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1 **Isolation and characterization of a new *DUR3*-like gene, *PyDUR3.3*, from**  
2 **the marine macroalga *Pyropia yezoensis* (Rhodophyta)**

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27 **Abstract**

28

29 DUR3 proteins mediate high-affinity transport of exogenous and endogenous  
30 urea. Although two *DUR3*-like genes (*PyDUR3.1/3.2*) have been identified in  
31 *Pyropia yezoensis*, a BLAST search using these sequences against the *P. yezoensis*  
32 EST database suggested the existence of another *DUR3*-like gene (*PyDUR3.3*). In  
33 this study, the *PyDUR3.3* gene was isolated and characterized and compared to  
34 *PyDUR3.1/3.2* genes. The predicted length of *PyDUR3.3* was 679 amino acids,  
35 which included 15 transmembrane domains. An amino acid sequence alignment of  
36 algal, plant, and yeast *DUR3* proteins showed that *PyDUR3.3* was more similar to  
37 *PyDUR3.2* than to other *DUR3* proteins including *PyDUR3.1*. Exon-intron  
38 structures of *PyDUR3.3* and *PyDUR3.2* were also closely related to each other,  
39 which clearly differed from that of *PyDUR3.1*. Expression analysis showed that  
40 *PyDUR3.3* mRNA levels were extremely high in sporophytes, regardless of the  
41 nutrient condition, compared to gametophytes. On the other hand, expression of  
42 *PyDUR3.2* and *PyDUR3.1* was high in the gametophytes and sporophytes,  
43 respectively, and induced by nutrient starvation. These results suggest that  
44 expression of *PyDUR3.3/3.2/3.1* depends on the life history phase as well as the  
45 nutrient conditions, and that *PyDUR3.3* and *PyDUR3.2* are paralogues specifically  
46 differentiated in function and life history phase.

47

48

49 **Keywords:** cDNA cloning • *DUR3* • gene expression • *Pyropia yezoensis* •  
50 Rhodophyta • sodium solute symporter • urea transporter

51

## 52 **Introduction**

53

54 The rhodophyte genus *Pyropia* represents a unique heteromorphic and digenetic  
55 life cycle that consists of a leafy gametophyte and a filamentous sporophyte [1].  
56 These developmental generations, in which *Pyropia* species form a thallus and a  
57 conchocelis, respectively, show differences in physiological and structural features,  
58 such as optimum growth conditions, cell wall composition, and chloroplast and  
59 chromosome numbers [2–7]. Since the heteromorphic life cycle can be reproduced  
60 within a few months in the laboratory, *P. yezoensis* has been utilized as a valuable  
61 model organism for fundamental and applied marine macroalgal research [5, 8].

62 In Japan, *P. yezoensis* is one of the most important algal species used as food,  
63 and approximately 350,000 tons (wet weight) of the gametophytic thalli are  
64 produced in *Pyropia* cultivation farms every year, most of which are processed to  
65 dried sheets of “*nori*” (e-Stat, portal site of official statistics of Japan:  
66 <http://www.e-stat.go.jp/SG1/estat/eStatTopPortal.do> “Accessed 24 Jun 2015”).  
67 The yield and quality of the cultivated *Pyropia* thalli are frequently affected by  
68 environmental factors. In particular, growth, development, and quality of *Pyropia*  
69 thalli are influenced by changing dissolved inorganic nitrogen (DIN) content, which  
70 consists of nitrate-N ( $\text{NO}_3\text{-N}$ ), nitrite-N ( $\text{NO}_2\text{-N}$ ), and ammonium-N ( $\text{NH}_4\text{-N}$ )  
71 [9–11]. Although these inorganic-N sources are essential for growth and  
72 development in photosynthetic eukaryotes, some plants and algae, including  
73 *Pyropia*, can efficiently utilize not only these types of inorganic-N but also  
74 organic-N such as urea-N and amino acid-N (AA-N) [9, 12–18]. In order to  
75 investigate the molecular mechanisms for inorganic/organic-N uptake and  
76 assimilation in *Pyropia* thalli, a subtracted cDNA library was constructed from *P.*  
77 *yezoensis* thalli grown in different nutrient conditions, and transcripts differentially  
78 expressed have been analyzed. To date, three *P. yezoensis* genes, a nitrate  
79 transporter gene, *PyNRT2*, and two urea transporter genes, *PyDUR3.1* and  
80 *PyDUR3.2* (previously designated as *PyUT1* and *PyUT2*, respectively), which may  
81 be involved in uptake and transport of nitrate and urea, respectively, have been  
82 identified [19]. In *P. yezoensis* thalli, the transcripts of both *PyDUR3* genes were  
83 markedly up-regulated under N-deficient conditions, suggesting that these proteins  
84 are associated with N-acquisition pathways for the direct utilization of urea from  
85 the environment.

86 *DUR* (Degradation of URea) genes were first characterized in yeasts. In the  
87 yeast *Saccharomyces cerevisiae*, the cytoplasmic enzymes urea carboxylase (*DUR1*)  
88 and allophanate hydrolase (*DUR2*), catalyzing degradation of urea to ammonia and  
89 carbon dioxide, were originally considered to be two separate genes, but were later  
90 found to be encoded by a single polypeptide and thus renamed *DUR1,2* [20, 21].  
91 The *DUR3* gene family encodes membranous urea transporter proteins which are  
92 structurally related to the sodium solute symporter (SSS) superfamily. SSS  
93 proteins are integral membrane proteins containing 11 to 15 putative transmembrane  
94 domains (TDs), which mediate the transport of wide variety of substrates (e.g., AAs,  
95 inositols, sugars, and urea) across cytoplasmic membranes [16, 18, 22]. In  
96 photosynthetic eukaryotes, *AtDUR3* from the model land plant *Arabidopsis thaliana*  
97 has been well characterized. *AtDUR3* (previously named At5g54380) was  
98 identified as a putative urea transporter gene by a genome-wide *in silico* search  
99 using various urea transporter sequences from mammals and microbes against the  
100 *Arabidopsis* genome, showing approximately 40% sequence identity to the yeast  
101 functional urea transporter gene, *S. cerevisiae* *ScDUR3* [23, 24]. *AtDUR3* is  
102 expressed in roots, shoots, and leaves, and up-regulated under N-deficiency in roots,  
103 during early germination in seeds and leaf senescence, indicating that *AtDUR3*  
104 provides pathways not only for direct uptake of soil urea but also for retranslocation  
105 or recycling of endogenous urea [24–26]. In addition, genome sequence analysis  
106 indicates that model land plants including *A. thaliana* have only a single *DUR3* gene  
107 [18, 24, 25, 27–29]. On the other hand, two *DUR3* genes have been identified in  
108 some algal species, including *P. yezoensis* [19, 30, 31], although the molecular  
109 characteristics remain unclear.

