

## EVALUATION OF *ARTHROSPIRA (SPIRULINA) PLATENSIS* PRODUCTION TRAIT USING CPCHID OPERON

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### Abstract

*Arthrospira (Spirulina) platensis* is one of the most cultivated commercial microalgae. Many genotypes of *A. platensis* have been identified, while not all are suited for economic exploitation because of low productivity or poor vitality. The cpCHID (C-phycoerythrin rod linker polypeptide H, I and D) operon encodes the phycobilisome linker peptides involved in the photosynthesis, and may directly correlate to the production trait. Here, ten strains of *A. platensis* with known production traits from traditional procedure were selected and characterized by cpCHID sequences. With the help of phylogenetic analyses, three commercially cultivated strains with high growth yields were in evolutionarily close relationship forming a single cluster, while others with varying low growth yields formed the other cluster. Simultaneously, eight market products were also classified into the cluster with high growth yield. We thus gave the conclusion that production trait from traditional procedure is consistent with the cluster analysis of cpCHID sequence. Furthermore, the cluster-specific residues of CpcHID are revealed, and these residues may involve in the interaction with allophycocyanin or other functions. CpcD structure is predicted and modeled on the allophycocyanin-linker complex, showing the direct interaction between cluster-specific residues and chromophores. In comparison with normal tedious, time and labor-consuming procedure for evaluating the production trait, cluster analysis based on cpCHID sequences is undoubtedly a reasonable way to carry out the high throughput evaluation of this trait and discover new strains for mass production.

### Introduction

*Arthrospira*, previously called *Spirulina*, is the most widely exploited economic microalgae (Ciferri, 1983; Mosulishvili *et al.*, 2002). Because it's rich in protein contents, essential fatty acids, vitamins, minerals (Tokusoglu & Unal, 2003), and polysaccharides (Zhang *et al.*, 2010), *Arthrospira* was claimed to be an ideal food and dietary supplement in the 21<sup>st</sup> century by Food and Agriculture Organization of the United Nations and World Health Organization. Recently, *Arthrospira* attracts more interests on its potential medical and biodiesel application (Khan *et al.*, 2005; Bermejo-Bescos *et al.*, 2008; Bachstetter *et al.*, 2010; Cheong *et al.*, 2010; Khola & Ghazala, 2012).

The typical morphology of *Arthrospira* is characterized by its regularly helical coiling or spirals, which have been used as important taxonomic criteria and in the rank of product quality (Lewin, 1980; Ciferri, 1983; Belay, 1997; Wang & Zhao, 2005). Many strains (genotypes) of *Arthrospira* have been identified while not all these strains are commercially exploitable due to their different productivities and qualities. Some common features have been found for the present commercial strains of *Arthrospira*, like the regular coiled helix, the blue-green color, high growth rate, and high adaptability to the shift of circumstances (Li & Wang, 2002; Muhling *et al.*, 2006). However, there was still not a high throughput method for evaluating production traits of various genotypes.

Baurain *et al.*, (2002) tried to discover the possible common phylogenetic characters of the economical strains collected from market using ITS (internal transcribed spacer) analysis, while the result was amphibolous, possibly for the high conservation of ITS sequences below species level. Later, Yang *et al.*, (2006) indicated

the correlation between the phylogenetic places and productivities of several *A. platensis* genotypes based on the analyses of their cpCHID (C-phycoerythrin rod linker polypeptide H, I and D) operon. However, only limited strains and incomplete sequences were analyzed in the research, which undoubtedly attenuated the certainty of such a correlation. As a kind of ancient genes, cpCHID operon encodes the phycobilisome (PBS) rod linker peptides that are involved in the assembly and function of PBS (Pepper, 1998; Nomsawai *et al.*, 1999; David *et al.*, 2011). As the light harvesting machinery of cyanobacteria, any changes in PBS would affect the photosynthetic efficiency (Reuter *et al.*, 1999) and thus affect the final productivity. The polymorphism of cpCHID may reflect the differences of productivities. Furthermore, cpCHID is more divergence among *A. platensis* strains compared with ITS and 16S rRNA (Scheldeman *et al.*, 1999; Mao *et al.*, 2001; Baurain *et al.*, 2002; Ballot *et al.*, 2004), which could supply more accurate information in phylogenetic analysis for *A. platensis* genotypes.

