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Isolation and Characterization of Squalene Synthase Gene in Three Species of *Achillea,* a Rich Source of Saponins

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ABSTRACT

Squalene synthase (SQS, EC.2.5.1.21) is a key enzyme involved in the biosynthesis pathway of triterpenoid and steroidal saponins. The present study aimed to collect molecular information about the *SQS* gene in *Achillea* species, the medicinal plants rich in saponins. For this reason, genomic DNA was isolated from leaves of three *Achillea* species, including *A. millefolium*, *A. wilhelmsii*, and *A. vermicularis* in Iran, then partial *SQS* gene was amplified through PCR and sequenced (NCBI accession numbers: AmSQS KX589055, AwSQS KX685330, and AvSQS KX685331). AmSQS was 800 bp, containing four exons and three introns; AwSQS and AvSQS were 510 bp and 500 bp, respectively, containing three exons and two introns. Phylogenetic analysis demonstrated that the isolated SQS sequences were significantly similar to each other, and to *Artemisia annua*, another species of the genus *Achillea*. Furthermore, in the phylogeny tree, the SQS gene sequences of dicots and monocots were located in separate clades. The deduced amino acid sequences obtained from the isolated *SQS* gene had also a high similarity to each other and other organisms SQSs (>73% similarity to higher plants and more than 57% and 47% to the yeast and human). The deduced amino acid sequences included two regions overlapping with domains B and C of SQS, comprising an important motif of aspartate-rich (DYLED) for substrate binding via Mg^{2+}-bridge. Data resulting from this study was the first report of *SQS* gene isolation and characterization in *Achillea* species, which also showed the ability of this gene in taxonomic classification.

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Introduction

Triterpenes ($C_{30}H_{48}$) and sterols are natural compounds in plants and animals (Lee *et al.*, 2004). They are a group of plant secondary metabolites produced by the fusion of repetitive C5 units (Buchanan *et al.,* 2000). Saponins, a major derivate group of triterpenoids and steroids, are important pharmaceutical compounds with anti-inflammatory, antitumor, anti-HIV, and antiviralproperties (Hill and Connolly, 2013; Thimmappa *et al.,* 2014; Ogbe *et al.*, 2015). Squalene is a key precursor in the saponin biosynthetic pathway, produced by squalene synthase (SQS, EC.2.5.1.21). SQS, a membrane-associated protein in the endoplasmic reticulum, catalyzes the conversion of two farnesyl pyrophosphate (FPP) molecules to squalene (Kim *et al.*, 2011). An increase in gene expression of SQS causes raised terpenoid production in plants (Zhao *et al.,* 2015). However, *SQS* genes have been characterized in several plants and other organisms. There is no information about the enzymes in *Achillea* species, a group of plant-rich triterpenoids and saponins.

The genus *Achillea* L. (yarrow), belonging to Asteraceae, has about 130 species worldwide, spreading in Europe, Asia, and some temperate regions of North Africa and America (Podlech, 1986; Mozaffarian, 2005; Si *et al.*, 2006; Saeidnia *et al.*, 2011). Previous studies have reported triterpenoid compounds, including α-amyrin, β-amyrin, taraxasterol, and pseudo-taraxasterol, also β-sitosterol, stigmasterol, campesterol, and cholesterol in *Achillea* species (Chandler *et al.*, 1982). *Achillea millefolium* is a well-known medicinal plant classified as a saponin-rich species. However, few studies have been done on the biosynthesis pathways of saponins in this genus. Further studies could provide an overview of the regulation of saponin biosynthesis and metabolism pathways in the plants and make them more productive. Because there is very little information about the structure of genes involved in the biosynthesis of the *Achillea* terpenoids, the present study tries to isolate and characterize partial sequences of the *SQS* genes in three species of *Achillea,* including *A. millefolium*, *A. wilhelmsii*, and *A. vermicularis*. Furthermore, this study will compare some of the isolated SQS genes and the deduced peptides to understand the structure of the *SQS* gene and the enzyme in the studied species of *Achillea*.

Materials and Methods

Genomic DNA extraction

Three Achillea species, including *A. millefolium*, *A. wilhelmsii*, and *A. vermicularis* were collected from natural regions of Iran (Polour, Kashan, and Taleghan in Tehran, Isfahan, and Alborz provinces, respectively). Each seed was sterilized for two hours by running tap water, 70% ethanol (2×1 min), and 1% hypochlorite sodium (10 min). They were subsequently washed by distilled water. After that, the seeds were cultured on the basic Murashige and Skoog medium (1962). Cultures were kept at 23±2 ˚C under 16h light/8h dark photoperiod. After two months, seedlings were harvested and the leaves were subjected to DNA extraction.

