage to palmelloid.

Experimental stress conditions for astaxanthin induction (the second phase of the experiment) were evaluated in a preliminary experiment using six strains (see Supporting information Figs. S2 and S3). Cultures cultivated under conditions described above were transferred to four different stress-inducing conditions by omitting several nutrients from the basal BBM-3 N medium as following: a) without nitrate, b) without phosphate, c) without nitrate and phosphate and d) without