### Materials and Methods

**Organisms and market products:** The strains used in this study are listed in Table 1, and their morphologies are shown in Fig. 1. Regularly coiled ZJU0103, ZJU0104 and ZJU0105 were commercial strains widely cultivated in mass production. All strains were axenically and clonally cultivated in Zarrouk's medium (Zarrouk, 1966) in a thermostatic chamber with alternating 12 h illumination at 54  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  and 12 h darkness. The cultivation temperature was 28°C under light and 20°C under darkness. The morphologies of filaments were observed under OLYMPUS CH-30 optical microscope. Other samples including eight market products are listed in Table 2.

**Table 1. Location and growth features of *A. platensis* strains used in this study.**

ZJU number <sup>a</sup>	Previous number <sup>b</sup>	Source and obtained time	Origin	The day of straight filaments observed	Average daily yield (g·m <sup>-2</sup> ·d <sup>-1</sup> ) <sup>c</sup>	GenBank accession number
ZJU0101	Sp-1	Y. L. Mao, 1994	Lake Texcoco, Mexico		5.25	EF583825
ZJU0102	Sp-2	T. Q. Gu, 1994	Lake Chad, Chad	105 <sup>th</sup>	5.01	EF583826
ZJU0103	Sp-3	Y. H. Wen, 1994	Lake Chad, Chad	112 <sup>nd</sup>	7.38	EF583827
ZJU0104	Sp-4	J. L. Jiang, 1995	Lake Chad, Chad	-	7.12	EF583828
ZJU0105	Sp-5	G. Y. Dong, 1995	Unknown	-	6.94	EF583830
ZJU0106	Sp-6	W. C. Du, 2002	Unknown	-	4.76	EF583829
ZJU0109/RH <sup>d</sup>	Sp-9	Obtained from ZJU0109/S, 2002	Unknown		2.81	EF583833
ZJU0110	Sp-10	Q. J. Yan, 1996	Unknown	49 <sup>th</sup>	4.84	EF583834
ZJU0111	Sp-11	W. D. Yi, 2002	Unknown	98 <sup>th</sup>	6.16	EF583841
ZJU0118	Sp-18	Jiangang Plant, 2004	Lake Chad, Chad	-	5.17	EF583840

<sup>a</sup> ZJU=Culture Collection of Algae at Zhejiang University, Hangzhou, China.

<sup>b</sup> Used in our previous papers and GenBank records.

<sup>c</sup> the cultivation was conducted from April 10th of 2008 to November 10th of 2008 at Jiangang Microalgae Plant, Zhejiang, China. The average daily yield was the total dry powder mass divided by the pond area and culture days. The cultivation of ZJU0109/RH was terminated at the 91st day because its filaments were seriously shortened and straightened and very hard to harvest, while other 9 strains were cultivated for 245 days using continuous cultivation.

<sup>d</sup> Obtained from a reverse change of straight ZJU0109/S strain, which was a gift from Mr. W. C. Du (RH=revert to helical, S=straight).

