

# Combined QTL and GWAS Analysis to Identify the Growth-Related Gene in *Rhopilema Esculentum* with the Help of 2b-RAD Sequencing

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## Research Article

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# Abstract

*R. esculentum* is a popular seafood in Asian countries and an economic marine fishery resource in China. However, the high-resolution genetic map and growth-related molecular markers still lack, hindering the process of the genetic breeding of *R. esculentum*. Therefore, we firstly used the 2b-RAD method to sequence 152 *R. esculentum* specimens, identified 9100 single nucleotide polymorphism (SNP) markers and constructed a high-resolution genetic map with a marker interval of 0.58 cM, covering 98.68% of the genome. Then, we separately detected four and three quantitative trait loci (QTLs) associated with umbrella diameter and body weight based on the linkage map, which is located on linkage group (LG) 4, 13, 14 and 15. Finally, 27 genes were found both associated with umbrella diameter and body weight of *R. esculentum* by genome-wide association study (GWAS), in which one gene named *RE13670* containing calcium-binding EGF-like domain may play an important role in controlling the growth. This study will be beneficial for underlying the growth mechanism of *R. esculentum* and also provide background knowledge for guiding its genetic breeding.

## Introduction

Edible jellyfish *R. esculentum* distributes in the northwest Pacific Ocean and is a popular seafood in Asian countries, especially in China<sup>1,2</sup>. The edibility, nutritional value and medicinal properties of *R. esculentum* make it an economical fish resource and widely farmed in aquaculture systems of China<sup>2</sup>. Although morphological development, environmental physiology and culture techniques have been well investigated in *R. esculentum*<sup>2</sup>, the molecular marker studies still lag in the genetic breeding industry.

The advance of the 2b-RAD approach attracts many researchers' attention and has accelerated the identification of SNP markers in aquatic fishes, such as *Carassius auratus*<sup>3</sup>, *Cyprinus carpio haematopterus*<sup>4</sup>, *Hypophthalmichthys nobilis*<sup>5</sup> and *Hemibagrus wyckioides*<sup>6</sup>. Based on the 2b-RAD approach, the high-resolution genetic linkage maps were constructed with a marker interval varying from 0.44 to 0.87 cM<sup>3-6</sup>. The genetic linkage map helped mapping QTLs of interested growth traits in aquatic species for genetic breeding<sup>3-7</sup>. Many QTLs related to body weight, body length, nutritional metabolisms and sex were identified in economic fishes through QTL analysis<sup>5,6,8,9</sup>. In addition to QTL analysis, GWAS has been a supplementary method for localizing the target genes in QTLs and successfully applied in aquatic animals, such as *Litopenaeus vannamei*<sup>10</sup>, *Atlantic salmon*<sup>11</sup>, rainbow trout<sup>12</sup> and catfish<sup>13</sup>.

Although the genomic information of *R. esculentum* has been released<sup>14</sup>, the high-resolution linkage map of *R. esculentum* is still lacking and the growth-related genes have not been reported yet. For *R. esculentum*, body weight and umbrella diameter are the main growth traits. Therefore, we integrated QTL and GWAS analyses to identify the genes that related to body weight and umbrella diameter of *R. esculentum* with the help of 2b-RAD sequencing.

## Results

## Phenotyping

The body weight and umbrella diameter of *R. esculentum* F1 offsprings varied from 2.7 to 33.5 g and 3.2 to 7 cm, respectively (Figure 1). The body weight of most *R. esculentum* was between 5 and 8 g and the umbrella diameter of most *R. esculentum* was between 3 and 5 cm (Figure 1A, B). Figure 1C showed that the body weight and umbrella diameter are positively correlated in *R. esculentum* and the correlation between them was also supported by the value of CORREL function (0.90) (Supplementary Table 1). In addition, the value of the coefficient of variation (C.V) for body weight in *R. esculentum* offsprings is higher than that of umbrella diameter (Supplementary Table S1).

## 2b-RAD sequencing

Total 1539 million raw reads were generated by 2b-RAD sequencing, including 19 million from the male and female parents and 1520 million from the F1 offspring (Table 1). The ratio of clean reads to raw reads is higher than 93.63% and the alignment rate of the clean reads to *R. esculentum* genome was close to 63% (Table 1). The tag number of female and male parents was both higher than that of F1 offspring while the tag depth was both lower than that of F1 offspring.

