RESEARCH ARTICLE

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cDNA SEQUENCES OF HOUSEKEEPING GENES *ACT*, *GAPDH*, AND *UBQ* FROM PANDAN PLANT (*Benstonea* sp.) ORIGINATING FROM RIAU, INDONESIA

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SUMMARY

Actin (*ACT*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and ubiquitin (*UBQ*) genes, together with other housekeeping genes, are frequently used as internal controls to normalize the expression profiles of candidate genes. This research aimed to isolate three housekeeping genes, namely, *ACT*, *GAPDH*, and *UBQ*, from pandan (*Benstonea* sp.) from Riau, Indonesia. Methods included plant sampling from Kajuik Lake, Riau, Indonesia; planting; mRNA extraction; total cDNA synthesis; housekeeping gene amplification via PCR technique; electrophoresis; amplicon cloning into pTA2 vector; sequencing; and finally cDNA sequence analysis. cDNA fragments with the sizes of 625, 565, and 413 bp were obtained for *ACT*, *GAPDH*, and *UBQ* with the registration numbers MG836259.1, MG836258.1, and MG836261.1, respectively. These cDNA sequences were predicted to encode 208, 188, and 137 deduced amino acids. All of the *Benstonea* sp. cDNA sequences had a similarity of 81.24%–90.13% with cDNA sequences from several plants that belonged to families other than *Benstonea* sp. The *Benstonea* sp. *ACT* cDNA sequence contained two exons that flanked one intron. Moreover, six out of 10 conserved residues and catalytic domains were found in the *Benstonea* sp. *GAPDH* amino acid sequence. Furthermore, the *Benstonea* sp. *UBQ* cDNA sequence was a polyubiquitin that consisted of three ubiquitin monomers. All cDNA sequences obtained in this study were the first reported from the *Benstonea* genus. This study underlined the need for the additional exploration of underutilized species by plant breeders and geneticists.

Keywords: Actin, *Benstonea* sp., cDNA sequence, GAPDH, housekeeping genes, pandan, ubiquitin

Key findings: Three cDNA sequences were isolated for the first time from the *Benstonea* genus. The sequences are indispensable for analyzing the expression of genes, especially genes that are responsible for the flooding stress tolerance of this plant, such that underlying physiological and molecular mechanisms can be

understood. The present findings can be used as a basis to develop crops with flooding stress tolerance.

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INTRODUCTION

Benstonea sp. is a type of pandan plant that grows in Lake Kajuik, Riau Province, Indonesia. This plant, together with other plants, maintains the survival of fauna in the lake (Roslim *et al*., 2016a, 2016b; Roslim, 2018). *Benstonea* sp. survives constant submergence by up to 1.5 m below the surface of the water of the lake for weeks during the rainy season. In the last weeks of the rainy season when flooding has diminished, this plant actively flowers and bears fruits, indicating that its flowering process is induced by the abundance of water in the lake (Roslim, 2017). The lake has an acidic pH, which the plant can also survive (Elvyra and Yus, 2010). This situation shows that that *Benstonea sp.* possesses numerous biotic stress-tolerant genes that enable it to survive flooding and other abiotic stresses. Studying the actions of these genes via expression analysis methods, such as qRT-PCR, requires a reference gene that acts as an internal control (Dean *et al*., 2002; Paolacci *et al*., 2009; de Andrade *et al*., 2017).

The existence of a gene that acts as an internal control in gene expression analysis is indispensable for obtaining credible gene expression data or avoiding bias caused by human error (Radonic *et al*., 2004). Internal controls are generally derived from the housekeeping gene group, which is a group of genes that is expressed in abundant amounts in eukaryotic cells and whose expression is not influenced by developmental stages or certain conditions (Thellin *et al*., 1999; Sinha *et al*., 2015). Some examples of housekeeping genes are the genes encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Yang *et al*., 2019), actin (*ACT*), ubiquitin (*UBQ*), beta-tubulin (*TUB*), and elongation factor 1 alpha (*EF1a*) (Qu *et al*., 2019). The *ACT*, *GAPDH* and *UBQ* genes are frequently used as internal controls in the gene expression analysis of plants (de Andrade *et al*., 2017; Tang *et al*., 2017; Joseph *et al*., 2018).

The *ACT*, *GAPDH*, and *UBQ* genes encode proteins that are involved in every eukaryotic organism's cellular process (Pickart and Eddins, 2004; Dominguez and Holmes 2011; Tristan *et al*., 2011). Actin proteins are a subunit of the actin filament that constitutes the cytoskeleton of all eukaryotic cells. The constituent monomer of actin protein includes the subunits of alpha-actin and beta-actin (Dominguez and Holmes, 2011). In plants and animals, the actin-encoding gene has many copies that constitute a multigene family (McDowell *et al*., 1996; Dominguez and Holmes, 2011). The plant *Melastoma malabathricum* has four isoforms of the *actin* gene (Hannum *et al*., 2010). *Arabidopsis thaliana* has 10 *actin* gene isoforms. The actin cDNA of *Arabidopsis thaliana* has a length of 377 bp and consists of two introns and three exons

(McDowell *et al*., 1996). A sequence similar to actin mRNA has been isolated from *Pandanus fascicularis* (Vinod *et al*., 2010).

Meanwhile, *GAPDH* genes are key enzymes in glycolysis. GAPDH enzymes catalyze the catabolism of glyceraldehyde-3-phosphate (G3P) into 1.3-biphosphoglycerate with the help of NAD and inorganic phosphate (Sirover, 2011). GAPDH enzymes exist in all organisms. In wheat plants (*Triticum aestivum*), 22 *GAPDH* genes have been identified and are categorized into four groups, namely, *gapA/gapB, gapC, gapCp*, and *gapN* (Zeng *et al*., 2016). Given that their expression is more stable than the expression of other housekeeping genes, GAPDH genes are more frequently used as an internal control in the gene expression analysis of plants (Sirover, 2011; Kozera and Rapacz, 2013).

Ubiquitin is a protein that plays a role in controlling various biological processes, including transcription, protein degradation, DNA repair, immunity, endocytosis, and autophagy (Buchberger, 2002; Husnjak and Dikic, 2012). Similar to any actin gene, some members of the ubiquitin family have been reported in plants, animals, humans, and yeast (Callis *et al*., 1995; Furukawa *et al*., 2000; Dittmar *et al*., 2002; Azad *et al*., 2013). In *Arabidopsis thaliana*, 14 members of the ubiquitin family are grouped into three groups, namely, polyubiquitin, ubiquitin-like, and ubiquitin extension genes (Callis *et al*., 1995). A sequence similar to the ubiquitin-conjugating enzyme-8 mRNA sequence has been isolated from *Pandanus fascicularis* (Vinod *et al*., 2010).

Previously, the DNA, but not cDNA, of some housekeeping genes, such as the partial DNA sequences of 18S rRNA, ubiquitin (Roslim *et al*., 2018a), and actin (Roslim and Herman, 2019) have been isolated from *Benstonea* sp. Their expression profiles at the cDNA level are needed to study the mechanism underlying the plant tolerance of abiotic stress. Therefore, this study aimed to analyze the cDNA sequences of the *ACT*, *GAPDH*, and *UBQ* genes of *Benstonea* sp. from Riau.