**Table 2. List of eight market products and strain C1 of *A. platensis* with the results obtained by sequences analyses of *cpCHID*.**

Sample designation	Source	<i>cpCHID</i> analyses	
		Cluster assignment	Corresponding <i>cpCHID</i> accession No.
MP1	Shunchang Tianshun Spirulina Co., Ltd., China	Cluster II	EF583838
MP2	Zhejiang Jiangshan Kangpu Biotechnology Co., Ltd., China	Cluster II	JN792589
MP3	Yangzhou Kanghua Biotechnology Co., Ltd., China	Cluster II	EF583837
MP4	Hangzhou Jiangang Biotechnology Co., Ltd., China	Cluster II	EF583831
MP5	Guangdong By-Health Biotechnology Co., Ltd., China	Cluster II	EF583839
MP6	Ningbo Zhenhai Mingte Blue Algae Co., Ltd., China	Cluster II	JN792590
MP7	Ningbo Zhenhai Mingte Blue Algae Co., Ltd., China	Cluster II	EF583832
MP8	DIC Corporation, Japan	Cluster II	GQ206143
Strain C1	Lake Bodou, Kanem, Chad	Cluster I	AF164139

MP: market products.

**The condition and productivity of mass cultivation:** The mass cultivation was conducted in a 300m<sup>2</sup> raceway pond covered with transparent plastic film at Jiangang Microalgae Plant, Zhejiang Province, China, with a media depth of 35 cm, and were circulated at a speed of 15m/min by means of paddle-wheels. A modified Zarrouk's medium was used in this large-scale cultivation, which contained 50g·m<sup>-3</sup> EDTA-Na<sub>2</sub>, 10 g·m<sup>-3</sup> FeSO<sub>4</sub>·7H<sub>2</sub>O, 20 g·m<sup>-3</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 100 g·m<sup>-3</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 1000 g·m<sup>-3</sup> NaCl, 500 g·m<sup>-3</sup> K<sub>2</sub>SO<sub>4</sub>, 200 g·m<sup>-3</sup> KCl, 1200 g·m<sup>-3</sup> NaNO<sub>3</sub>, 300 g·m<sup>-3</sup> K<sub>2</sub>HPO<sub>4</sub> and 6500 g·m<sup>-3</sup> NaHCO<sub>3</sub>. When the OD<sub>560</sub> of cultures was up to 0.9, filaments were harvested by pumping the cultures into a vibrating screen followed by filtering through a membrane of 62 μm. The filtrate was pumped back to the ponds, while the algal slurry was washed with drinking water and then applied to a spray-drier (SHEN2 GZL-100, China) to get the powder products. Harvesting was paused when the OD<sub>560</sub> of cultures

was down to 0.2 for a continuous cultivation, and nutrient concentration was adjusted to initial level. The productivity was shown as the average daily yield in table 1.

**PCR amplification of *cpCHID* operon:** The total DNAs of the ten strains listed in Table 1 were extracted separately according to the description of Li and Wang (Li & Wang, 2002), and were used for PCR. For the market products, the templates were prepared as previously described (Baurain *et al.*, 2002). PCR was performed in 25-μl reaction mixture by using forward primer PF (5'-CAATACATCTTCGCCGATTT-3') and reverse primer PR (5'-CGTATTATCGGTAGT CATC GG-3'). The PCR procedure was as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 60 s, and 72°C for 2 min, plus a final extension at 72°C for 8 min.