110 In *P. yezoensis*, a large-scale expressed sequence tag (EST) analysis has been  
111 carried out and an EST database, including approximately 10,600 and 10,200 EST  
112 sequences from the gametophytic and sporophytic generations, respectively, has  
113 been made available [32, 33]. Interestingly, a BLAST search using *PyDUR3.1* and  
114 *PyDUR3.2* cDNA nucleotide (nt) sequences [19] against the EST database suggested  
115 the possibility that *P. yezoensis* possesses another *DUR3*-like gene (*PyDUR3.3*)  
116 predominantly expressed in the sporophytic generation. The present study was  
117 undertaken to clone and sequence the *P. yezoensis* *PyDUR3.3* cDNA and genomic  
118 DNA (gDNA). In addition, in order to understand the differences in physiological  
119 and molecular characteristics among the three *PyDUR3* genes, *PyDUR3.3*,

120 *PyDUR3.2*, and *PyDUR3.1*, their sequence similarities, genomic organization, and  
121 level of transcript accumulation between the heteromorphic generations were  
122 analyzed.

123

## 124 **Materials and methods**

125

### 126 Materials and cultivation conditions

127

128 *Pyropia yezoensis* (strain FA-89), which was originally isolated by selective  
129 breeding and maintained by the Fukuoka Fisheries and Marine Technology Research  
130 Center, Japan [34, 35], was used in this study. Leafy gametophytes (thalli) and  
131 filamentous sporophytes (conchocelis) of the *P. yezoensis* were maintained using  
132 one-fifth strength Provasoli's enriched seawater (1/5 PES) medium [36] in the  
133 laboratory. The culture medium was changed every 2 days for the thalli and 7 days  
134 for the conchocelis throughout the experiments. The thallus culture was aerated  
135 with air filtered through a FP30/0.2PTFE-S filter (Whatman, Dassel, Germany),  
136 irradiated with 50  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  light on a 10:14 h (light/dark) cycle  
137 (10L/14D photoperiod), and maintained at 10°C. The conchocelis culture was  
138 maintained statically, irradiated with 10  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  light on a 14L/10D  
139 photoperiod at 18°C. Thalli and conchocelis cultures were inoculated into the  
140 experimental seawater medium upon reaching an average length of 3 cm or an  
141 average wet weight of 100 mg, respectively.

142 Dissolved inorganic nitrogen (DIN) content in natural seawater for preparation  
143 of each experimental seawater medium was analyzed using a TRAACS 2000  
144 (Bran+Luebbe, Norderstedt, Germany). The average DIN concentration of the  
145 natural seawater was 257.6  $\mu\text{g/L}$  ( $\text{NO}_3\text{-N} = 240.4 \mu\text{g/L}$ ,  $\text{NO}_2\text{-N} = 4.5 \mu\text{g/L}$ , and  
146  $\text{NH}_4\text{-N} = 12.7 \mu\text{g/L}$ ).

147

### 148 Total RNA and gDNA extraction

149

150 Total RNA extraction from fresh *P. yezoensis* thalli and conchocelis was  
151 performed using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). After  
152 DNase treatment using the MessageClean Kit (GenHunter, Tennessee, USA), the  
153 purified total RNA was stored at -80°C until single-stranded (ss) cDNA synthesis

154 for 3' or 5' rapid amplification of cDNA ends (RACE) and quantitative-polymerase  
155 chain reaction (qPCR) was performed.

156 Genomic DNA (gDNA) extraction from fresh *P. yezoensis* thalli was performed  
157 according to a previously-reported method [37, 38]. The extracted gDNA was  
158 further purified using the DNeasy Plant Mini Kit (Qiagen). The gDNA obtained  
159 was used for isolation of partial gDNA clones of *PyDUR3.3*, *PyDUR3.2*, and  
160 *PyDUR3.1*.

161

162 *PyDUR3.3* cDNA cloning

163

164 Initially, for isolation of *P. yezoensis PyDUR3.3* cDNA fragments, 3' RACE was  
165 used. **Five micrograms** of total RNA were used for ss cDNA synthesis using the 3'  
166 RACE System for Rapid Amplification of cDNA Ends (Invitrogen, California, USA).  
167 The PCR amplification of *PyDUR3.3* cDNA was performed using gene-specific  
168 primer PyDUR3.3-EP1, which was designed based on the nt sequences of ESTs  
169 AU192239 (542 bp) and AU194122 (490 bp) in the *P. yezoensis* EST database  
170 (*Porphyra* EST Index, Kazusa DNA Res. Inst., Chiba, Japan:  
171 <http://est.kazusa.or.jp/en/plant/porphyra/EST/> “Accessed 24 Jul 2013”) [32, 33],  
172 and the adapter primer AUAP from the kit (Table 1). The cDNA fragment  
173 amplified by PCR was subcloned into a pT7Blue T-vector (Novagen, Wisconsin,  
174 USA) and sequenced. Subsequently, gene-specific primers for *PyDUR3.3* were  
175 synthesized for 5' RACE.

176 In order to determine the 5' end of the *PyDUR3.3* cDNA, the 5' RACE System  
177 for Rapid Amplification of cDNA Ends (Invitrogen) was used. Single-stranded  
178 cDNA for 5' RACE was synthesized from 5 µg of total RNA using the gene-specific  
179 primer PyDUR3.3-SP1 (Table 1). After dC-tail addition to the 3' end of the cDNA,  
180 the PCR amplification of the dC-tailed cDNA was performed using the gene-specific  
181 primer PyDUR3.3-SP2 (Table 1) and the adapter primer AAP from the kit. The  
182 nested PCR amplification was performed using a set of the gene-specific primer  
183 PyDUR3.3-SP3 and the adapter primer AUAP from the kit (Table 1), and the  
184 amplified cDNA fragments were subcloned into a pT7Blue T-vector and sequenced.

185

186 *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1* gDNA cloning

187

188 Genomic copies of *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1* was amplified by  
189 Long and Accurate-PCR (LA-PCR, Takara Bio, Kyoto, Japan) according to the  
190 supplier's instructions using three sets of gene-specific primers, PyDUR3.3-GF/GR  
191 for *PyDUR3.3*, PyDUR3.2-GF/GR for *PyDUR3.2*, and PyDUR3.1-GF/GR for  
192 *PyDUR3.1* (Table 1), synthesized based on the nt sequences of the *PyDUR3.3*,  
193 *PyDUR3.2*, and *PyDUR3.1* cDNAs, respectively. The gDNA fragments amplified  
194 by LA-PCR were subcloned into a pT7Blue T-vector and sequenced.