MATERIALS AND METHODS

Materials and methods

A *Benstonea* sp. plant sample was collected from Kajuik Lake, Langgam, Pelalawan Regency, Riau Province, Indonesia. It was then planted in the Genetics Laboratory, Department of Biology, University of Riau for RNA isolation purposes. Primer pairs for amplifying the *GAPDH*, *ACT*, and *UBQ* genes were designed on the basis of Gantasala *et al*. (2013), the actin genomic DNA sequences of pandan (Roslim and Herman, 2019), and Roslim *et al*. (2018a), respectively (Table 1).

RNA was isolated from leaves, stems, and roots by using Zymo Plant RNA (Zymo Research). Given that the concentration of RNA isolated from stems was higher than that of RNA isolated from leaves and roots, stem RNA was then used as a template for total cDNA synthesis with Agilent Affinity Script Multi Temperature (Agilent). Subsequently, the total cDNA was used as a template in PCR by using a Hercuvan thermocycler to amplify housekeeping genes. The PCR components used were 1× PCR buffer ($+Mg^{2+}$), 0.1 mM dNTPs, 2.4 µM forward primer, 2.4 µM reverse

Table 1. Primers for the amplification of the partial-length cDNA of *ACT*, *GAPDH*, and *UBQ g*enes.

Primers	5'----------3'	Annealing Temperatures (°C)	Regions
P_act_F2	AGT GGT TGT GAA CGA GTA GC	53.7	*ACT*
P_act_R2	GGCACCACACTTTCTACAAT		
GAPDH_F	AACCGGTGTCTTCACTGACAAGGA	52.4	*GAPDH*
GAPDH_R	GCTTGACCTGCTGTCACCAACAAA		
Ub_F1	GCYAARATHCARGAYAAGGA	51.7	*UBQ*
Ub_R1	TGDAGDGTKGAYTCCTTCT		

primer, 2 U dream *Taq* DNA polymerase, 1319 ng of total cDNA, and 16.5 µl of ddH_2O to bring the volume of the PCR reaction to 50 µl (Porebski *et al*., 1997; Roslim, 2017). The PCR program consisted of a pre-PCR cycle at 94 °C for 5 min; 35 cycles consisting of three stages, namely, denaturation at 94 °C for 45 s, annealing at a certain annealing temperature (Table 1) for 1 min, and elongation at 68 °C for 1 min and 30 s, and a post-PCR cycle at 68 °C for 10 min. The PCR product was then cloned into pTA2 vector for the sequencing requirement by following the procedure performed by Roslim *et al.* (2018b). Sequencing was carried out by 1st Base, Malaysia through PT Genetika Science Indonesia as the Indonesian agency.

Data analysis

The cDNA sequences were analyzed by using the BLASTn program at https://blast.ncbi.nlm.nih.gov/ (Altschul *et al*., 1990). Tables 2, 3, and 4 show 18 accessions with BLASTn results with query cover values of ≥97% and identities of ≥81%. For phylogenetic tree construction, the amino acid sequences of the 18 accessions of each gene and additional full-length, well-characterized amino acid sequences, such as three *Arabidopsis thaliana ACT* (*At-ACT2*, *At-ACT8*, and *At-ACT9*) genes, one *Triticum aestivum GAPDH* (*Ta-GAPC*) gene, and three *A. thaliana UBQ* (*At-UBQ3, At-UBQ10* and *At-UBQ11*) genes, were selected from GenBank database for comparison (Callis *et al*., 1995; McDowell *et al*., 1996; Zeng *et al*., 2016). The trees were created with MUSCLE multiple alignment, Poisson model, and neighbor-joining methods by using MEGA software version 6.06 (*Molecular Evolutionary Genetics Analysis;* Build#: 6140226) (Edgar and Robert; 2004; Tamura *et al*., 2013).

The *ACT* amino acid sequences of three monocots, such as *Cymbidium faberi* (*Cf-ACT1*/*2*/*3*/*5*), *Musa acuminata* (*Ma-ACT1*/*2*), and *Oryza sativa* (*Os-ACT*), that appeared in BLASTn results (Table 2), and one dicot, namely *A. thaliana* (*At-ACT2*/*8*/*9*), were selected to determine the intron–exon position in the *Benstonea* sp. *ACT* amino acid sequence by using MEGA software version 6.06 with MUSCLE multiple alignment. The *At-ACT* genes were used for comparison because the *At-ACT* gene has been characterized extensively. *At-ACT2*/*8* is closely related to monocot actin genes, and *At-ACT9* is an actin pseudogene (McDowell *et al*., 1996). Moreover, conserved regions in the *Benstonea* sp. *GAPDH* amino acid sequence were

Table 2. BLASTn alignment analysis results based on the *ACT* cDNA sequence of *Benstonea* sp.

Species	Gene	Query Cover (%)	E value	Identity (%)	Family
Freesia hybrid cultivar	*ACT*	98	0.0	90.13	Iridaceae
Gladiolus hybrid cultivar	*ACT*	98	0.0	89.00	Iridaceae
Ornithogalum longebracteatum	*ACT3*	98	0.0	88.96	Hyacinthaceae
Betula platyphylla	*ACT*	98	0.0	88.83	Betulaceae
Cocos nucifera	*ACT*	98	0.0	88.67	Arecaceae
Musa acuminata	*ACT1*	98	0.0	88.31	Musaceae
Populus trichocarpa	*ACT*	98	0.0	88.31	Salicaceae
Lycoris longituba	*ACT*	98	0.0	88.19	Amaryllidaceae
Musa acuminata	*ACT2*	98	0.0	88.19	Musaceae
Betula luminifera	*ACT*	98	0.0	88.19	Betulaceae
Oryza sativa	*ACT*	98	0.0	88.15	Poaceae
Cymbidium ensifolium	*ACT*	98	0.0	87.82	Orchidaceae
Cymbidium faberi	*ACT3*	98	0.0	87.70	Orchidaceae
Hevea brasiliensis	*ACT*	98	0.0	87.66	Euphorbiaceae
Cymbidium faberi	*ACT2*	98	0.0	87.54	Orchidaceae
Cymbidium faberi	*ACT1*	98	0.0	87.54	Orchidaceae
Cymbidium faberi	*ACT5*	97	0.0	87.97	Orchidaceae
Gossypium hirsutum	*ACT7*	98	0.0	87.50	Malvaceae

Table 3. BLASTn alignment analysis results based on the *GAPDH* cDNA sequence of *Benstonea* sp.