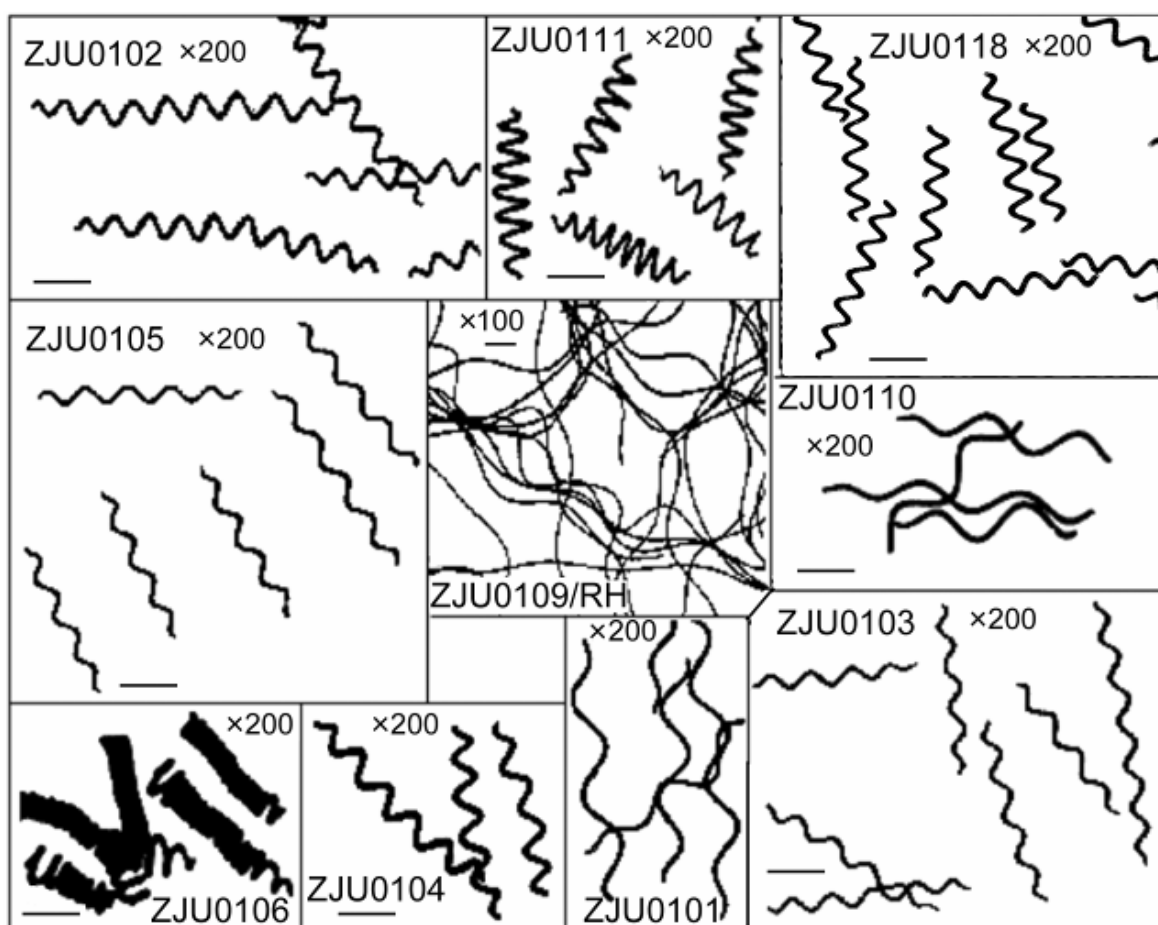


Fig. 1. The morphologies of ten *A. platensis* strains. Scale bars, 100  $\mu\text{m}$ .

**Sequence determination and analysis:** PCR products were purified from 1% agarose gel, and then were cloned into T-cloning site of pMD18-T vector (TaKaRa CO, Dalian, China) according to the manufacturer's instruction. Ligated plasmids were transformed into *Escherichia coli* TG1 competent cells, and transformants were selected on the LB/Amp<sup>+</sup> plate. Subsequently, plasmids from three recombinant colonies containing the amplified fragment were prepared by using a plasmid miniprep kit (Qiagen CO, Beijing, China). DNA sequence was determined using an Applied Biosystems 3730 sequencer (Applied Biosystems, USA). The GenBank accession numbers of determined cpcHID sequences are listed in Table 1.

Sequences under investigation were aligned by ClustalX software (version 1.81). Phylogenetic and molecular analyses were undertaken by using the Molecular Evolutionary Genetics Analysis software (MEGA version 5.0) (<http://www.megasoftware.net>). Phylogenetic trees were constructed by using neighbor-joining (NJ) method (Saitou & Nei, 1987). The robustness of the branching pattern was tested by bootstrap analyses through 1,000 replications.