**Table 1.** Summary of 2b-RAD sequencing.

|                     | Raw reads | Clean reads | Alignment rate | Tag number | Tag depth |
|---------------------|-----------|-------------|----------------|------------|-----------|
| Male parent         | 9594032   | 8982914     | 62.95%         | 36765      | 154       |
| Female parent       | 9594032   | 9109329     | 62.17%         | 36503      | 155       |
| F1 offspring (mean) | 10556728  | 10056703    | 63.13%         | 35624      | 178       |

## Linkage mapping

Total 9100 SNP markers were generated with the transition to transversion (TS/TV) ratio of 1.48 and 6674 SNP markers can be used for constructing the linkage map after filtering. A total of 1427 and 1460 SNP markers were separately selected for constructing female and male-specific linkage maps (Figure 2). The number of LGs corresponded to the chromosome number of *R. esculentum*, named LG1-21 (Figure 2). The genetic lengths of the female and male-specific maps were 1360.29 and 1200.62 cM with a marker interval of 0.95 cM and 0.82 cM, respectively (Figure 2).

The consensus linkage map of *R. esculentum* was constructed using 2508 SNP markers, covering 98.68% of *R. esculentum* genome (Figure 3, Supplementary Table S2). The genetic length of the consensus linkage map was 1456.34 cM with a marker interval of 0.58 cM (Figure 3, Supplementary Table S2). The longest LG is LG14 with a genetic length of 88.9 cM, 1.75-fold higher than that of the shortest LG20 (Figure 3).

## QTL mapping

For *R. esculentum*, four and three QTLs were detected associated with the umbrella diameter and body weight, respectively (Table 2). These QTLs are located on LG 4, 13, 14 and 15 and the LOD value floated between 3.0 and 4.4 (Table 2). For umbrella diameter and body weight, two QTLs in the similar regions separated existed on LG 14 and 15, explaining 9.4 to 13.0% variation (Table 2).

**Table 2.** QTLs associated with umbrella diameter (UD) and body weight (BW) in *R. esculentum*.

| Traits | Linkage group | Location/cM | Chromosome | LOD value | Explained variation(%) |
|--------|---------------|-------------|------------|-----------|------------------------|
| UD     | 13            | 42.40       | REGS429    | 3.1       | 9.6                    |
| UD     | 13            | 42.41       | REGS429    | 3.1       | 9.6                    |
| UD     | 14            | 48.73       | REGS695    | 3.68      | 11.3                   |
| UD     | 15            | 40.4        | REGS285    | 4.3       | 13.0                   |
| BW     | 4             | 15.34       | REGS515    | 3.22      | 9.9                    |
| BW     | 14            | 49.45       | REGS695    | 3.4       | 10.5                   |
| BW     | 15            | 41.4        | REGS285    | 3.06      | 9.4                    |

### Candidate genes related to umbrella diameter and body weight

GWAS analysis indicated that the candidate genes related to umbrella diameter and body weight located on LG 14 and 15, following the results of QTL analysis (Figure 4A, Table 2). In *R. esculentum*, 28 and 35 candidate genes were identified associated with umbrella diameter and body weight, respectively (Supplementary Table S3). Of these candidate genes, 27 genes were overlapped and one overlapped gene *RE13670* (Appendix 1) that located on LG14 may play the key role in controlling the growth of *R. esculentum* (Figure 4B, Supplementary Table S3). *RE13670* was annotated as EGF and pentraxin domain-containing protein 1-like and contained six types of conserved domains, including DUF5011 super family, Calcium-binding EGF-like (EGF\_CA), Ephrin\_rec\_like, FXa\_inhibition, IG\_like and PLAT (Figure 4C). In addition, *RE13670* was homologous to *Acropora digitifera* with 30.36% similarity after NCBI-blast analysis.

## Discussion

Growth shows a moderate heritability in aquaculture animals and plays an important role in production output, therefore, making it a primary target for selective breeding<sup>15,16</sup>. Our study showed the growth traits body weight and umbrella diameter are positively correlated in *R. esculentum*, manifesting any trait can independently represent the growth. Excluding growth-related phenotype, molecular marker shows potential for investigating the growth of aquaculture animals in the genetic breeding industry<sup>17</sup>. SNP markers as the molecular marker were genotyped easily to construct genetic linkage maps for the genetic breeding of aquaculture animals<sup>18</sup>.