Species	Gene	Query Cover (%)	E value	Per. Ident (%)	Family
Stemona collinsiae	GAPDH	100	0.0	86.73	Stemonaceae
Cocos nucifera	GAPDH	99	1e−167	83.66	Arecaceae
Ananas comosus	GAPDH	99	4e−166	83.48	Bromeliaceae
Elaeis guineensis	GAPDH	100	1e−161	82.83	Arecaceae
Oncidium hybrid cultivar	GAPDH	100	1e−160	82.65	Orchidaceae
Phyllostachys edulis	GAPDH	100	1e−160	82.65	Poaceae
Cymbidium sinense	GAPDH2	100	6e−158	82.30	Orchidaceae
Cymbidium sinense	GAPDH1	100	6e−158	82.30	Orchidaceae
Cymbidium goeringii	GAPDH1	100	6e−158	82.30	Orchidaceae
Cymbidium faberi	GAPDH5	100	6e−158	82.30	Orchidaceae
Cymbidium faberi	GAPDH4	100	6e−158	82.30	Orchidaceae
Cymbidium faberi	GAPDH3	100	6e−158	82.30	Orchidaceae
Cymbidium faberi	GAPDH2	100	6e−158	82.30	Orchidaceae
Cymbidium faberi	GAPDH1	100	3e−156	82.12	Orchidaceae
Cymbidium faberi	GAPDH	100	3e−156	82.12	Orchidaceae
Shorea beccariana	GAPC	100	3e−155	81.95	Dipterocarpaceae
Cymbidium goeringii	GAPDH2	100	3e−155	81.95	Orchidaceae
Triticum aestivum	GAPC1	100	9e−150	81.24	Triticeae

Table 4. BLASTn alignment analysis results based on the *UBQ* cDNA sequence of *Benstonea* sp.

Species	Gene	Query Cover (%)	E value	Per. Ident (%)	Family
Micromonas commoda	UBQ	100	4e−139	87.17	Mamiellaceae
Oryza sativa	Polyubiquitin	100	1e−134	86.41	Poaceae
Saccharum hybrid cultivar	ubi9	100	1e−134	85.96	Poaceae
Panicum virgatum	UBQ2	100	1e−133	86.20	Paniceae
Micromonas pusilla	UBQ	100	1e−132	85.96	Mamiellaceae
Avena fatua	Tetraubiquitin	100	1e−132	85.96	Poaceae
Triticum aestivum	WubiG	99	5e−132	85.92	Triticeae
Trametes versicolor	UBQ	100	6e−131	85.71	Polyporaceae
Brachypodium distachyon	Polyubiquitin	100	6e−131	85.71	Poaceae
Schizophyllum commune	ubi4	100	6e−131	85.71	Schizophyllaceae
Guzmania hybrid cultivar	UBQ	100	6e−131	85.71	Bromeliaceae
Oryza sativa	RUBQ1	100	7e−130	85.47	Poaceae
Sporobolus stapfianus	Polyubiquitin	100	7e−130	85.47	Poaceae
Zea mays	Polyubiquitin	100	3e−128	85.23	Poaceae
Oryza sativa	Rub1	100	3e−128	85.23	Poaceae
Zea mays	MubG1	100	3e−128	85.23	Poaceae
Zea mays	MubC5	100	3e−128	85.23	Poaceae
Coccomyxa subellipsoidea	Hexaubiquitin	100	1e−127	85.19	Trebouxiophyceae

determined by aligning the amino acid sequences of the 18 accessions in *GAPDH* BLASTn (Table 3) plus the *Ta-GAPC* sequence for comparison because *GAPDH* genes in *T. aestivum* have been characterized extensively (Zeng *et al*., 2016). Furthermore, the number of ubiquitin monomers in *Benstonea* sp. was determined by using the aligned amino acid sequences of *Zea mays* (*Zm-MubG1*/*polyubiquitin*/*MubC5*) and *O. sativa* (*Os-polyubiquitin*) because these sequences are complete. In addition, *A. thaliana* (*At-UBQ3*/*10*/*11*) was used for comparison because *UBQ* genes in *A. thaliana* have been characterized well (Callis *et al*., 1995). Primer pairs for the RT-PCR evaluation of each gene were designed by using primer3 at the primer3.ut.ee website (Untergasser *et al*., 2012; Koressaar *et al*., 2018).

RESULTS

The partial-length cDNA amplification of the *Benstonea* sp. housekeeping genes was successfully conducted, and PCR products with a size of 625 bp for *ACT*, 565 bp for *GAPDH*, and 413 bp for *UBQ* were obtained (Figure 1). The sequences have been registered and released in the GenBank database using by the registration numbers MG836259.1, MG836258.1, and MG836261.1. The cDNA sequences were predicted to encode 208, 188, and 137 deduced amino acids.

Analysis of the partial-length cDNA sequence of *ACT*

The BLASTn analysis of the *ACT* cDNA sequence showed that the *Benstonea* sp. *ACT* cDNA sequence had 87.50%–90.13% similarity with the *ACT* cDNA

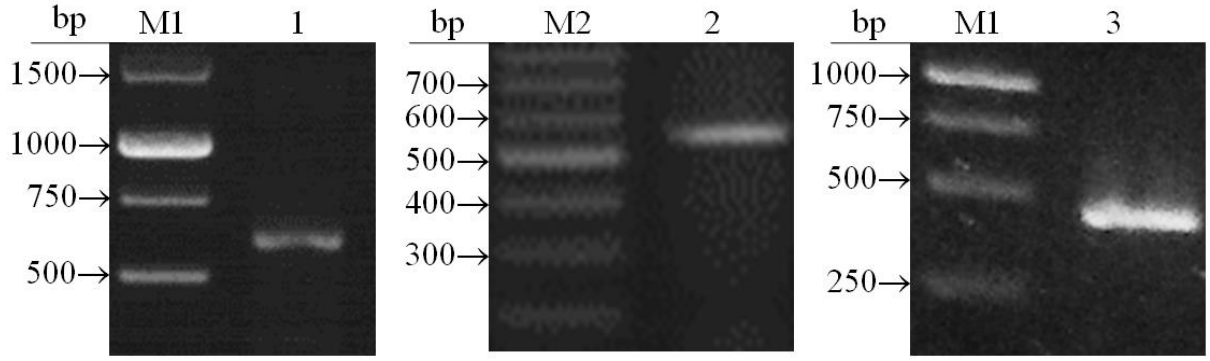

Figure 1. cDNA fragments of (1) *ACT*, (2) *GAPDH*, and (3) *UBQ* of *Benstonea* sp. separated on 1% agarose gel. The DNA ladders of (M1) 1 kb and (M2) 100 bp (Thermo Scientific) were used.

sequences of several plant accessions that are available in the GenBank database with query coverages of 97% to 98% and E-values of 0.0 for all (Table 2). The *Benstonea* sp. *ACT* cDNA sequence was the most similar to that of *Freesia* hybrid cultivar (90.13%) followed by that of the *Gladiolus* hybrid cultivar (89.00%). Both of these plants are members of the Iridaceae family, dicot class. Furthermore, the *Benstonea* sp. *ACT* cDNA sequence shared the lowest similarity (87.50%) with the *ACT* cDNA of *Gossypium hirsutum*, which originated from the Malvaceae family. This species is classified as a dicot, whereas *Benstonea* sp. is a member of the monocot class. The BLASTn result also showed the absence of an *ACT* cDNA sequence from the Pandanaceae family (Table 2). Moreover, the overall mean distance value based on the *ACT* cDNA sequence for several accessions was 0.137 ± 0.011.