Genomic DNA was isolated according to the Khanuja *et al*. (1999) method. The quantity and quality of the isolated DNA were evaluated using a NanoDrop spectrophotometer (Thermoscientific 2000, USA) and 1% agarose gel electrophoresis, respectively.

Isolation of *SQS* genes

Since the sequence of the *SQS* gene was not previously identified in *Achillea* species, primers were designed according to the conserved regions of known *SQS* genes in other plants amongst *Arabidopsis thaliana* (AF04560), *Nicotiana tabacum* (U59683), *Bupleurum chinease* (GQ889268), *Artemisia annua* (AF405310) and *Aster amellus* (HQ131826). Two pairs of specific primers were designed using Gene Runner 5.1 software (www.generunner.com/) (Table 1) and synthesized by Sinaclon Company (SinaClon, Iran).

Table 1. The primers used in PCR analysis for amplification of partial *SQS* genes in *Achillea* species.

Primer name		Sequence (5′→3′)	Tm (°C)	PCR products
A1SQS	Forward	5′-ATGTTTCTACTGCCTTTCTGG-3′	58	840
	Reverse	5′-GCACAAAACCTGAAGATGGC-3′		
A2SQS	Forward	5′-TATGTTGCGGGACTTGTTGG -3′	58	600
	Reverse	5′-GCACAAAACCTGAAGATGGC-3′		

PCR reactions comprised 50 ng DNA template, 0.5 µL dNTP mix (10 mM), 2.5 µL PCR buffer10X, 0.5 µL each forward and reverse primers (10 pM), 1.25 µL $MgCl_2$ (25 mM) and 1.25 U Taq DNA polymerase in a final volume of 25 µL. The PCR was performed in a DNA thermal cycler (MJ Mini, BIO-RAD, US) in terms of initial denaturation at 95 ˚C (10 min), 35 cycles at 94 ˚C for 60 s, 58 ˚C for 60 s and 72 ˚C for 60 s, and a final extension at 72 ˚C (10 min). The amplified sequences were checked out by gel electrophoresis (1% agarose) and sequenced by Pishgam Biotech Company.

Bioinformatics analysis

The resulting nucleotide sequences were identified through NCBI Blastn. Exons and introns were determined with ORF (open reading

frame) finder (https://www.ncbi.nlm.nih.gov/). The phylogenetic tree was drawn using the neighbor-joining method by MEGA 5 (Biodesign Institute, Tempe, AZ, USA; bootstrap of 500 replicates). To determine conserved domains, the deduced amino acid sequences with eukaryote SQSs were aligned by DNAMAN (Lynnon Biosoft, Quebec, QC, Canada).

Results

In the present study, three partial SQS genes were isolated and identified from leaves of three *Achillea* species (*A. millefolium, A. wilhelmsii,* and *A. vermicularis*). Amplification was performed by two pairs of specific primers, as *A. millefolium* DNA was successfully amplified with *A1SQS* primers, while DNA of two other species (*A. wilhelmsii* and *A. vermicularis*) were amplified by A2SQS primers (Fig. 1).

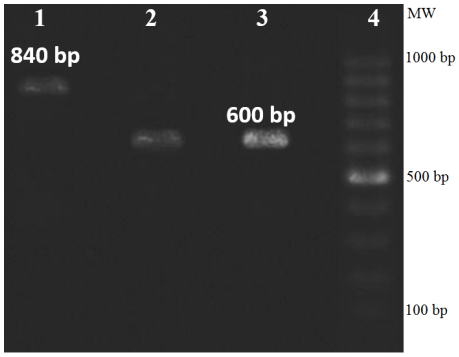

Fig. 1. PCR products of the *SQS* genes on 1% agarose gel electrophoresis: A single DNA fragment derived from PCR of the template DNA extracted from the leaves of *A. millefolium* by using primer *AmSQS* (Lane 1), leaves of *A. wilhelmsii* (Lane 2), and leaves of *A. vermicularis* (Lane 3) by using primer *AwvSQS*; Lane 4 is DNA ladder in the range of 100-1000 bp.