Due to the simplicity and flexibility, the 2b-RAD method was extensively used for constructing high-density linkage maps for fish<sup>5,6,18-20</sup>. Zhu et al. constructed the high-density linkage map of *Pseudobagrus ussuriensis* with an interval of 0.357 cM via 2b-RAD method<sup>18</sup>. For *R. esculentum*, the marker interval of the linkage map is 0.58 cM at intermediate levels, higher than *H. nobilis*<sup>5</sup>, *H. wyckioides*<sup>6</sup> and *Channa argus*<sup>20</sup>, less than *C. auratus*<sup>3</sup>, *P. ussuriensis*<sup>18</sup>, *Larimichthys crocea*<sup>21</sup>. The difference of marker interval between *R. esculentum* and the other aquaculture animals may attribute to the SNP numbers used for constructing linkage maps and the depth of genetic studies. For *P. ussuriensis*, 7435 SNPs were used for constructing the linkage map, which is 1.96-fold higher than that of *R. esculentum*<sup>18</sup>. In addition, the genetic studies of growth and sex-determination were widely carried out in *P. ussuriensis*<sup>18,22,23</sup>, deeper than that of *R. esculentum*. However, this is the first report of a high-density linkage map in *R. esculentum*.

In aquatic animals, the high-density linkage map also plays an important role in performing QTL mapping and finding genes related to the growth traits<sup>3,5,6,8</sup>. Based on the high-density linkage map, numerous QTLs about growth traits, such as body weight, body length, sex as well as disease resistance were identified<sup>3,20,21,24-28</sup>. In *C. auratus* at 2 months, eight QTLs in eight chromosomes were discovered associated with the body weight, explaining 10.1–13.2% of the phenotypic variations<sup>3</sup>; In *Nibeia albiflora*, 22 and 13 QTLs were detected associated with growth (body weight, body height, body width and body length) and sex dimorphism, respectively<sup>24</sup>. For *R. esculentum*, three and four QTLs were separately identified concerning umbrella diameter and body weight, less than the QTL numbers found in *C. auratus* and *N. albiflora*<sup>3,24</sup>. This may be caused by the higher-level classification of *C. auratus* and *N. albiflora*, so the genomes may be more complex and genes locating on more chromosomes participated in regulating the growth. For *C. auratus*, eight QTLs distributing on five LGs were related to the body weight<sup>3</sup>; For *N. albiflora*, six QTLs distributing on six LGs were related to the body weight<sup>24</sup>. Additionally, two QTLs in similar regions are both associated with umbrella diameter and body weight in *R. esculentum*, further supporting the above inference and the positive correlation of the two growth traits.

QTL analysis often combines with GWAS for identifying growth-related genes in aquaculture animals<sup>29,30</sup>. For example, five QTLs were revealed associated with the body weight of catfish and the candidate genes in these regions were related to bone development and muscle growth<sup>29</sup>. In *Epinephelus fuscoguttatus*, 23 QTLs are detected corresponding to the growth and 19 candidate genes were detected<sup>30</sup>. Table 3 summarized the candidate genes related to growth traits of aquaculture animals by GWAS and different species showed the difference (Table 3). More than one gene about multiple functions was identified in relation to the growth of *C. carpio* L., *L. crocea* and *O. mykiss* while one candidate gene was identified controlling the growth in *P. yessoensis*, *E. fuscoguttatus* and *L. maculatus* (Table 3). In *R. esculentum*, RE13670 containing the EGF\_CA domain showed the most possibility in controlling the growth, which is in accordance with the growth-related genes reported in *C. auratus*<sup>3</sup>. EGF\_CA domain needs calcium for performing biological function and is present in extracellular (mostly animal) and membrane-bound<sup>31</sup>. Moreover, this domain has three main roles, including protein-protein

interactions, as a spacer unit and structural stabilization<sup>32</sup>. Nevertheless, the function of EGF\_CA domains has not been well understood in aquaculture animals. With the release of genomic information of *R. esculentum*<sup>14</sup> and the development of biotechnology, the gene function studies of *R. esculentum* will be improved and more genes will be investigated for genetic breeding.

Table 3

Summary details of the candidate genes related to growth of aquaculture animals by GWAS analysis.