The BLASTp analysis of the *ACT* amino acid sequences showed that the *Benstonea* sp. *ACT* amino acid sequence had more than 92.20% similarity to the *ACT* gene sequences of several plants. The highest similarity (99.51%) was found between the *ACT* amino acid sequence of *Benstonea* sp. and *Ornithogalum longebracteatum*, which are both monocots. The lowest similarity (74.02%) was observed between the *ACT* amino acid sequences of *Benstonea* sp. and *Arabidopsis thaliana* (*At-ACT9*). Moreover, the similarity between the *Benstonea* sp. *ACT* amino acid sequence and the other *A. thaliana ACT* amino acid sequences (*At-ACT2* and *At-ACT8*) was 92.20% (data not shown).

Phylogenetic tree analysis based on the *ACT* amino acid sequences showed that the *Benstonea* sp. *ACT* amino acid sequence formed one cluster with *ACT1* and *ACT2* of *M. acuminata.* This group then joined another group that consisted of the *ACT* sequence from *O. longebracteatum*, *F. hybrid cultivar*, and *G. hybrid cultivar*. *At-ACT2* and *At-ACT8* formed a cluster separately, and *At-ACT9* even separated from all monocots. In other words, the *ACT* amino acid sequences of monocots were grouped separately from those of dicots (Figure 2).

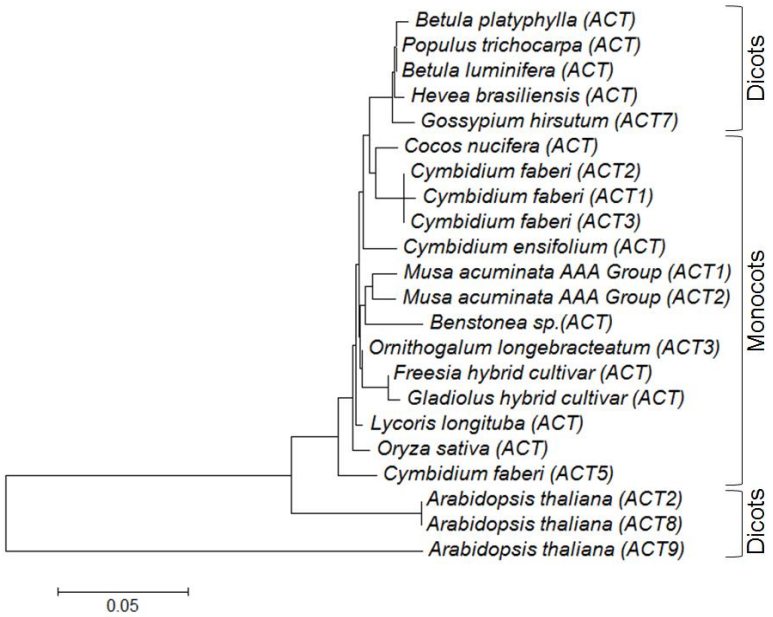

Figure 2. Phylogenetic tree of *ACT* genes generated on the basis of amino acid sequences via the neighbor-joining method.

The MUSCLE multiple alignment of the *ACT* amino acid sequences of *Benstonea* sp., *C. faberi*, *M. acuminata*, *O. sativa*, and *A. thaliana* showed that the *Benstonea* sp. *ACT* amino acid sequence spanned from amino acids number 47 to 254 relative to the complete sequences of *At-ACT8*, *Ma-ACT1*, and *Os-ACT*. Furthermore, intron-2 was located at amino acid 151 in the analyzed actin genes (Figure 3).

Analysis of the partial-length cDNA sequence of *GAPDH*

The *Benstonea* sp. *GAPDH* cDNA sequence had a resemblance of 81.24%–86.73% with the *GAPDH* cDNA sequences of several plant accessions deposited in the GenBank database (Table 3). The query coverage was 100% for all accessions, except for *Cocos nucifera* and *Ananas comosus* (99%). The lowest E-value (0.0) and the highest similarity were found with the *Stemona collinsiae GAPDH* cDNA sequence of the Stemonaceae family. This species is in the same order as *Benstonea* sp, i.e., Pandanales. The lowest similarity was found with the *T. aestivum GAPC1* cDNA sequence. *GAPC1* is a cytosolic *GAPDH* gene. Therefore, the *Benstonea* sp. *GAPDH* cDNA sequence obtained in this study was a cytosolic *GAPDH* gene. Moreover, BLASTn analysis showed one *GAPC* cDNA sequence that belonged to a dicot plant species, namely, *Shorea beccariana*, with low similarity (81.95%) with the *Benstonea* sp. *GAPDH* cDNA sequence (Table 3). The BLASTn analysis of the *Benstonea* sp. *GAPDH* cDNA sequence also showed no *GAPDH* cDNA sequence from the Pandanaceae family. Furthermore, the overall mean distance value based on the *GAPDH* cDNA sequence in some analyzed accessions was 0.208 ± 0.014.