195

#### 196 Sequence analysis

197

198 Plasmid DNAs including cDNA or gDNA fragments were isolated using the  
199 QIAprep Spin Miniprep Kit (Qiagen), and sequenced with the aid of a BigDye  
200 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and  
201 an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Database searches  
202 and similarity analyses of cDNA and gDNA nt sequences were performed with the  
203 BLASTN and BLASTX programs [39, 40] against public nt and protein databases,  
204 and a *P. yezoensis* genome database (Genome Assembly Ver. 1 Scaffold,  
205 [http://nrifs.fra.affrc.go.jp/ResearchCenter/5\\_AG/genomes/nori/index.html](http://nrifs.fra.affrc.go.jp/ResearchCenter/5_AG/genomes/nori/index.html)  
206 "Accessed 2 Sep 2015") [41].

207 Multiple alignment and neighbor-joining-based phylogenetic tree construction  
208 for the deduced AA sequences of the PyDUR3 proteins and the selected DUR3 and  
209 SSS superfamily proteins were performed using the MEGA6 software [42]  
210 incorporating the ClustalW program [43]. The alignment parameters are as  
211 follows: gap opening penalty, 10.0; gap extension penalty, 0.2; and percent identity  
212 delay, 30; protein weight matrix, Gonnet. Bootstrap resampling analysis from  
213 1,000 replicates was used to evaluate internal branches. Local bootstrap  
214 probabilities are indicated for branches with over 50% support. The  
215 DDBJ/EMBL/GenBank accession numbers of the deduced AA sequences analyzed  
216 are listed in Table 2. Prediction of TDs of the deduced AA sequence was carried  
217 out with the TMHMM Server v2.0 (The Center for Biological Sequence Analysis,  
218 Lyngby, Denmark: <http://www.cbs.dtu.dk/services/TMHMM/>). In addition, protein  
219 motif and transit peptide searches in the deduced AA sequence were carried out with  
220 the MOTIF (Kyoto University Bioinformatics Center:  
221 <http://www.genome.jp/tools/motif/>) and iPSORT (Human Genome Center, The



222 University of Tokyo: <http://ipsort.hgc.jp/>) [44] programs.

223

224 Expression analysis

225

226 **Five micrograms** of total RNA treated with DNase were used for ss cDNA  
227 synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied  
228 Biosystems). Quantitative-PCR (qPCR) with the ss cDNA as the template was  
229 performed using a 7300 Real-Time PCR System (Applied Biosystems) and FastStart  
230 Universal Probe Master (ROX) (Roche Diagnostics, Basel, Schweiz) according to  
231 the supplier's instructions. Three sets of forward and reverse primers and  
232 FAM-labeled TaqMan MGB probes were designed to specifically amplify 57, 71,  
233 and 55 bp fragments for *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1* mRNAs, respectively.  
234 The forward and reverse primers and the TaqMan MGB probe sets were  
235 *PyDUR3.3*-QPF/QPR/QPP for *PyDUR3.3* mRNA, *PyDUR3.2*-QPF/QPR/QPP for  
236 *PyDUR3.2* mRNA, and *PyDUR3.1*-QPF/QPR/QPP for *PyDUR3.1* mRNA (Table 1).  
237 Each standard curve of qPCR for *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1* mRNAs was  
238 obtained from PCR amplification with diluted plasmid including the respective  
239 cDNA as the template. Normalization of the mRNA level of each *PyDUR3* gene  
240 was performed with an index of 18S rRNA detected using the TaqMan Eukaryotic  
241 18S rRNA Endogenous Control (Applied Biosystems) including the VIC-labeled  
242 MGB probe. The data were analyzed **by one-way analysis of variance (ANOVA)**  
243 **and Tukey's post-hoc test**, with  $p < 0.01$  between two experimental **groups regarded**  
244 as significant.

245

## 246 **Results**

247

248 Cloning and sequence analysis of *PyDUR3.3* cDNA

249

250 A *PyDUR3.3* cDNA fragment (approximately 1.7 kbp) was amplified by PCR  
251 based on 3' RACE procedure using a combination of primers, *PyDUR3.3*-EP1 and  
252 AUAP (Table 1). BLAST analysis showed that the deduced AA sequence for the  
253 cDNA fragment was homologous to *P. yezoensis* *PyDUR3.2* [19]. The poly(A) tail  
254 was not included in the cDNA fragment, because the cDNA contained an AUAP at  
255 the 3'-terminus. In order to isolate the 3' end of the *PyDUR3.3* cDNA, 3' RACE

256 PCR amplification was performed using the gene-specific primer PyDUR3.3-EP2  
257 (Table 1), which was synthesized on the basis of the nt sequence of the  
258 AUAP-containing *PyDUR3.3* cDNA, and AUAP. A *PyDUR3.3* cDNA fragment  
259 (approximately 0.7 kbp), including a poly(A) tail and overlapping with the  
260 AUAP-containing *PyDUR3.3* cDNA, was successfully amplified. Next, by the PCR  
261 amplification for 5' RACE, the cDNA fragments (approximately 0.6 kbp) were  
262 cloned. Finally, based on three overlapping clones obtained by the 3' and 5' RACE  
263 procedures, the nt sequence of 2,416 bp, which includes the entire coding region of  
264 2,040 bp, was determined (DDBJ/EMBL/GenBank accession number AB931115)  
265 (Table 2, Fig. 1).