| Species                 | Gene  | Growth traits  | Reference  |
|-------------------------|---|--|------------|
| <i>P. yessoensis</i>    | <i>E2F transcription factor 3</i>   | Shell length and height, body weight and adductor muscle | 33         |
| <i>C. carpio</i> L.     | BR serine/threonine-protein kinase 2 and eukaryotic translation-initiation factor 2-alpha kinase 3    | Body weight  | 34         |
| <i>E. coioides</i>      | Neuropeptide Y receptor Y2  | Body weight and total length                             | 35         |
| <i>E. fuscoguttatus</i> | <i>bmp2k</i>  | Body weight, length, height and thickness                | 30         |
| <i>L. crocea</i>        | <i>fgf18, fgf1, nr3c1, cyp8b1, fabp2</i> and so on  | Growth and body shape                                    | 36         |
| <i>L. maculatus</i>     | <i>fgfr4</i>  | Body weight and length                                   | 37         |
| <i>L. vannamei</i>      | Protein kinase C delta type and ras-related protein Rap-2a  | Body weight and length                                   | 38         |
| <i>L. vannamei</i>      | Class C scavenger receptor  | Body weight  | 10         |
| <i>O. mykiss</i>        | Genes involved growth factors and development of skeletal muscle, bone tissue and nutrient metabolism | Body weight  | 39         |
| <i>R. esculentum</i>    | <i>RE13670</i>  | Body weight and umbrella diameter                        | This study |

## Conclusion

In this study, a high-density linkage map of *R. esculentum* was constructed with a marker interval of 0.58 mM using the 2b-RAD method. Based on the linkage map, seven QTLs were identified associated with the growth of *R. esculentum* and one candidate gene *RE13670* encoding EGF\_CA protein may play the key role in controlling the growth of *R. esculentum* by QTL mapping and GWAS analysis. Both the constructed linkage map and identified candidate genes will expand the knowledge of molecular markers of the genetic breeding industry in *R. esculentum*.

## Methods

## Mapping family and phenotyping

A full-sib family of *R. esculentum* was generated from a breeding pond in Yingkou City, Liaoning province, China. Total 152 specimens of *R. esculentum* including two parents and 150 F1 offsprings at seven-month were collected from the mapping family for measuring the body weight and umbrella diameter.

## DNA extraction

The *R. esculentum* specimens were collected for extracting genomic DNA and the DNA extraction process referred to our previous method<sup>14</sup>. The DNA quality was measured by Qingdao OE Biotech Co., Ltd.

## 2b-RAD library construction, sequencing and analysis

After removing the low-quality DNA samples, 2b-RAD libraries of 144 specimens were constructed at Qingdao OE Biotech Co., Ltd., following the published method<sup>40</sup>. Firstly, 100-200 ng genomic DNA was digested by 1 U BsaXI (New England Biolabs). Secondly, the ligation reaction was conducted to add specific adaptors to the digested genomic DNA. Thirdly, the ligation products were amplified in MyCycler thermal cyclers (Bio-Rad). Fourthly, the PCR products were purified using a MinElute PCR Purification Kit and digested using Sapl (New England Biolabs). Fifthly, the digested products were transferred to the tube containing magnetic beads for incubation and then transferred the supernatant to a new tube for ligation using T4 DNA ligase (New England Biolabs). After that, the ligation products were purified and barcodes were introduced by PCR using barcode-bearing primers. Finally, PCR products were purified and pooled for sequencing using the Illumina Novaseq 6000 PE150 sequencing platform.

The raw data of 2b-RAD sequencing were trimmed for getting the high-quality data, and then the high-quality data were aligned to reads. The reads with the BsaXI site were extracted and aligned to the reference reads using SOAP (version 2.21)<sup>41</sup>. The reference reads were extracted from *R. esculentum* genome (NCBI Genome ID: 56778) after electronic digestion using the BsaXI enzyme. The maximum likelihood method was used for SNP typing<sup>42</sup>. The generated SNPs were filtered and were annotated using the software SnpEff (version 4.1)<sup>43</sup>.

## Linkage map construction

SNPs with minor allele frequency (MAF) equal to 0.05 and the maximum missing rate equal to 0.2 were used to construct the linkage maps by software JoinMap 4.0 version<sup>44</sup>, setting the logarithm of odds (LOD) threshold values between 2 and 15. The male-specific linkage map was constructed using paternal heterozygous genotype and maternal heterozygous and homozygous genotype. The female-specific linkage map was constructed using maternal heterozygous genotype and paternal heterozygous and homozygous genotype. The consensus genetic linkage map was constructed by merging male and female-specific linkage maps using the software MergeMap<sup>45</sup>. Marker distances were calculated using Kosambi's mapping function<sup>46</sup>.

## QTL mapping analysis

Based on the consensus linkage map, QTL mapping analyses of body weight and umbrella diameter in *R. esculentum* were performed by software MapQTL 6<sup>47</sup>. The interval mapping method was used for QTL analysis and every one cM on each LG was scanned for searching the possible QTL. LOD threshold value at 95% level was calculated via 1000 permutation tests for each trait and QTL. LOD score of QTL that was greater than the LOD threshold value at 95% level was declared significant.