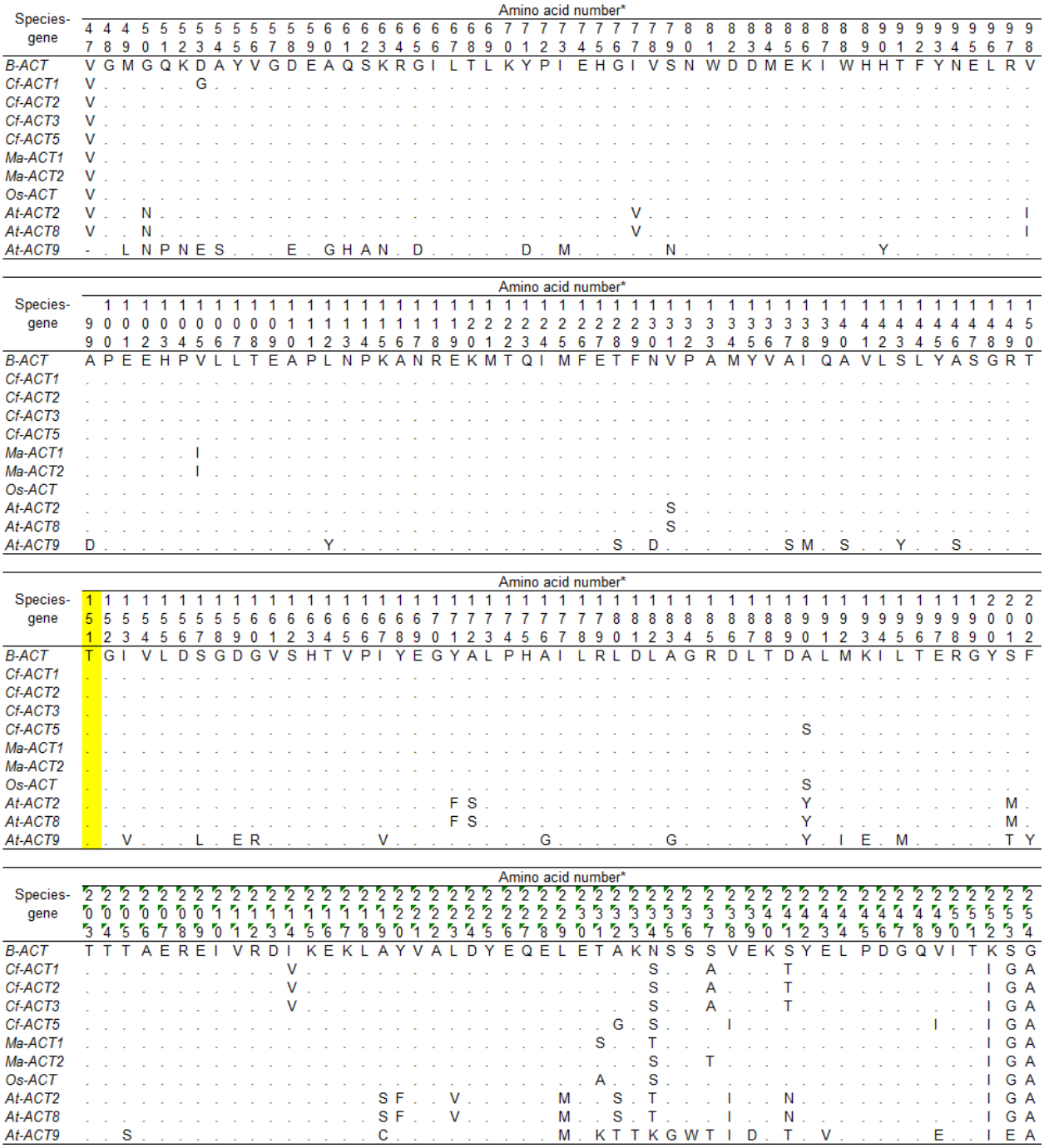

Figure 3. Intron-2 position in the *ACT* amino acid sequence. Light yellow shows the position of intron-2 based on *Arabidopsis thaliana ACT* gene structure. *The numbers arranged vertically show the amino acid numbers referring to the *Ma-ACT1*, *Os-ACT*, *At-ACT2,* and *At-ACT8* sequences. Dots (.) indicate that an amino acid at a particular position is the same as that of *B-ACT.*

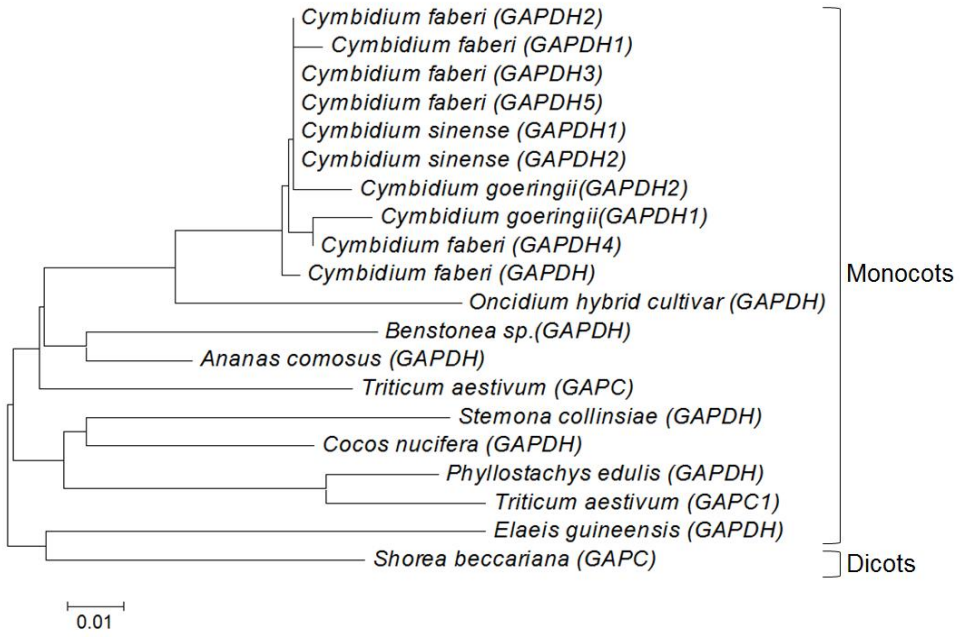

Figure 4. Phylogenetic tree of *GAPDH* genes generated on the basis of amino acid sequences via the neighbor-joining method.

The BLASTp analysis of the *GAPDH* amino acid sequences showed that the *Benstonea* sp. *GAPDH* amino acid sequence had 86.17%–93.62% similarity to the *GAPDH* genes of several plants. The highest similarity (93.62%) was found between the *GAPDH* amino acid sequences of *Benstonea* sp. and *A. comosus*. The lowest similarity (86.17%) was between the *GAPDH* amino acid sequences of *Benstonea* sp. and *Elaeis guineensis*. Moreover, the similarity of the *Benstonea* sp. *GAPDH* amino acid sequence to those of *T. aestivum* (*Ta-GAPC*) was 88.30% (data not shown).

Phylogenetic tree analysis based on the *GAPDH* amino acid sequences showed that the *Benstonea* sp. *GAPDH* amino acid sequence formed one cluster with monocot cytosolic *GAPDH* genes. The *Benstonea* sp. *GAPDH* amino acid sequence was in one group with *Ta-GAPC*. The *GAPDH* amino acid sequence of *S. beccariana* was in one cluster with that of the monocot *E. guineensis* but was separated far from the *Benstonea* sp. *GAPDH* amino acid sequence (Figure 4).

The MUSCLE multiple alignment of the *GAPDH* amino acid sequences of *Benstonea* sp. showed that the BLASTn result of *GAPDH* sequences of all 18 accessions (Table 3) plus *Ta-GAPC* indicated that the *Benstonea* sp. *GAPDH* amino acid sequence consisted of six conserved regions, namely, GAKKV, SNASCTTNCLAP, STGAAKAV, RVPT, VS, and DF. The conserved cysteine (C-157) and histidine (H-184) residues that were essential for substrate binding to the enzyme catalytic site were also found in the *GAPDH* amino acid sequences of *Benstonea* sp. All of the analyzed monocots and dicots had all the conserved regions (Figure 5).