266 The *PyDUR3.3* cDNA had an open reading frame (ORF) encoding a polypeptide  
267 of 679-AA residues (Fig. 1) with a molecular mass of 71.4 kDa. An AA sequence  
268 alignment of PyDUR3.3 with *P. yezoensis* PyDUR3.1 and PyDUR3.2 and with some  
269 published algal, higher plant, and yeast DUR3 proteins showed that the PyDUR3.3  
270 is more similar to PyDUR3.2 (80.7% identity) than to other DUR3 proteins  
271 including PyDUR3.1 (36.8–56.7% identities) (Fig. 2). Hydrophobicity plots  
272 calculated with the TMHMM Server v2.0 showed 15 TDs for PyDUR3.3, and the  
273 positions of 1st and 2nd TDs from the N-terminus of the PyDUR3.3 differed from  
274 those of PyDUR3.1 and PyDUR3.2. The DUR3 proteins belong to the SSS  
275 superfamily which catalyzes the uptake of a wide variety of solutes into cells of  
276 prokaryotes and eukaryotes [16, 18, 22, 24]. Protein sequence analysis by MOTIF  
277 and iPSORT programs indicated that PyDUR3.3 included a SSS family-specific  
278 domain (84th–494th AAs) as well as did PyDUR3.1 (92nd–499th AAs) and  
279 PyDUR3.2 (86th–496th AAs), and that no mitochondrial or chloroplast transit  
280 peptides were included in PyDUR3.3/3.2/3.1. To investigate the relationships of  
281 the predicted PyDUR3.3 protein to its counterparts, including different family  
282 members of the SSS superfamily, from other algae, land plants, and bacteria, a  
283 neighbor-joining-based phylogenetic tree was constructed on the basis of multiple  
284 alignment of AA sequences from the selected SSS superfamily proteins (Fig. 3).  
285 Phylogenetic analysis indicated that PyDUR3.3 fell into the red algal DUR3 cluster,  
286 and that PyDUR3.3 was more similar to DUR3 proteins, including PyDUR3.1 and  
287 PyDUR3.2, from red algae (56.7–80.7% identities) than to those from other algae  
288 (37.0–54.0% identities), land plants (49.2–50.4% identities), and yeasts  
289 (30.3–37.6% identities).

290

291 Determination of *PyDUR3* gene structures

292

293 Genomic copies of three *PyDUR3* genes were amplified by LA-PCR using three  
294 primer sets (Table 1), designed on the basis on the corresponding *PyDUR3* cDNA nt  
295 sequences, with gDNA as the template. Partial gDNA fragments, 2,755 bp for  
296 *PyDUR3.3* (DDBJ/EMBL/GenBank accession number AB933540), 2,573 bp for  
297 *PyDUR3.2* (AB933541), and 4,201 bp for *PyDUR3.1* (AB933542), were isolated.  
298 Each gDNA fragment included 5' and 3'-untranslated regions (UTR) of exons  
299 encoding translational start and stop codons, respectively. In *PyDUR3.3/3.2*, three  
300 exons (E1–3) were assigned to the coding region by comparison with the  
301 corresponding cDNA nt sequences (Fig. 4). Lengths of the ORF coding regions in  
302 E1–3 were 900, 22, and 1,118 bp, respectively, for *PyDUR3.3*, and 906, 22, and  
303 1,115 bp, respectively, for *PyDUR3.2* (Table 3). In contrast, the coding region of  
304 *PyDUR3.1* was split into eight exons (E1–8) (Fig. 4) in which lengths of the ORF  
305 coding regions were 252, 100, 70, 457, 157, 5, 199, and 983 bp, respectively (Table  
306 4). Introns were located at codons specifying the 300th/301st (I1) and 308th (I2)  
307 AAs for *PyDUR3.3*, 302nd/303rd (I1) and 310th (I2) AAs for *PyDUR3.2*, and  
308 84th/85th (I1), 118th (I2), 141st (I3), 293rd/294th (I4), 346th (I5), 347th/348th (I6),  
309 and 414th (I7) AAs for *PyDUR3.1*. The consensus GT donor and AG acceptor  
310 sequences at the 5'- and 3'-termini of the introns [45, 46], respectively, were found  
311 in all introns except for the I5 in *PyDUR3.1*. **BLAST analysis identified discrete**  
312 **contigs corresponding to *PyDUR3.3* (contig\_34496), *PyDUR3.2***  
313 **(contigs\_30224/23844), and *PyDUR3.1* (contig\_8966) (Fig. 4), suggesting that**  
314 ***PyDUR3.3/3.2/3.1* genes were encoded separately in the nuclear genome of *P.***  
315 ***yezoensis*. The nt sequences of the *PyDUR3.3/3.2* gDNA fragments were identical**  
316 **to those of the corresponding contigs, whereas slight differences in the nt sequences**  
317 **of introns I1 and I2 (90.7–99.5% identities) were found between *PyDUR3.1* gDNA**  
318 **fragment and contig\_8966.**

319

320 Expression of *PyDUR3* genes in *P. yezoensis* thalli and conchocelis

321

322 The mRNA levels for *PyDUR3.3/3.2/3.1* in *P. yezoensis* thalli and conchocelis  
323 were examined by qPCR using three combinations of gene-specific primers and

324 TaqMan MGB probes (Table 1, Fig. 4). For the expression assay, *P. yezoensis*  
325 thalli and conchocelis maintained in 1/5 PES media were transferred to 0 PES  
326 (natural seawater) media and cultivated for 72 h (thalli) or 168 h (conchocelis) until  
327 discoloration was observed. Although the DIN of the initial 0 PES media  
328 corresponding to 0 h cultivation (Fig. 5, sample N) was approximately 258 µg/L, the  
329 DIN decreased to approximately 10 µg/L after 24 h cultivation and remained at that  
330 level until end of the culture period, indicating that thalli and conchocelis  
331 cultivated for 72 h and 168 h (Fig. 5, sample D), respectively, were exposed to a  
332 N-deficient condition and almost the same physiological condition. In order to  
333 compare *PyDUR3* gene expression profiles between the heteromorphic generations,  
334 the mRNA level of the *PyDUR3* genes was normalized using *18S rRNA*, which is a  
335 reliable internal standard in plants under various growth stages, cultivars, and  
336 stressful conditions [47], as a reference gene. *PyDUR3.3* mRNA were present at  
337 lower levels in thalli, whereas the mRNA levels were extremely high in the  
338 conchocelis (approximately 340–480-fold that of the initial thalli), regardless of the  
339 nutritional conditions. In contrast to *PyDUR3.3* expression, remarkable  
340 differences were observed in *PyDUR3.2* expression. The relative mRNA level of  
341 *PyDUR3.2* was approximately 27.6 times higher in the thalli cultivated for 72 h than  
342 in the initial thalli, and was extremely low (approximately 0.2-fold that of the  
343 initial thalli) in the conchocelis cultivated for 0 h and 168 h. On the other hand,  
344 the relative mRNA levels of *PyDUR3.1* in the thalli and conchocelis cultivated  
345 under the N-deficient condition changed approximately 3.0 and 1.5-fold compared  
346 to those in the initial thalli and conchocelis, respectively.