## Genome-wide association analysis

The genome-wide association analysis of body weight and umbrella diameter and SNP locus on the linkage map was performed by software plink<sup>48</sup>. The genome-wide significant threshold value was determined by the formula:  $p \text{ value} = 0.05/N$ , where N represents the marker numbers for association analysis<sup>49</sup> and the threshold of  $-\log_{10}(p \text{ value})$  was set as 3. Manhattan plots were generated by the R software package 'MVP'.

## Candidate genes associated with body weight and umbrella diameter

The genes located on the up-and down-stream 500 Kb distance of the associated genomic region of body weight and umbrella diameter were detected<sup>14,50</sup>. We ascertained the candidate genes by combining their function annotation and analyzed results via online software NCBI-blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Conserved domain search service (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

## Declarations

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## Contributions

B.L. Chen: data analysis, writing-the original article. Y.L. Li: sample collection, article editing. M.L. Tian: sample collection, measurement of the phenotypes. S. Hao: sample collection. W. Sun: sample collection. Y.F. Li: experimental design and analysis.

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## Ethics declarations

Competing interests statement

The authors declare no competing interests.

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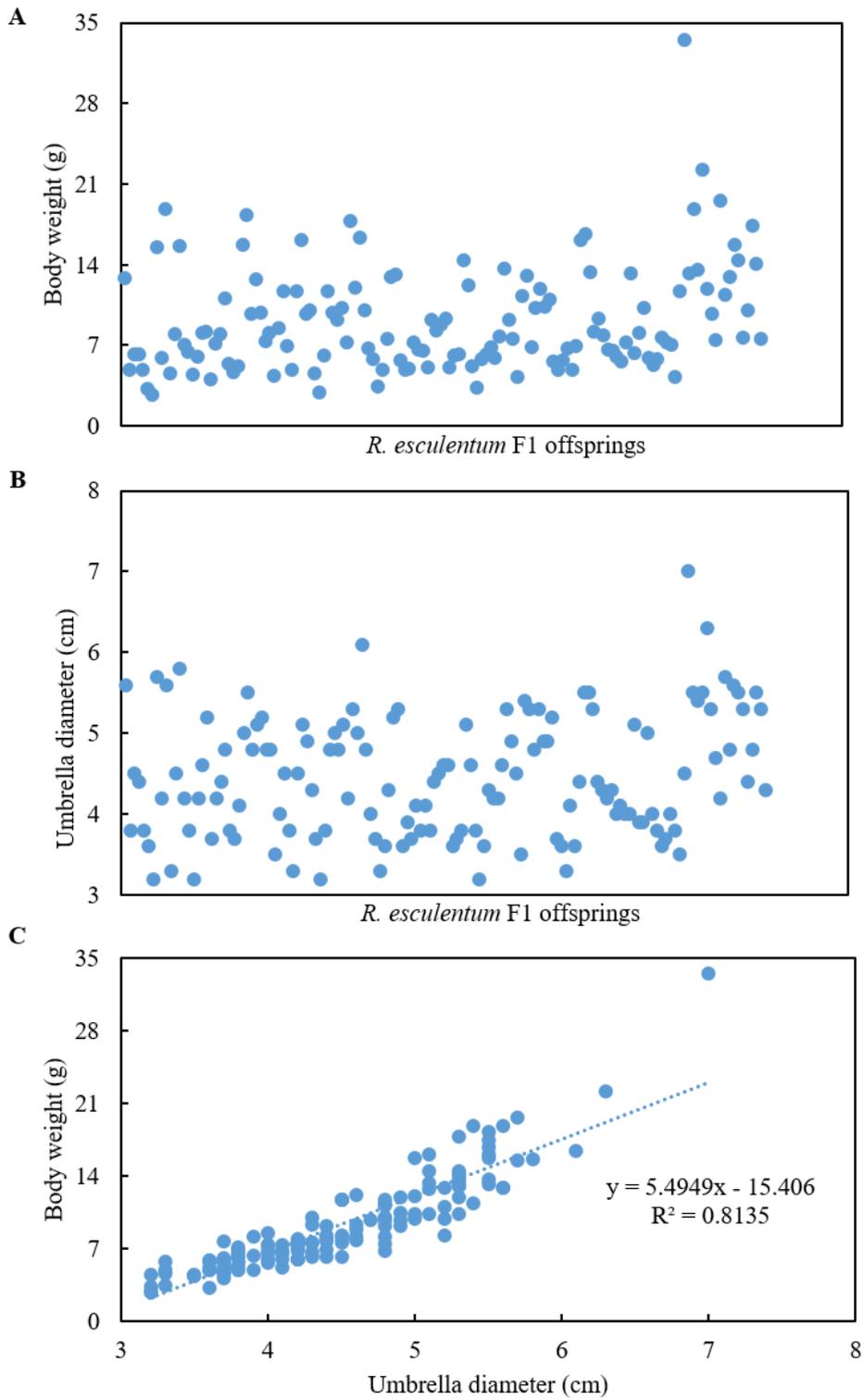
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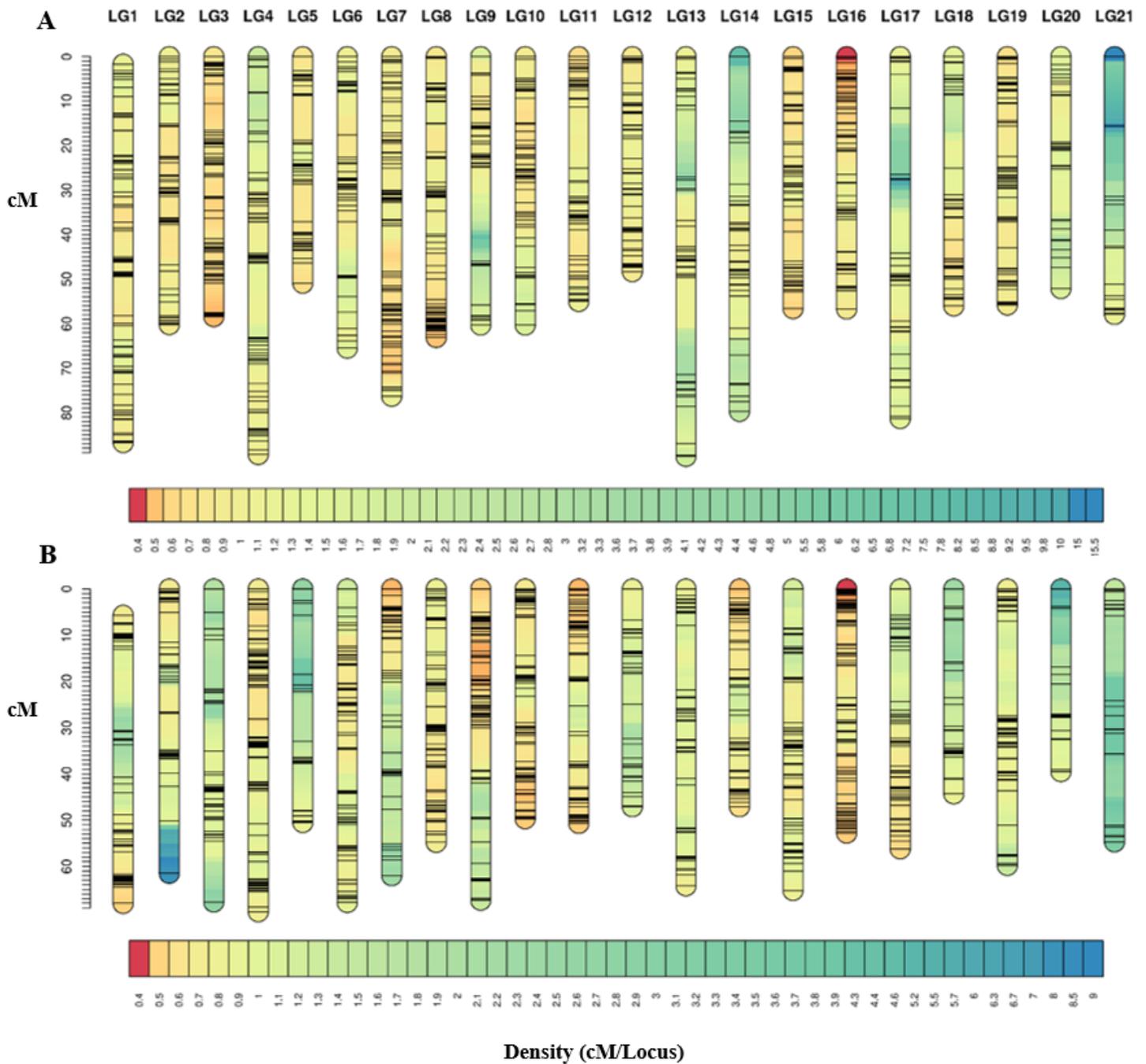
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## Figures