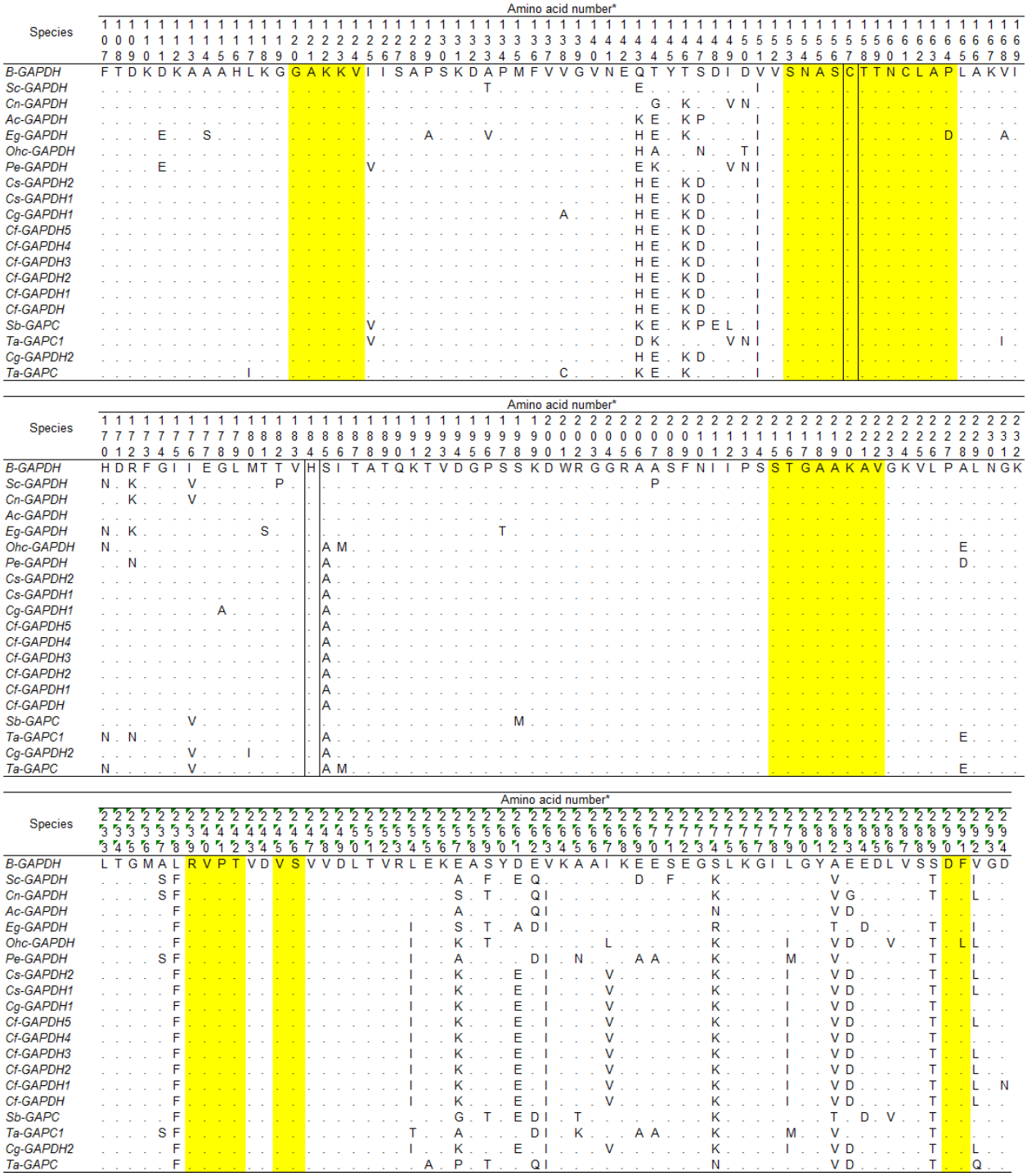

Figure 5. Six conserved regions in the amino acid glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) genes. Yellow light refers to conserved regions. C-157 and H-184 in the boxes indicate conserved cysteine (C) and histidine (H) residues for substrate binding in the enzyme catalytic site. *Numbers arranged vertically show the amino acid numbers referring to the *Cf-GAPDH* and *Sb-GAPC* sequences. Dots (.) indicate that the amino acid at a particular position was the same as that of *B-GAPDH.*

Analysis of the partial-length cDNA sequence of ubiquitin

The BLASTn analysis of the *Benstonea* sp. *UBQ* cDNA sequence showed that the *Benstonea* sp. *UBQ* cDNA sequence had 85.19%–87.17% similarity to the *UBQ* cDNA sequences of several monocot, fungus, and microalgal accessions deposited in the GenBank database with a query coverage of 100% for all accessions, except for *T. aestivum* (99%), and E-values exceeding 0.0 (Table 4). The *Benstonea* sp. *UBQ* cDNA sequence was the most similar to the *Micromonas commoda* sequence (87.17%). The lowest similarity was found with *Coccomyxa subellipsoidea* (85.19%). Both are microalgae. Furthermore, the BLASTn analysis result for the *UBQ* cDNA sequences showed no *UBQ* cDNA sequence from the Pandanaceae family (Table 4). The overall mean distance value based on the *UBQ* cDNA sequence in some analyzed accessions was 0.123 ± 0.012.

The BLASTp analysis of *UBQ* amino acid sequences showed that the *Benstonea* sp. *UBQ* amino acid sequence had more than 96.35% similarity to several *UBQ* genes of monocots, fungi, and microalgae. The highest similarity (99.27%) was found between the *UBQ* amino acid sequence of *Benstonea* sp. and several monocots and microalgae. The lowest similarity (96.35%) was between the *UBQ* amino acid sequences of *Benstonea* sp. and *C. subellipsoidea* microalgae. The *UBQ* amino acid sequences of two fungi, i.e., *Trametes versicolor* and *S. commune*, had 97.81% similarity with that of *Benstonea* sp. (data not shown).

Phylogenetic tree analysis based on the *UBQ* amino acid sequences showed that the sequences was very similar among taxa such that the tree displayed in Figure 6 was a consensus tree. The tree showed that the *Benstonea* sp. *UBQ* amino acid sequence was closely related to *Zm-MubC5*. Moreover, the *UBQ* amino acid sequence of *Benstonea* sp. formed one cluster with accessions from Poaceae and Bromeliaceae families. Furthermore, no clear grouping among plant, fungi, and microalgae was observed (Figure 6).

The MUSCLE multiple alignment of the *UBQ* amino acid sequences of *Benstonea* sp., *Z. mays*, *O. sativa*, and *A. thaliana* showed that the *Benstonea* sp. *UBQ* amino acid sequence comprised two partial ubiquitin monomers and one full-length ubiquitin monomer. As inferred from the *Zm-MubG1* and *Zm-polyubiquitin* amino acid sequences, the *Benstonea* sp. *UBQ* amino acid sequence started at amino acid 52 of ubiquitin monomer-1 and ended at 36 of ubiquitin monomer-3. The *UBQ* amino acid sequences of *Z. mays* and *O. sativa* terminated at the same C-terminal additional amino acid, such as glutamine, whereas *At-UBQ3* contained two additional C-terminal residues, namely serine phenylalanine. The additional C-terminus of *At-UBQ10* was phenylalanine (Figure 7).