**CpcD modeling with allophycocyanin:** Linker peptide CpcD from ZJU0103 showed 34% similarity with the core linker of allophycocyanin-linker complex AP·L<sub>C</sub><sup>7,8</sup> (pdb ID:

1B33). CpcD structure predicted using (PS)<sup>2</sup> server (Chen *et al.*, 2006) superposed the linker chain in AP·L<sub>C</sub><sup>7,8</sup> to obtain a model of CpcD-allophycocyanin complex.

## Results

**Morphological characteristics and product yields of *A. platensis*:** The appearances of 10 *A. platensis* strains were observed through optical microscope (Fig. 1). Strains ZJU0102 and ZJU0111 were fusiform-shaped with different helix pitch and trichome length. ZJU0106 showed the most tightly coiled trichomes, and ZJU0109/RH had the least degree of spiralization and longest filament. Other strains were in so-called regular spirals but showing various trichome lengths, helix diameters and helix pitches, except that the much more similar morphologies between ZJU0103 and ZJU0105, and between ZJU0101 and ZJU0110.

As listed in Table 1, after 245 days continuous cultivation, while 91 days for ZJU0109/RH, the ten strains could be divided into 4 classes according to their average daily yields. Commercial strains ZJU0103, ZJU0104 and ZJU0105 were in the first class with the highest daily yield of around 7g·m<sup>-2</sup>; the second class member of ZJU0111 showed a higher daily yield of 6.16g·m<sup>-2</sup>; strain ZJU0109/RH was in the last class with the least daily yield of only 2.8g·m<sup>-2</sup>. Other 5 strains were

in the third class for their similar average daily yield of around  $5\text{g}\cdot\text{m}^{-2}$ . In agreement with above classification, morphological changes (straight filaments) that showed no competence to the helical trichomes were observed firstly in ZJU0109/RH and then in some strains belonging to the third class (Table 1). No shape changes were found in the first and second class members during the whole cultivation period.

**Phylogenic analysis of *cpcHID* sequences:** An alignment of the amplified complete *cpcHID* sequences were analyzed, and used for the construction of phylogenic tree. As shown in Fig. 2, the ten strains were mainly grouped into two clusters. Commercial strains ZJU0103, ZJU0104 and ZJU0105 were classified into one single cluster, named cluster II. The distances of other strains in cluster I were mostly consistent with above classification according to their growth yields. This indicated that some features may exist in *cpcHID* and could be used as an indicator for the productivity of *A. platensis* genotypes.

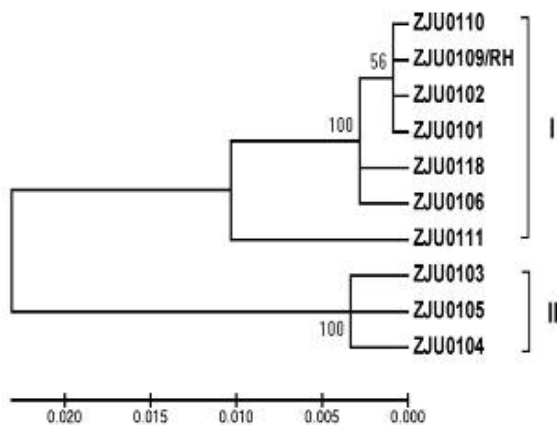


Fig. 2. Phylogenetic placements of ten *A. platensis* based on the analyses *cpcHID* sequences. Numbers around each node are confidence levels (%) generated from 1,000 bootstrap trees. The scale is in the units of the number of base differences per site.

The peptides alignment of CpcHID revealed several cluster-specific sites (Fig. 3). Here, 5 cluster-specific residues in CpcH, 6 in CpcI, and 3 in CpcD were observed to classify the strains into cluster I or cluster II. Nevertheless, more variables among the ten strains were also indicated by figure 3, which were useful to differentiate these strains.