347

## 348 Discussion

349

350 In this study, a new gene, *PyDUR3.3*, which is homologous to plant and yeast  
351 high-affinity urea transporter genes, was isolated from *P. yezoensis* by RACE-PCR.  
352 The *PyDUR3.3* cDNA encoded a putative protein of 679 AAs (Fig. 1), and the  
353 primary structure of the *PyDUR3.3* differed from those of the *PyDUR3.1* and  
354 *PyDUR3.2* [19]. Structurally, the *DUR3* proteins belong to the SSS superfamily,  
355 which includes more than one hundred members of prokaryotic and eukaryotic  
356 origin and is divided into 11 subfamilies [22]. In addition, proteins of the SSS  
357 superfamily contain 11 to 15 putative TDs in an  $\alpha$ -helical conformation, some of

358 which has been described to mediate uptake and transport of various solutes (e.g.,  
359 AAs, inositols, nucleosides, sugars, and urea) across cytoplasmic membranes [16,  
360 18, 24, 48]. Among all SSS proteins, the DUR3 members are closest to the  
361 bacterial sodium/pantothenate symporters (PanF) and sodium/proline symporters  
362 (PutP). Sequence analysis of the deduced AA sequence of PyDUR3.3 showed that  
363 PyDUR3.3 contained 15 putative TDs found in most of the DUR3 proteins from  
364 photosynthetic eukaryotes and yeasts, although the AA sequences were quite varied  
365 in length (653–818 AAs) (Table 2) and identity (36.8–80.7%) (Fig. 2) among the  
366 DUR3 protein family. Phylogenetic analysis indicated that the DUR3 proteins  
367 from photosynthetic eukaryotes and yeasts roughly fell into the corresponding  
368 clusters distinguished with each division, and that the DUR3 proteins from  
369 Rhodophyta (56.0–80.7% identities among their AA sequences) were more similar  
370 to those from Heterokontophyta (45.5–54.0% identities) and Chlorophyta  
371 (40.6–50.5% identities) than those from Haptophyta (29.1–43.7% identities) and  
372 Ascomycota (27.5–37.6% identities) (Fig. 3). In the Rhodophyta cluster, the  
373 PyDUR3.3 and PyDUR3.2 were phylogenetically closely related to each other  
374 (80.7% identity), whereas the PyDUR3.1 was clearly distinguished from  
375 PyDUR3.3/3.2 and more similar to a *Chondrus crispus* CcDUR3.1 (60.6% identity)  
376 than the PyDUR3.3/3.2 (56.0–56.7% identities). These results suggest that  
377 *PyDUR3.3/3.2* differs considerably from *PyDUR3.1* in ancestry and evolution.

378 To date, the *DUR3* gene in land plants has been investigated and found to be a  
379 single copy gene on the basis of EST and gDNA database searches. In addition, *A.*  
380 *thaliana* AtDUR3, *Oryza sativa* OsDUR3, and *Zea mays* ZmDUR3 proteins have  
381 been functionally characterized to mediate urea/proton cotransport at low external  
382 urea concentrations by functional complementation analysis of these plant *DUR3*  
383 genes in a yeast *DUR3*-knockout mutant, as well as physiological analysis of these  
384 plant *DUR3*-insertion transgenic lines [16, 24, 27–29]. On the other hand, two  
385 *DUR3* homologues have been identified in algae such as *C. crispus* (Rhodophyta),  
386 *Emiliana huxleyi* (Haptophyta), and *Chlamydomonas reinhardtii* (Chlorophyta)  
387 with the completion of whole genome sequencing [30, 31, 49], although the  
388 functional characteristics of their products are still unclear. In *P. yezoensis*, the  
389 *PyDUR3* genes are also encoded by multigene families [19], and the exon-intron  
390 structures of *PyDUR3.3/3.2* were closely related to each other, which clearly  
391 differed from that of *PyDUR3.1* (Fig. 4). In the yeasts *S. cerevisiae* and

392 *Schizosaccharomyces pombe*, multicellular red alga *C. crispus*, and unicellular  
393 green alga *Ostreococcus tauri*, an ancient member of the terrestrial green lineage  
394 [50], no introns were found in ORFs of the *DUR3* genes, whereas ORFs of the *DUR3*  
395 genes in other algae and land plants studied is interrupted by two (e.g., Os*DUR3*) to  
396 nine (e.g., *EsDUR3* and *AtDUR3*) introns with taxon-nonspecific locations (Table 3).  
397 Interestingly, in the unicellular algae *E. huxleyi* and *C. reinhardtii*, each having two  
398 *DUR3* genes, the exon-intron structure of one *DUR3* gene (*EhDUR3.1* and  
399 *CrDUR3B*) is more complicated than that of the other *DUR3* gene (*EhDUR3.2* and  
400 *CrDUR3A*). In *P. yezoensis* *PyDUR3* genes, the same structural feature was  
401 observed between *PyDUR3.3/3.2* and *PyDUR3.1* (Table 3, Fig. 4), strongly  
402 supporting that *PyDUR3.3* might be a paralogue of *PyDUR3.2*. Amino acid  
403 sequence and phylogenetic analyses also support this conclusion (Figs. 2 and 3).

404 Generally, *Pyropia* thalli can utilize inorganic nitrogen and organic solutes like  
405 AAs and urea in growth and development [9, 12–15]. In addition, N-deficiency in  
406 seawater leads *Pyropia* thalli and conchocelis to discoloration with degradation of  
407 phycobiliproteins, the predominant photosynthetic pigments in red algae [9–11].  
408 Therefore, multiple *PyDUR3* genes in *P. yezoensis* may be responsible for uptake of  
409 a large variety of solutes in seawater and for intracellular transport of endogenous  
410 urea liberated via degradation of phycobiliproteins, as urea metabolism in plant  
411 cells [18]. In land plants, a *DUR3* protein is specific for urea and acts as a major  
412 high-affinity urea transporter not only for direct uptake of exogenous urea in roots  
413 but also for retranslocation or recycling of endogenous urea liberated via  
414 degradation of storage proteins in germinating seeds and mature leaves [24–27, 29].  
415 Land plant *DUR3* gene expression is up-regulated in roots under N-deficiency, in  
416 seeds during early germination, and in mature leaves during senescence. In  
417 contrast, yeast *S. cerevisiae* *ScDUR3* has been reported to mediate uptake of  
418 extracellular urea and polyamines [51]. Compared to the information for land  
419 plant and yeast *DUR3* genes, far less is known about molecular characteristics of  
420 algal *DUR3* genes. Therefore, changes in mRNA levels of the three *PyDUR3*  
421 homologs in thalli and conchocelis were examined under different nutrient  
422 conditions (Fig. 5), and the results suggested that expression of the *PyDUR3.3* gene  
423 is constitutive and specific to the conchocelis, whereas *PyDUR3.2* gene expression  
424 is specific to the thalli and significantly affected by exposure to nitrate starvation,  
425 and that *PyDUR3.1* gene expression is positively regulated by nitrate starvation in

426 both thalli and conchocelis.