DISCUSSION

The first step in studying patterns and gene expression levels with the qRT-PCR technique is the isolation of housekeeping genes. Therefore, the cDNA sequences of the housekeeping genes that were acquired in this study

must be selected and validated to determine which ones can be used either as an internal control or as a reference gene in the gene expression analysis of plants given the absence of a reference gene that is universally applicable to all organisms and treatment conditions (Vandesompele *et al*., 2002; Gantasala *et al*., 2013; Kozera and Rapacz, 2013; Tang *et al*.,

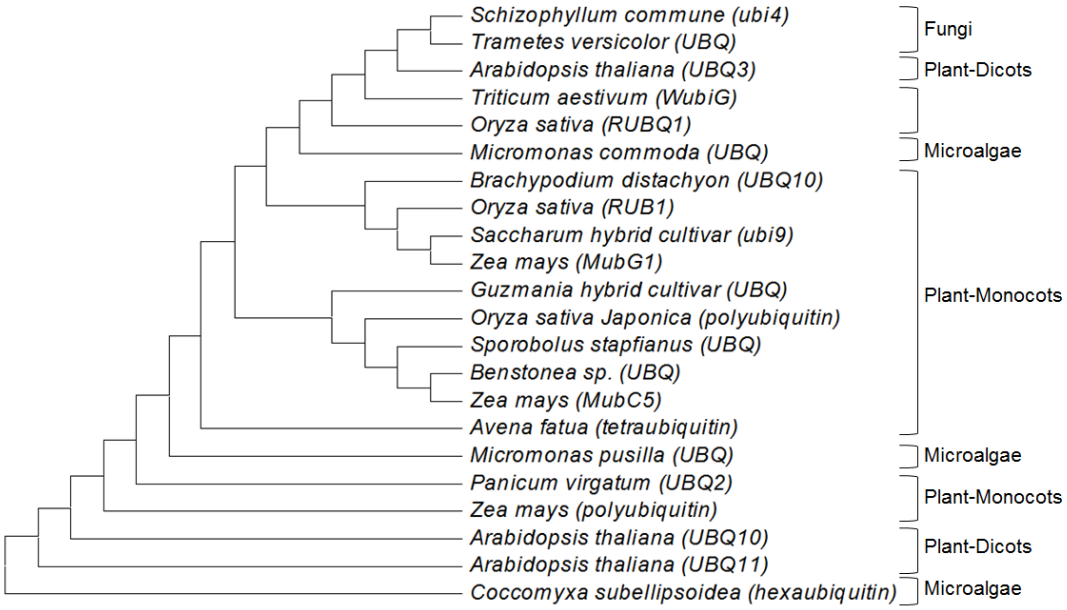

Figure 6. Phylogenetic consensus tree generated on the basis of *UBQ* amino acid sequences via the neighbor-joining method.

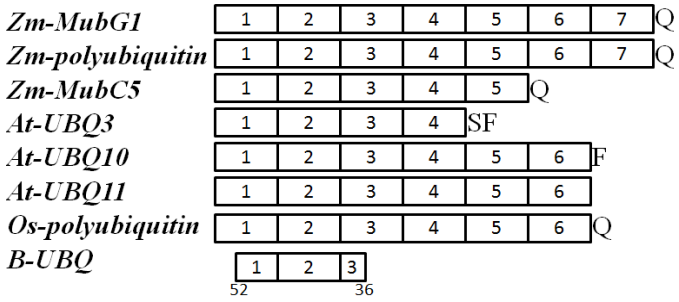

Figure 7. Diagram of *UBQ* coding regions based on amino acid sequences. *Zm* = *Zea mays*, *At* = *Arabidopsis thaliana*, *Os* = *Oryza sativa*, and *B* = *Benstonea* sp. 1, 2, 3, 4, 5, 6, and 7 are the numbers of ubiquitin monomers. In *B-UBQ*, 52 and 36 represent amino acid numbers in reference to *Zm-MubG1* and *Zm-polyubiquitin*. The *B-UBQ* sequence obtained in this study contains one full length (number 2) and two partial monomers (numbers 1 and 3). The letter outside the box represents the C-terminal additional amino acid in a complete ubiquitin gene. Q = glutamine, S = serine, F = phenylalanine.

2017; Joseph *et al*., 2018; Zhao *et al*., 2019). Moreover, at least two reference genes are needed for the normalization of gene expression data (Vandesompele *et al*., 2002; Martins *et al*., 2017; Tang *et al*., 2017). Some housekeeping genes, such as *ACT*, *GAPDH*, *TUB*, *18S rRNA*, and *UBQ* (Hashemi *et al*., 2016; Martins *et al*., 2017; Luo *et al*., 2018), have been selected and validated as reference genes in some plants.

In this research, the *ACT* cDNA sequence of *Benstonea* sp. was successfully isolated and shown to have higher similarity to the sequences of other plant species that are members of monocots (13 genes from nine accessions) than to those of dicots (five genes from five accessions). Moreover, the *ACT* amino acid sequence of *Benstonea* sp. had a close relationship with *Ma-ACT1* and *Ma-ACT2*, formed a group with the same monocots, and was clearly separated from dicot *ACT* genes. These results showed that the monocot *ACT* gene was different from the dicot *ACT* gene and was in accordance with the results of McDowell *et al*. (1996), who stated that plant *ACT* genes are classified into two major groups, such as the monocot and dicot *ACT* groups.

In plant cells, the actin protein is encoded by the actin gene family. The analysis of the *Benstonea* sp. *ACT* cDNA sequence performed in this study revealed four *ACT* genes that originated from *C. faberi* (*Cf-ACT1*/*2*/*3*/*5*) and two others that belonged to *M. acuminata* (*Ma-ACT1*/*2*). Moreover, the results for the *A. thaliana ACT* amino acid sequences (*At-ACT2/8/9*) that were included in phylogenetic tree analysis were in accordance with those of McDowell *et al*. (1996), who stated that *At-ACT2/8* forms one group with ACT genes from monocots, such as *Z. mays* and *O. sativa*. Meanwhile, *At-ACT9* was located in the basal position relative to all plant *ACT* genes.

The obtained *Benstonea* sp. *ACT* cDNA sequence contained one intron (intron-2) and two exons (exon-2 and -3). The intron position in the *Benstonea* sp. *ACT* cDNA sequence was inferred by aligning the *ACT* amino acid sequences of *Benstonea* sp. and several accessions, especially *At-ACT8*, due to its full-length sequence (1805 bp mRNA and 377 amino acids) and close relationship to monocots. Generally, *At-ACT* genes comprise four exons and three introns. Intron-2 is inserted in codon 151 (McDowell *et al*., 1996).