**Cluster assignment of market products:** Eight market products of *A. platensis* named MP1-8 were collected with unknown genotypes, and their *cpcHID* sequences were determined. Except that the *cpcHID* sequences of MP2 and MP6 were new in GenBank, the *cpcHID* sequences of MP8, MP1, MP3, MP4, MP5 and MP7 were the same as that of NJ1999 and our laboratory strains Sp-16, Sp-15, Sp-7, Sp-17 and Sp-8 (table 2), respectively. Phylogenic analyses (Fig. 4) together with *A. platensis* strain C1 and samples in table 1 intriguingly assigned those market products, together with commercial strains in Fig. 2, into cluster II while strain C1 with known poor

vitality in cluster I. Further investigation of their CpcHID, the cluster-specific residues were also found in market products just as shown in Fig. 3. That is, *cpcHID*, especially some specific residues, does correlate to the productivity of *A. platensis*.

**CpcD interacts with chromophores in allophycocyanin-linker complex of phycobilisome:** We show the cluster-specific residues that may contribute to the various productivities between clusters. The crystal structure of allophycocyanin-linker complex (pdb 1B33), AP-L<sub>C</sub><sup>7,8</sup>, from phycobilisomes of *Mastigocladus laminosus* (Reuter *et al.*, 1999) allowed us to see the exact interaction of linker and allophycocyanin. The 34% similarity of linker in AP-L<sub>C</sub><sup>7,8</sup> (linker of *M. laminosus*) with CpcD of *A. platensis* supported a successful prediction of CpcD tertiary structure, and therefore modeled CpcD in the linker site of trimeric allophycocyanin core as shown in Fig. 5. Unlike the low sequence similarity, the predicted CpcD structure showed high structural similarity with the linker of *M. laminosus* consisting one  $\alpha$ -helix and three-stranded  $\beta$ -sheet in an elongated shape. Based on this model, the three cluster II specific residues lie in the surface of CpcD, while two of them, Ile27 and Gln60 are in the interface and interact directly with the allophycocyanin residues. Moreover, the two residues are in close contact with the chromophores by hydrogen bond ( $\delta\text{NH}_2$  of Gln60 with OB of CYC) or by hydrophobic interaction (Ile27 with BLA). Obviously, Met27 and Arg60 substitutions in cluster I may interfere in their contacts with chromophores, and then may lead to some structure changes that affect energy transfer in phycobilisome.

## Discussion

Mass cultivation is an important procedure in *Arthrospira* industry. First of all, productivity is the essential factor to be investigated. Traditionally, the production trait of any *Arthrospira* genotypes was obtained by carefully examining their growth condition, physiological and growth characters, trichome shapes and color, productivity, etc., through the whole growth period with several repeats. We have observed and confirmed that ZJU0101, ZJU0102, ZJU0106, ZJU0110 and ZJU0118 showed not only low adaptability upon the shifts of environmental factors (such as temperature, light intensity and pH), but also high possibility of abnormal conversion of trichome structures. In addition, at the early growing stage from a clonal culture, these strains were in a yellowish-green color, and needed much more time to reach to the logarithmic growth phase. On the contrary, ZJU0103, ZJU0104 and ZJU0105, together with corresponded strains of MP1, MP3, MP4, MP5 and MP7 in Table 2, showed a high growth rate and better adaptability upon environmental shifts, and therefore are applied widely in mass cultivation. Another *S. platensis* strain C1 (*Arthrospira* sp. PCC 9438) is not applied to mass production for its very short trichomes and the high frequency of morphological structure change from helical to straight (Deshnium *et al.*, 2000; Hongsthong *et al.*, 2007). Considering the productivities shown in table 1 and the cluster II assignment of market products (Fig. 3), the intriguing consistency of production trait from traditional way with that from phylogenic analyses can be clearly concluded.

CpcH

Table with 3 columns: Strain ID (ZJU0106 to ZJU0105), Peptide sequence, and Score (100 or 99).