427 The *Pyropia* life cycle alternates between two growth phases, a leafy thallus as  
428 the haploid gametophyte and a filamentous conchocelis as the diploid sporophyte  
429 [1]. The *Pyropia* thalli appear in nature as free-living organisms during the winter  
430 with low temperature and short day length, and the conchocelis burrows into  
431 calcium carbonate ( $\text{CaCO}_3$ ) substrates such as oyster shells and grows during the  
432 summer with high temperature and long day length. Shellfish excrete urine  
433 including nitrogenous compounds such as  $\text{NH}_4\text{-N}$ , urea-N, and AA-N [52]. In  
434 addition, biogenic polyamines accelerate the formation of  $\text{CaCO}_3$  in seawater [53],  
435 suggesting that a similar phenomenon may be involved in the calcification process  
436 in shellfish. *PyDUR3.3* was constitutively expressed in the conchocelis (Fig. 5),  
437 suggesting a possibility that PyDUR3.3 plays an especially important role in the  
438 uptake of nitrogenous compounds excreted from shellfish. In plants and algae,  
439 polyamines are ubiquitous cellular components and play important roles in  
440 fundamental cellular processes, including adaptive/defensive responses to various  
441 environmental stresses [54–58]. Expression of *PyDUR3.2* and *PyDUR3.1* genes  
442 were induced by N-deficiency in both generations (Fig. 5), suggesting that these  
443 PyDUR3 proteins play especially important roles in the uptake of nitrogenous  
444 compounds in N-deficient conditions, as well as in transport of endogenous urea  
445 produced from degradation of proteins in the discoloration process and polyamines  
446 involved in stress responses.

447 In this study, three *PyDUR3* genes could be characterized on the basis of the  
448 sequence similarities, genomic organization, and expression profiles in  
449 heteromorphic generations. Experimental results suggest that *PyDUR3.3* and  
450 *PyDUR3.2* are paralogues specifically differentiated in function and life history  
451 phase, which clearly differed from *PyDUR3.1* in ancestry and evolution. However,  
452 transport mechanisms including substrate specificity and subcellular localization of  
453 each PyDUR3 remain unclear. Plant *DUR3* genes have been characterized by  
454 functional complementation analysis using a yeast *DUR3*-knockout mutant,  
455 electrophysiological and radiotracer analyses of *DUR3*-injection *Xenopus* oocytes,  
456 and physiological analysis of *DUR3*-insertion transgenic lines [16, 24, 27–29].  
457 Further experiments for the functional characterization of these *PyDUR3* genes are  
458 currently being carried out using the tools mentioned above.

459



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461

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470



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472

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683

684 **Figure legends**

685

686 **Fig. 1** Nucleotide sequence of the *P. yezoensis* PyDUR3.3 cDNA. The deduced  
687 AA sequence is shown below the nt sequence. *Numbers* in the margin represent nt  
688 and AA residues from the 5'-end and N-terminus, respectively. The deduced AA  
689 sequence is shown in *single letter code*. The stop codon (TAG) is marked by an  
690 *asterisk*. *Bold small letters* in the AA sequence indicate the putative TDs.  
691 *Underlined* and *dashed underlined* nt sequences correspond to primers for  
692 RACE-PCR (PyDUR3.3-EP1/EP2 and PyDUR3.3-SP1/SP2/SP3) and LA-PCR  
693 (PyDUR3.3-GF/GR), and for qPCR (PyDUR3.3-QPF/QPP/QPR), respectively, listed  
694 in Table 1. *Arrowheads* with the primers indicate nt sequence direction from 5' to  
695 3'-ends. The *double underlined* nt sequence shows high similarity to the AUAP  
696 primer used for 3' RACE. Refer to Table 2 for the DDBJ/EMBL/GenBank  
697 accession number of the PyDUR3.3 cDNA sequence.

698

699 **Fig. 2** Comparison of AA sequences of DUR3 proteins from *P. yezoensis* with  
700 those of other algae, higher plants, and yeasts. The deduced AA sequence of *P.*  
701 *yezoensis* PyDUR3.3 (identified in this study) is shown on the upper line in *single*  
702 *letter code*. The AA sequences of *P. yezoensis* PyDUR3.2 and PyDUR3.1 [19], *E.*  
703 *siliculosus* EsDUR3 [59], *C. reinhardtii* CrDUR3A [30] *A. thaliana* AtDUR3 [24],  
704 and *S. cerevisiae* ScDUR3 [23] are shown below. *Numbers* in the margin represent  
705 AA residues from the N-terminus. Identical and gapped AAs are shown by *periods*  
706 and *dashes*, respectively. Below the AA sequences, *asterisks* indicate AA residues  
707 conserved in all sequences, and *double/single dots* denote highly/moderately  
708 conservative replacements. *Underlined bold small letters* in each AA sequence  
709 indicate the putative TDs. After each AA sequence, percentage AA sequence  
710 identity relative to PyDUR3.3 and numbers of TDs are given in *parentheses*. Refer  
711 to Table 2 for the DDBJ/EMBL/GenBank accession numbers of the DUR3 protein  
712 sequences.

713

714 **Fig. 3** Neighbor-joining-based phylogenetic tree of DUR3 and SSS superfamily  
715 proteins obtained using the ClustalW and MEGA6 programs [42, 43]. *Boxes*  
716 indicate *P. yezoensis* PyDUR3 proteins. The bootstrap values with 1,000 replicates  
717 over 50% are indicated at the nodes of the tree. Refer to Table 2 for the



718 DDBJ/EMBL/GenBank accession numbers of the DUR3 and SSS superfamily  
719 protein sequences analyzed.

720

721 **Fig. 4** Schematic representation of gDNA clones encoding the *PyDUR3* genes.  
722 *Horizontal black bars* and *boxes* represent introns and exons, respectively. *Shaded*  
723 *boxes* and *open/light-shaded boxes* represent UTRs of exons and *PyDUR3* coding  
724 exons, respectively. In *boxes*, *light-shaded regions* indicate TD coding exons.  
725 The partial gDNA fragments, 2,755 bp for *PyDUR3.3* (DDBJ/EMBL/GenBank  
726 accession number AB933540), 2,573 bp for *PyDUR3.2* (AB933541), and 4,201 bp  
727 for *PyDUR3.1* (AB933542), were isolated. *Horizontal gray bars represent*  
728 *scaffolds in the P. yezoensis genome database [41] corresponding to the PyDUR3*  
729 *gDNA fragments.* *Arrows and arrowheads* indicate the *PyDUR3* gene-specific  
730 primers for LA-PCR (*PyDUR3.3*-GF/GR, *PyDUR3.2*-GF/GR, and  
731 *PyDUR3.1*-GF/GR) and qPCR (*PyDUR3.3*-QPF/QPP/QPR,  
732 *PyDUR3.2*-QPF/QPP/QPR, and *PyDUR3.1*-QPF/QPP/QPR), respectively, listed in  
733 Table 1.