The amplified products were sequenced, annotated, and submitted to the NCBI GenBank database by accession numbers of AmSQS KX589055, AwSQS KX685330, and AvSQS KX685331 for *A. millefolium, A. wilhelmsii,* and *A. vermicularis*, respectively. AmSQS was 800 bp, including an open reading frame (ORF) with 396 bp at the positions of 72...141bp, 298...441 bp, 533...637 bp, 721...798 bp <, encoding a 132-amino acid peptide. AwSQS was 510 bp and had a 342 bp ORF at the positions of >1...103 bp, 193...297 bp, 375...510 bp <, encoding a 114-amino acid peptide. Also, AvSQS was 500 bp and had a 330 bp ORF at the positions of >1...89 bp, 179...283 bp, 361...500 bp < encoding a 110-amino acid peptide.

Fig. 2 shows the deduced amino acid sequences of the partial *Achillea* SQS genes. Exons and introns were identified based on the universal eukaryotic codes where introns commenced with GT and terminated with AG dinucleotide (Sharp and Burge, 1997) (Fig. 3). The blast results indicated that *Artemisia annua* (AF405310, Asteraceae) is a close relative species to the studied *Achillea* species since the alignment of the AmSQS with the *SQS* gene in *A. annua* showed 99% query coverage and 90% identity, and AwSQS and AvSQS had 99-100% query coverage and 91-92% identity as well. The AmSQS had 4 exons overlapping with exons 5 to 8 of *A. annua* SQS, and each AwSQS and AvSQS sequence contained 3 exons overlapped (Fig. 3).

A phylogenetic tree was constructed using the three partial SQS genes of Achillea species and other plants' known SQS genes (Fig. 4). As shown in Fig. 4, eight species were clustered into two main clades, the two monocots located in one clade (with 85% similarity) whereas the dicots located in another one. In the dicot clade, *A. wilhelmsii* and *A. vermicularis* had the most similarities to each other, and subsequently *to A. millefolium*. The partial SQS genes of the *Achillea* species had the most similarity to *Artemisia annua* (about 91-92%) and *Aster amellus* (69-70% similarity) SQS genes. Every three species belong to the Asteraceae family. Other studied species had about 53-58% similarities, among which *Arabidopsis thaliana* is located in the farthest clade (Fig. 4). The deduced amino acid sequences of the partial SQS genes of the three *Achillea* species were aligned with SQSs in several other organisms such as yeast, humans, and some plants (Fig. 5).

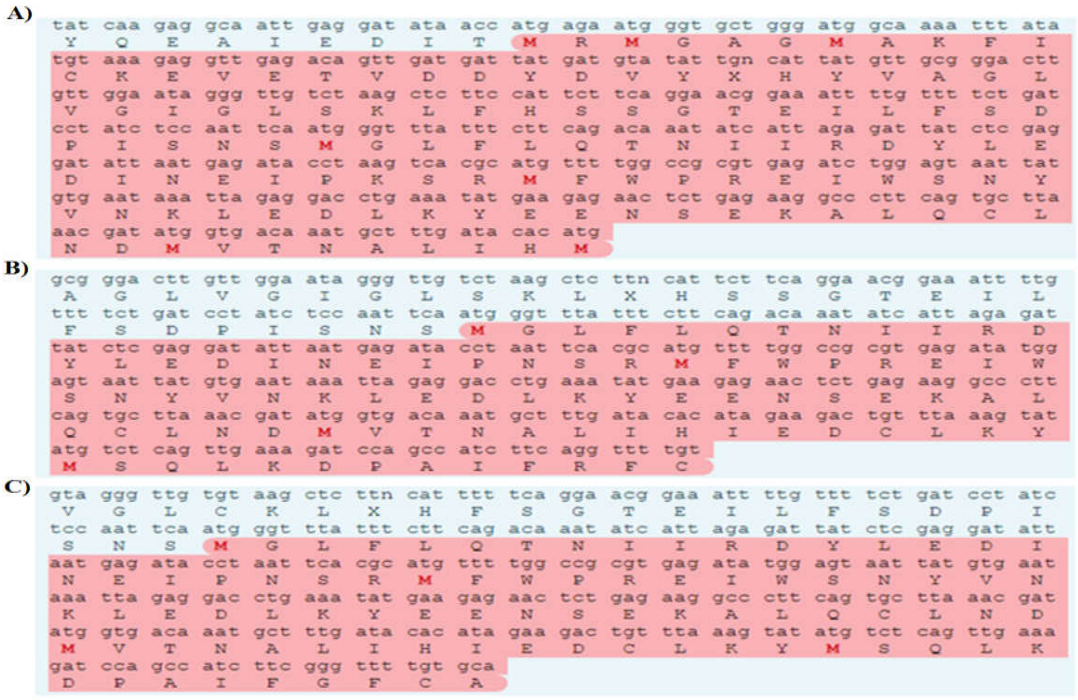

Fig. 2. Comparison of the nucleotide and deduced amino acid sequences of *Achillea* SQS genes: A) KX589055 (*A. millefolium*); B) KX685330 (*A. wilhelmsii*); C) KX685331 (*A. vermicularis*).