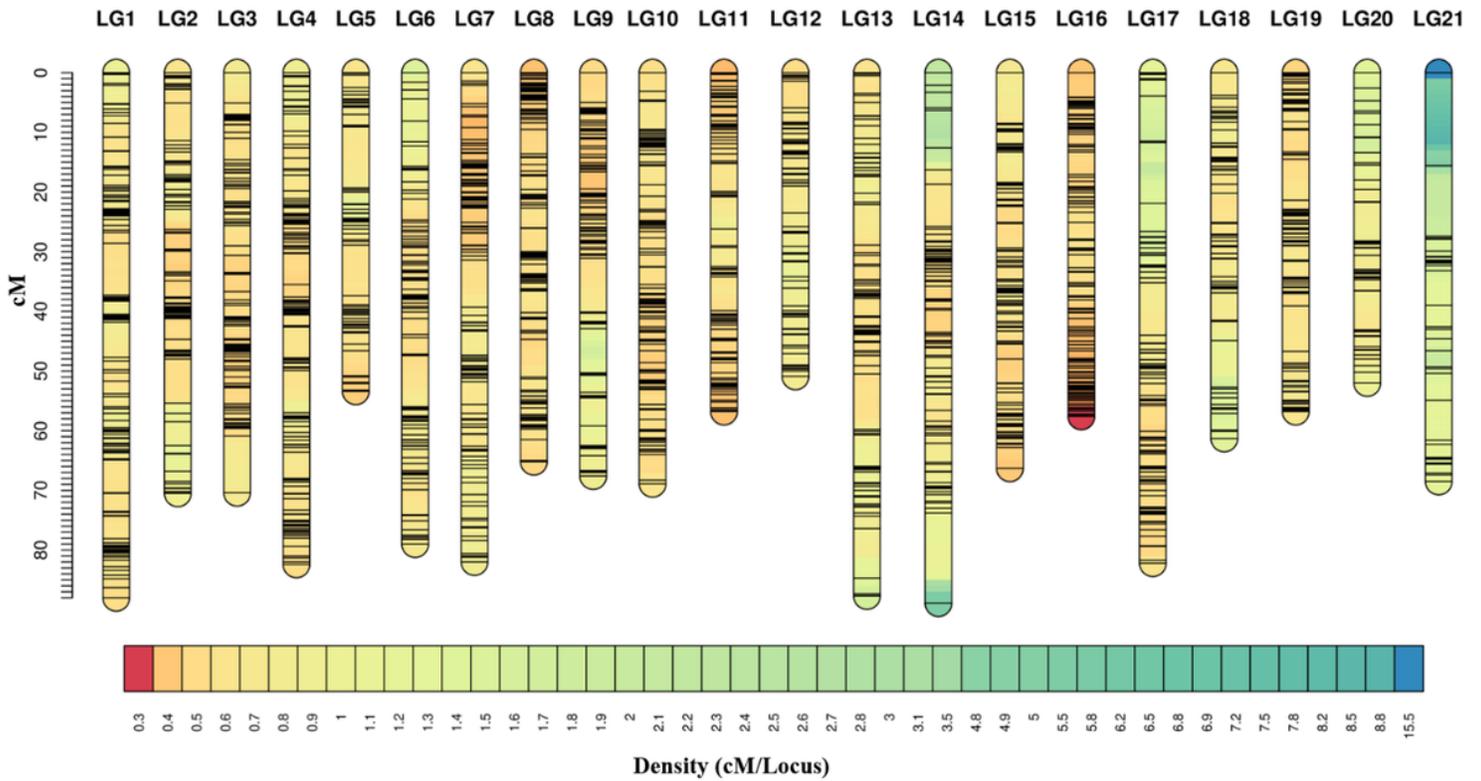
**Figure 1**

Analysis of body weight and umbrella diameter in *R. esculentum* F1 offsprings. (A) Body weight, (B) Umbrella diameter, (C) Linear relation between umbrella diameter and body weight.