The selection and validation of housekeeping genes to obtain a reference gene that can be used as an internal control for the study of gene expression can be performed by using qRT-PCR technique. This technique is intentionally used because it can provide reliable, sensitive, reproducible and accurate results. Some factors can affect the sensitivity of the qRT-PCR technique; one of these factors is genomic DNA contamination in a cDNA solution (Tavares *et al*., 2011; Laurell *et al*., 2012; Hashemipetroudi *et al*., 2018). One easy way to detect genomic DNA contamination in a cDNA solution is by performing a PCR with a template that separates cDNA and DNA molecules. PCR is performed by using a primer that attaches to two exons, and the exons then clamp an intron (Hannum *et al*., 2010). If a PCR with a cDNA template produces more than one DNA band, among which one is the same size as the DNA band produced by a PCR with a DNA template, then the cDNA solution is positive for genomic

DNA contamination (Hannum *et al*., 2010; Hashemipetroudi *et al*., 2018). A genomic-DNA-contaminated cDNA solution cannot be used as a template for gene expression analysis because the data obtained will be biased and inaccurate.

Previously, the DNA sequence of *ACT Benstonea* sp*.* was isolated with a length of 1403 bp. The alignment of the DNA and cDNA sequences of *ACT Benstonea* sp. acquired in this study showed that the obtained cDNA sequences clamp an intron that is 784 bp in length (Roslim and Herman, 2019). This result indicates that the *ACT* gene in *Benstonea* sp. can be feasibly considered as an indicator of genomic DNA molecule contamination in isolated cDNA solution. Furthermore, a primer pair that can be used in the qRT-PCR is forward 5′-CCA GTC CAA AAG AGG TAT CC-3′ and reverse 5′-CTC CTG CTC ATA ATC AAG GG-3′ with a 505 bp amplicon.

Moreover, the *GAPDH* gene in plant cells is classified into three groups; one of these genes is a cytosolic *GAPDH* gene (also called *GAPC*) that is categorized into subfamily III. Five cytosolic *GAPDH* genes, namely Ta-GAPDH4/6/8/10/12, have been found in *T. aestivum* (Zeng *et al*., 2016). In this study, BLASTn analysis showed six types of GAPDH (*Cf-GAPDH*/*1*/*2*/*3*/*4*/*5*) in *C. faberi* and two types in *C. sinense* (*Cs-GAPDH1*/*2*) and *C. goeringii* (*Cg-GAPDH1*/*2*). In addition, almost similar to the *ACT* cDNA sequence, the *Benstonea* sp. *GAPDH* cDNA sequence was more similar to monocot sequences than to dicot sequences. The *Benstonea* sp. *GAPDH* cDNA sequence had the highest similarity to *S. collinsiae*, which is also a monocot and member of Pandanales.

The *GAPDH* protein contains 10 conserved residues. The two highly conserved regions are important to the coenzyme-binding domain ("NGFGRIGR") and catalytic domain (cysteine and histidine residues) (Zaffagnini *et al*., 2013; Zeng *et al*., 2016). In this study, six of the 10 conserved residues and a catalytic domain were found in the *Benstonea* sp. *GAPDH* amino acid sequence.

BLASTn analysis showed that in contrast to the *Benstonea* sp. *ACT* and *GAPDH* cDNA sequences, the *Benstonea* sp. *UBQ* cDNA sequence shared similarity with monocot, fungal, and microalgal sequences. Moreover, the *Benstonea* sp. *UBQ* amino acid sequence was more closely related to *Z. mays* (*MubC5*) sequences than to monocot sequences.This result indicated that the *UBQ* gene is almost identical among taxa likely because it is composed of tandem repeats of ubiquitin-coding regions (monomers) without spaces between them. An ubiquitin monomer has 228 nucleotides encoding 76 amino acids with an additional C-terminal residue (Finley *et al*., 1989).

Fourteen *A. thaliana UBQ* genes were characterized and divided into three ubiquitin gene types, such as polyubiquitin, ubiquitin-like, and ubiquitin extension genes. Polyubiquitin genes encode the same amino acid sequence (protein), and they are different from each other in terms of the nature of the additional C-terminal amino acids and the number of ubiquitin-coding regions. Four ubiquitin genes, namely, *At-UBQ3*/*10*/*11*/*14*, are an example of *A. thaliana* polyubiquitin (Callis *et al*., 1995). The *Benstonea* sp. *UBQ* cDNA sequence obtained in this study was a polyubiquitin that consisted of three ubiquitin monomers, two of which

were partial sequences and one was a full-length ubiquitin monomer.

Furthermore, primer pairs for the amplification of the *Benstonea* sp. *GAPDH* and *UBQ* genes were designed for qRT-PCR analysis. The *GAPDH* primer pair was forward 5′-AAG GTC ATC ATT TCT GCT CC-3′ and reverse 5′-ACC AAA TCT TCT TCG GCA TA-3′ with an amplicon that was 494 bp in size. The *UBQ* primer pair was forward 5′-CAC GCT TGC TGA CTA TAA TAT-3′ and reverse 5′-ATT TGC ATA CCA CCT CGA AG-3′ with an amplicon that was 303 bp in size.

The BLASTn analysis of cDNA sequences showed that the highest identity value was found for *ACT* (90.13%) followed by that for *UBQ* (87.17%) and *GAPDH* (86.73%). These results showed that both sequences (*ACT* and *UBQ* sequences) have high resemblance between species and organisms. In other words, compared with the GAPDH sequence, the *ACT* and *UBQ* sequences are more conserved among organisms. Moreover, the *GAPDH* sequence is more variable among plant species than the *ACT* and *UBQ*.

This result is in line with the lower overall mean distance value of *ACT* and *UBQ* compared with that of *GAPDH*. This value refers to the proportion of different nucleotides caused by mutations in all compared sequences (Tamura and Kumar, 2002; Tamura *et al*., 2013). Hug *et al.* (2016) and Jayaswal *et al.* (2017) reported that *ACT* and *UBQ* genes together with other housekeeping genes, such as the *TUB* gene and the gene encoding large and small RNA ribosomal subunits, have a high similarity or are highly conserved among plant species. The high level of similarity will facilitate designing primers to isolate housekeeping genes from other plant species.

None of the three cDNA sequences used in BLASTn analysis originated from the *Benstonea* genus. Therefore, the three isolated cDNA sequences in this research are the first cDNA sequences isolated from the *Benstonea* genus. These sequences are necessary for gene expression analysis in *Benstonea* sp. with related phenotypes. In the future, these three sequences will be selected and validated to determine which is appropriate to be used as an internal control in studies on gene expression analysis in plants, for example, the expression analysis of genes related to flooding stress, such that underlying physiological and molecular mechanisms can be understood. These results can then be used as a basis for developing crops with tolerance for flooding stress.

CONCLUSION

The cDNA fragments of three housekeeping genes were successfully isolated from *Benstonea* sp. with a size of 625 bp for *ACT*, 565 bp for *GAPDH*, and 413 bp for *UBQ* with the registration numbers MG836259.1, MG836258.1, and MG836261.1, respectively. These cDNA sequences were predicted to encode 208, 188, and 137 deduced amino acids. The cDNA sequences had 81.24%–90.13% similarity to cDNA sequences from several plants originating from different families of *Benstonea* sp. The *Benstonea* sp. cDNA sequences obtained in this study are the first reported from this genus. The three *Benstonea* sp. housekeeping cDNA sequences obtained in this study have the characteristics of each gene, such

as conserved area, domain, intron, and monomer number.

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