Table with 3 columns: Strain ID (ZJU0106 to ZJU0105), Peptide sequence, and Score (200 or 199).

Table with 3 columns: Strain ID (ZJU0106 to ZJU0105), Peptide sequence, and Score (270 to 269).

CpcI

Table with 3 columns: Strain ID (ZJU0106 to ZJU0105), Peptide sequence, and Score (100).

Table with 3 columns: Strain ID (ZJU0106 to ZJU0105), Peptide sequence, and Score (200).

Table with 3 columns: Strain ID (ZJU0106 to ZJU0105), Peptide sequence, and Score (287).

CpcD

Table with 3 columns: Strain ID (ZJU0106 to ZJU0105), Peptide sequence, and Score (86).

Fig. 3. The peptides alignment of CpcH, CpcI and CpcD for the ten A. platensis strains. Cluster-specific residues are shown at the bottom of alignment, and shading area showed the identical residues.

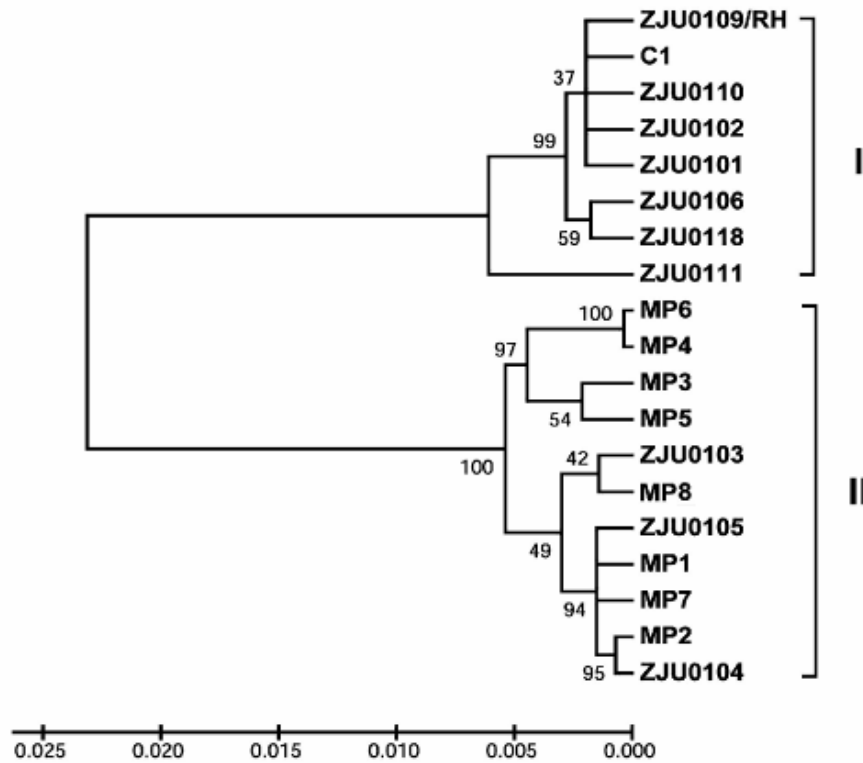


Fig. 4. Classification of *A. platensis* genotypes according to their *cpcHID* sequences.