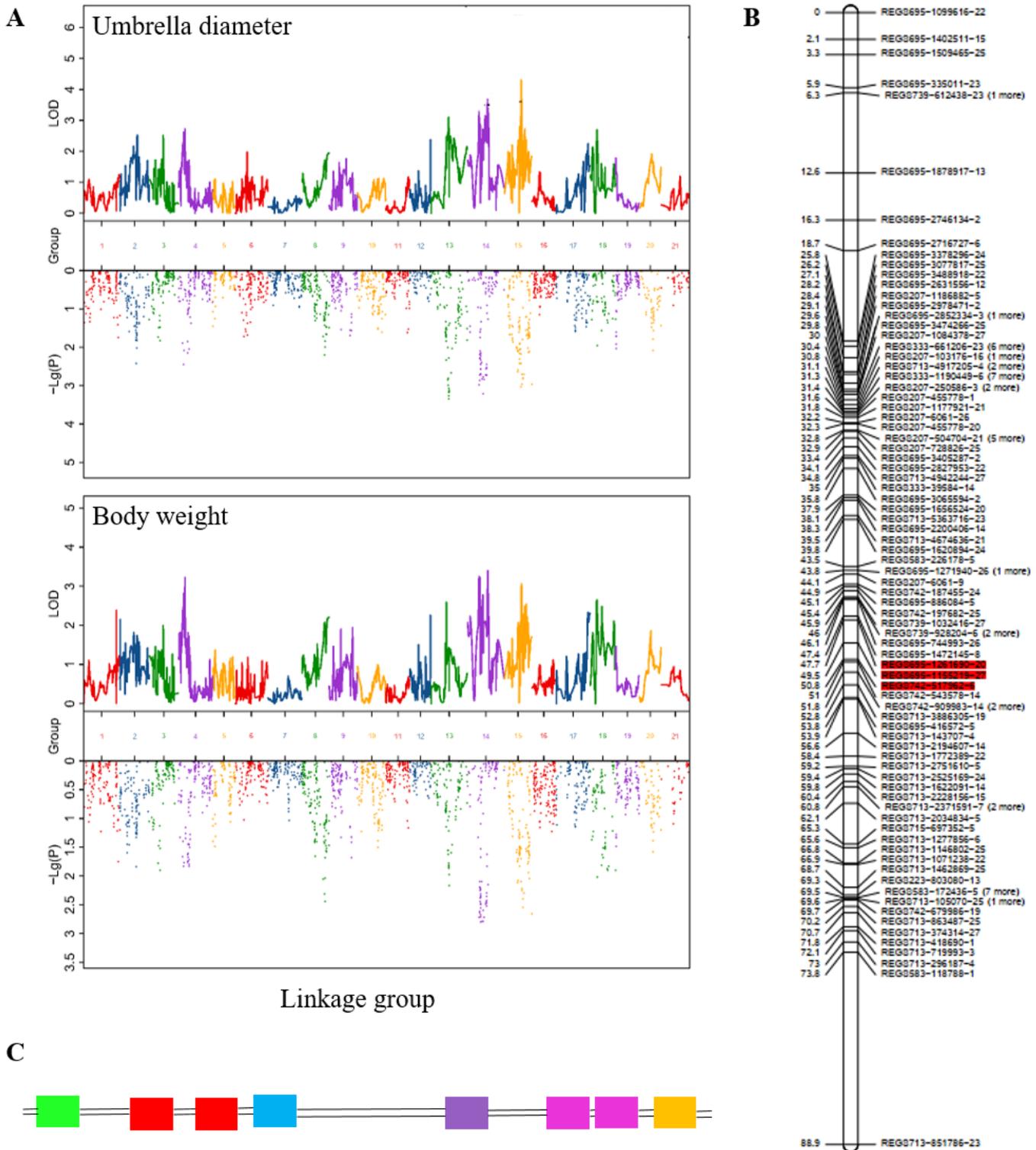
**Figure 2**

Genetic distance and marker distribution in two parental linkage maps. (A) The linkage map of the female parent, (B) The linkage map of the male parent. The scaleplate on the left indicates genetic distance (cM as a unit). The below color module represents the distribution density of the SNP marker per cM.



**Figure 3**

Genetic distance and marker distribution in the consensus linkage map of *R. esculentum*. The scaleplate on the left indicates genetic distance (cM as a unit). The below color module represents the distribution density of the SNP marker per cM.



**Figure 4**

Analysis of the candidate genes related to umbrella diameter and body weight in *R. esculentum* by QTL and GWAS analysis. (A) QTL and GWAS analysis for body weight and umbrella diameter, (B) SNP markers located on linkage map 14, (C) Conserve domain analysis of candidate gene RE13670. In figure 4B, the red background indicates the QTLs locating on LG 14, In figure 4C, the green rectangle indicates PLAT/LH2 domain, the red rectangle indicates EGF\_CA domain, the blue rectangle indicated

FXa\_inhibition domain, the purple rectangle indicates IG\_like domain, the pink rectangle indicates Ephrin\_rec\_like domain, the orange rectangle indicates DUF5011 super family domain.

## Supplementary Files

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