734

735 **Fig. 5** Expression of the *PyDUR3* genes in *P. yezoensis* thalli and conchocelis.  
736 *Pyropia* thalli and conchocelis maintained in 1/5 PES media were transferred to  
737 seawater without PES enrichment (DIN = 258 µg/L) (0 h, N) and cultivated for **72 h**  
738 or 168 h (D) in the case of the thalli or conchocelis, respectively. The relative  
739 mRNA levels of each *PyDUR3* gene were normalized with the amount of 18S rRNA  
740 and given as relative fold abundance compared to the initial thallus N sample.  
741 *Error bars* indicate the standard deviation **of triplicate experiments** ( $n = 3$ ).  
742 *Different letters* indicate significant differences ( $p < 0.01$ ).

743

744 **Table 1** Nucleotide sequences of primers for PCR amplifications

Primer name	Sequence
3' RACE for PyDUR3.3	
PyDUR3.3-EP1	5'-dTTTGACAACAAGCCCGTCCTGGACAAG-3'
PyDUR3.3-EP2	5'-dCAAGCCAGACAACCTGTGACTGGTC-3'
AUAP	5'-dGGCCACGCGTCGACTAGTAC-3'
5' RACE for PyDUR3.3	
PyDUR3.3-SP1	5'-dATGACAGCCGTATTGAAGTAG-3'
PyDUR3.3-SP2	5'-dTGCCAGGTTGGTACTGATACC-3'
PyDUR3.3-SP3	5'-dAACAATAACGTTGGTAGCCAGCGC-3'
LA-PCR	
PyDUR3.3-GF	5'-dATCCCAAGTCTCTCTTTCTGGTCGTAC-3'
PyDUR3.3-GR	5'-dCAACCTGCTATAGTGAACCAGCTTACC-3'
PyDUR3.2-GF	5'-dGTTCCACCATGGCGGACTTTGTCAAC-3'
PyDUR3.2-GR	5'-dAGGCCGCGCAAAAGACCGTCAGTCTAT-3'
PyDUR3.1-GF	5'-dCGGGAGGTGTTGGCGCAAAAATGGCGA-3'
PyDUR3.1-GR	5'-dCACAGCTCTACACCTACTCGCAACACG-3'
Quantitative-PCR	
PyDUR3.3-QPF	5'-dCTGCCGACGGCGAGTCTA-3'
PyDUR3.3-QPR	5'-dGCCACCTGTTTGC GTTGAT-3'
PyDUR3.3-QPP	5'-dACGAGTAGCCGTTGCG-3'
PyDUR3.2-QPF	5'-dCGAGAGCGACGGCATCTC-3'
PyDUR3.2-QPR	5'-dATCCGAGCCGACCCTCTT-3'
PyDUR3.2-QPP	5'-dCCGAGGTGGACGAGTAG-3'
PyDUR3.1-QPF	5'-dTGGAACCACCAGAGCCTTCT-3'
PyDUR3.1-QPR	5'-dCGCGCAACGGTGATCA-3'
PyDUR3.1-QPP	5'-dACCGCACATCGTTCT-3'

745

746

747 **Table 2** DDBJ/EMBL/GenBank accession numbers of DUR3 and SSS protein  
 748 sequences

Species	Proteins	Abbreviations	AAs	TDs <sup>a)</sup>	Accession No.
<b>Urea active transporter protein</b>					
<b>Rhodophyta</b>					
<i>Chondrus crispus</i>	DUR3.1	CcDUR3.1	675	15	HG001812
	DUR3.2	CcDUR3.2	669	15	HG001512
<i>Pyropia yezoensis</i>	DUR3.1	PyDUR3.1	740	15	AB359179
	DUR3.2	PyDUR3.2	680	15	AB359180
	DUR3.3	PyDUR3.3	679	15	AB931115
<b>Heterokontophyta</b>					
<i>Ectocarpus siliculosus</i>	DUR3	EsDUR3	668	15	FN647726
<i>Phaeodactylum tricornutum</i>	DUR3	PtDUR3	704	15	CM000612
<b>Haptophyta</b>					
<i>Emiliania huxleyi</i>	DUR3	EhDUR3.1	756	14	KB864035
	DUR3	EhDUR3.2	818	13	KB868914
<b>Chlorophyta</b>					
<i>Ostreococcus tauri</i>	DUR3	OtDUR3	710	15	Q00V32
<i>Chlamydomonas reinhardtii</i>	DUR3A	CrDUR3A	653	15	DS496184
	DUR3B	CrDUR3B	721	15	DS496184
<i>Physcomitrella patens</i>	DUR3	PpDUR3	713	15	DS545146
<i>Oryza sativa</i>	DUR3	OsDUR3	721	15	AY463691
<i>Zea mays</i>	DUR3	ZmDUR3	731	15	KM271989
<i>Arabidopsis thaliana</i>	DUR3	AtDUR3	694	14	AB018113
<i>Vitis vinifera</i>	DUR3	VvDUR3	710	15	XM_002263007
<b>Ascomycota</b>					
<i>Saccharomyces cerevisiae</i>	DUR3	ScDUR3	735	15	NC_001140
<i>Schizosaccharomyces pombe</i>	DUR3	SpDUR3.1	664	15	NC_003423
	DUR3	SpDUR3.2	661	15	NC_003424
	DUR3	SpDUR3.3	673	14	NC_003423
<i>Neurospora crassa</i>	DUR3	NcDUR3	704	15	CM002236
<b>Bacterial proline symporter</b>					
<i>Bacillus subtilis</i>	PutP	BsPutP	473	12	AL009126
<i>Corynebacterium glutamicum</i>	PutP	CgPutP	524	13	Y09163
<i>Helicobacter pylori</i>	PutP	HpPutP	496	13	AE000511
<i>Neisseria meningitidis</i>	PutP	NmPutP	508	13	AE002098
<i>Vibrio vulnificus</i>	PutP	VvPutP	497	12	AF454004
<b>Bacterial pantothenate symporter</b>					
<i>Bacillus cereus</i>	PanF	BcPanF	479	13	AAEK01000037
<i>Escherichia coli</i>	PanF	EcPanF	483	13	CP001855
<i>Fusobacterium nucleatum</i>	PanF	FnPanF	484	12	AE009951
<i>Salmonella enterica</i>	PanF	SePanF	483	13	CP000857
<i>Shigella flexneri</i>	PanF	SfPanF	483	13	CP000266
<i>Yersinia pestis</i>	PanF	YpPanF	486	13	AE009952
<b>Bacterial other sodium solute symporter</b>					
<i>Pseudomonas aeruginosa</i>	gpuP	PaGpuP	461	13	AE004091
<i>Ralstonia solanacearum</i>	SSS	RsSSS	479	13	AL646052
<i>Vibrio parahaemolyticus</i>	SGLT	VpSGLT	543	14	AF255301

<sup>a)</sup> TDs in the deduced AA sequences were predicted by TMHMM version 2.0.