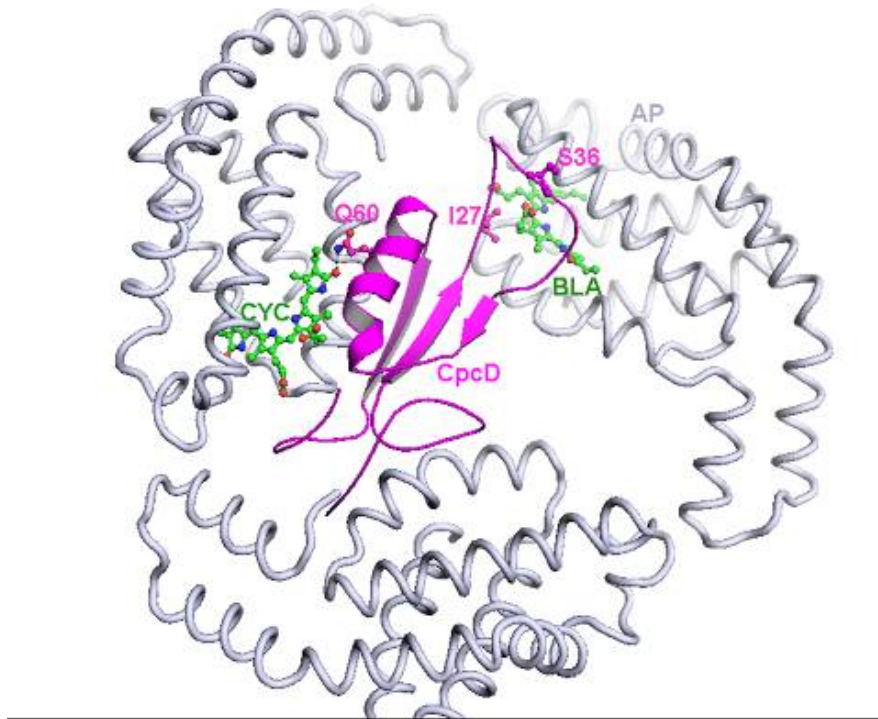


Fig. 5. Predicted ZJU0103-CpcD structure (pink cartoon) modeled with allophycocyanin (grey tube) based on AP-Lc<sup>7,8</sup> crystal structure (pdb 1B33). Cluster-specific residues and chromophores (in green color) were shown in ball and stick; I27 and Q60 lay in the interface of linker and allophycocyanin, and directly contact with the chromophores of biliverdins IX alpha (BLA) and phycocyanobilin (CYC) respectively.

The close relationship in phylogeny of some strains used in mass cultivation has been vaguely indicated by 16S rRNA or ITS analyses previously (Baurain *et al.*, 2002; Li & Wang, 2002). While the high-conserved character of 16S rRNA and ITS may limit the obtained phylogenetic information of *A. platensis* genotypes. The *cpcHID* operon encodes the phycobilisome linker peptides CpcH, CpcI and CpcD, and shows more divergence among various genotypes of *A. platensis* (Yang *et al.*, 2006). In this study, *cpcHID* were used to do cluster analyses, and cluster II members were confirmed to have high productivities in the first rank of production traits. As components of phycobilisomes, changes in the linker peptides may result in the changes of photosynthetic efficiency, and thus affect the final productivity. That is, using *CpcHID* to do production trait is reasonable.

The cluster-specific residues of *CpcHID* revealed here may relate to the key sites for the interaction with allophycocyanin or other function. The tertiary structure of CpcD of ZJU0103 was predicted and modeled with allophycocyanin. Based on present model, the cluster II-specific residues of I27 and Q60 directly interact with allophycocyanin, while S36 is in random coiled region. Most importantly, I27 and Q60 are in close contact with chromophores, suggesting their possible effect in the energy transfer within phycobilisome. Linker peptides are known early involving in the modulation of the chromophores' spectral properties (Fuglistaller *et al.*, 1987). Obviously, the consistency of productivity and *cpcHID* phylogeny is not just a coincidence; the inner mechanism and function of cluster specific residues are to be detected by more structural and biochemical experiments.

To evaluate production traits, phylogenetic analysis is undoubtedly a high throughput way to save much more time from the traditional way, and could help discovering more strains for *Arthrospira* commercial culture facilities. Cluster II members should be in first choice when breeding candidates for mass cultivation. However, it must be pointed out that not all cluster I members are lack of economic values. For example, ZJU0111 is also applied to mass cultivation for its high  $\gamma$ -linolenic acid content.

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