749

750

751 **Table 3** Gene structure of *DUR3* genes

<i>DUR3</i> genes	Lengths (bp) of ORF coding regions in exons									
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Rhodophyta										
<i>CcDUR3.1</i>	2028									
<i>CcDUR3.2</i>	2010									
<i>PyDUR3.1</i>	252	100	70	457	157	5	199	983		
<i>PyDUR3.2</i>	906	22	1115							
<i>PyDUR3.3</i>	900	22	1118							
Heterokontophyta										
<i>EsDUR3</i>	33	49	42	56	524	294	442	124	427	16
<i>PtDUR3</i>	221	64	7	55	675	1093				
Haptophyta										
<i>EhDUR3.1</i>	105	255	443	583	214	195	176	300		
<i>EhDUR3.2</i>	96	219	64	1404	674					
Chlorophyta										
<i>OtDUR3</i>	2133									
<i>CrDUR3A</i>	116	176	766	484	420					
<i>CrDUR3B</i>	67	27	70	176	776	320	154	105	114	357
<i>PpDUR3</i>	177	108	400	260	239	958				
<i>OsDUR3</i>	183	771	1212							
<i>ZmDUR3</i>	192	108	663	1233						
<i>AtDUR3</i>	168	108	96	83	221	263	239	431	204	272
<i>VvDUR3</i>	174	108	96	83	221	263	239	650	299	
Ascomycota										
<i>ScDUR3</i>	2208									
<i>SpDUR3.1</i>	1995									
<i>SpDUR3.2</i>	1986									
<i>SpDUR3.3</i>	2022									
<i>NcDUR3</i>	106	1664	345							

752



PyDUR3.3	MAT	<b>pavvnpqiaawgcaat-vvrnffl</b>	DPOYECRESFFDNKP	---	VLDKwvqvivlafqvfqiatvqvli	EORVLGRKMDSEFFNTAGRsvktGLTASVIVSQ	102							
PyDUR3.2		MADF...SVE.F.TTSP.T.....A.QA...GLADG-SAQ	Sra	.....	<b>is.....l</b>	R.I.N.P.....	104							
PyDUR3.1	MATSPA	IPGFNLPSHE..DSEAPW..ELDL..K.N...GDVSNP	THP		<b>Ai.t.fvivr.l.s...f...l</b>	AL.YLFSTV.T.Y.....s.....	110							
EsDUR3		MTSVPED.IDQD...GGE.P----	SEA	...	<b>v..i..if.sff.tfl.wld</b>	KFN.VSIT..O.....i.....	80							
CrDUR3A		MRPRSAGDTA	PEGL	<b>wf.ligl.av.alvptflwa</b>	DRKFG.TQA..N.A...	<b>i.a....cd.ak</b>	70							
AtDUR3		MATCPPDFSTKYIDG.GGCQRQS...	GGTTV	---	<b>QG...avi.g.avf.sfl.wl</b>	K.YV.ARHT..W.....N.....i.....	92							
ScDUR3		MGEFKP-----P	POGA	<b>..a...gl.av.agmm.lttyllk</b>	YQKEIITA.E.T.....v.aav..s		67							
PyDUR3.3	WTWAATLLQSSNVAFKYGV	<b>gpfwyasgat</b>	<b>lqiivfsl</b>	<b>llavqv</b>	KRRAPTAHTFLEI	IQARWGT <b>vahavflffalatn</b>	<b>ivtmsmlil</b>	<b>GGS</b>	<b>AVS</b>	<b>AL</b>	<b>tgistnlas</b>	<b>fliplg</b>	212	
PyDUR3.2												214		
PyDUR3.1												220		
EsDUR3												190		
CrDUR3A												180		
AtDUR3												202		
ScDUR3												177		
PyDUR3.3												302		
PyDUR3.2												304		
PyDUR3.1												310		
EsDUR3												300		
CrDUR3A												270		
AtDUR3												307		
ScDUR3												267		
PyDUR3.3												403		
PyDUR3.2												405		
PyDUR3.1												411		
EsDUR3												401		
CrDUR3A												371		
AtDUR3												408		
ScDUR3												377		
PyDUR3.3	LVAYDIYVP	IRRHMGHNPTG	<b>keiilvsriaivafqllmgvl</b>	<b>glia</b>	LNAIGVSLG	<b>wvylgm</b>	<b>gviigsavapvamsi</b>	<b>wAKCSGA</b>	<b>gaisgav</b>	<b>gglal</b>	<b>aiaswlg</b>	<b>gyast</b>	FDG-G	512
PyDUR3.2													514	
PyDUR3.1													517	
EsDUR3													507	
CrDUR3A													478	
AtDUR3													514	
ScDUR3													483	
PyDUR3.3	VNIANTGLD	<b>vmvgnlvailssavic-avvsfi</b>	KPDNCDWSATKAIALVDEVDN										566	
PyDUR3.2													568	
PyDUR3.1													571	
EsDUR3													561	
CrDUR3A													533	
AtDUR3													560	
ScDUR3													593	
PyDUR3.3													666	
PyDUR3.2													668	
PyDUR3.1													671	
EsDUR3													663	
CrDUR3A													636	
AtDUR3													664	
ScDUR3													703	
PyDUR3.3	ELAESADGESTNE												679 (----, 15 TDs)	
PyDUR3.2	GESDGISS.VDE												680 (80.7%, 15 TDs)	
PyDUR3.1	FHESGGGS.AGD	EGGYEVGYADGFAAARA	AVAAGSGMPLD	GELPDL	MGPDPGGHRL	VEPPEPSHRTS	F						740 (56.7%, 15 TDs)	
EsDUR3	QAPSA												668 (54.0%, 15 TDs)	
CrDUR3A	AHKGDHME	LPPAPANRA											653 (47.8%, 15 TDs)	
AtDUR3	R.RALTM	AVPEA.KIY	LELEKTKK	NDEEG									694 (49.3%, 14 TDs)	
ScDUR3	NSNPQDL	HVV.SQIS	ARHRQSS	HFGQV	DEII								735 (36.8%, 15 TDs)	