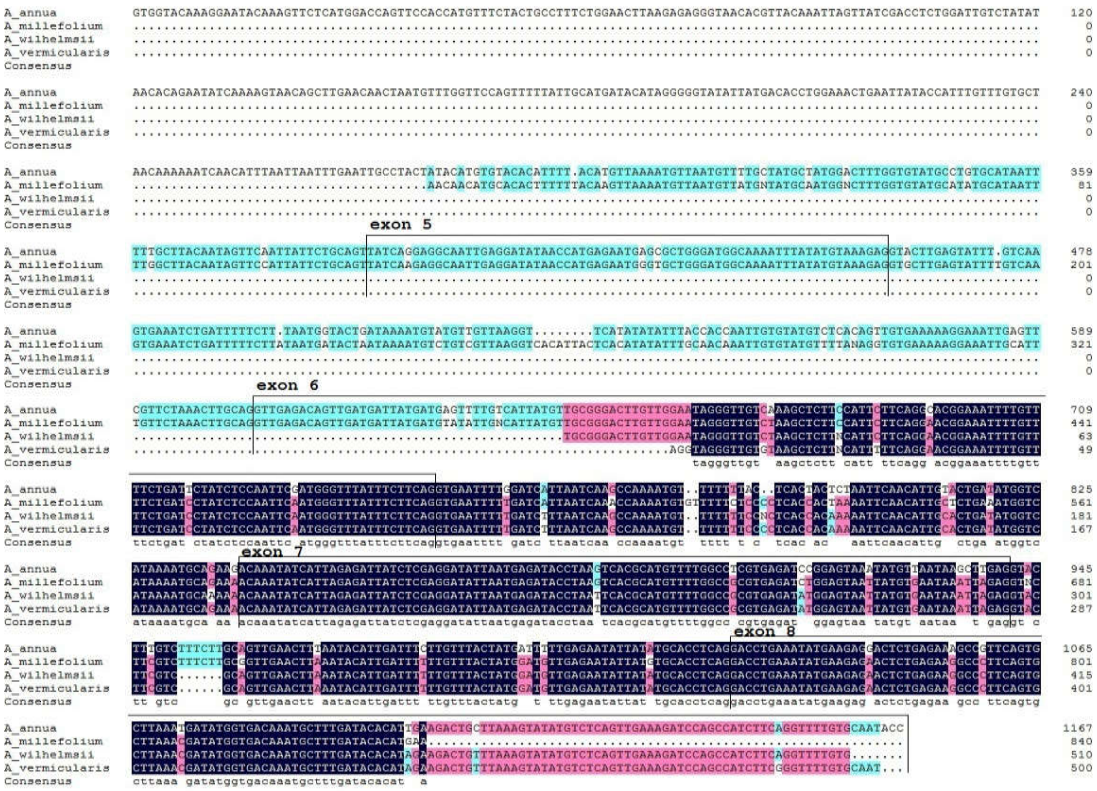

Fig. 3. Alignment of the partial nucleotide sequences of *SQS* gene from three *Achillea* species compared to *Artemisia annua*: The similarity of the nucleotides is shown by different colors among KX589055 (*A. millefolium*), KX685330 (*A. wilhelmsii*), KX685331 (*A. vermicularis*), and AF405310 (*A. annua*) using DNAMAN software. Letters of A, B, C, and D are represented exons 5 to 8 in *A. annua.*

Our results showed that predicted peptides in three *Achillea* species were much similar to each other (93% to 97%). However, they were more similar to SQSs in *Artemisia annua* (Q6SYC8) and *Aster amellus* (E5KHT), both belonging to Asteraceae family. The minimum similarity was observed between the predicted peptides and SQS of humans (P37268) and yeast (P29704) by 47-58% (Table 2). Given that in eukaryotic organisms, SQS proteins have four conserved domains (A-D) (Gu *et al*., 1998), a comparison of peptide fragments in three *Achillea* species to SQSs in other organisms revealed two regions overlapping with domains B and C (Fig. 5).

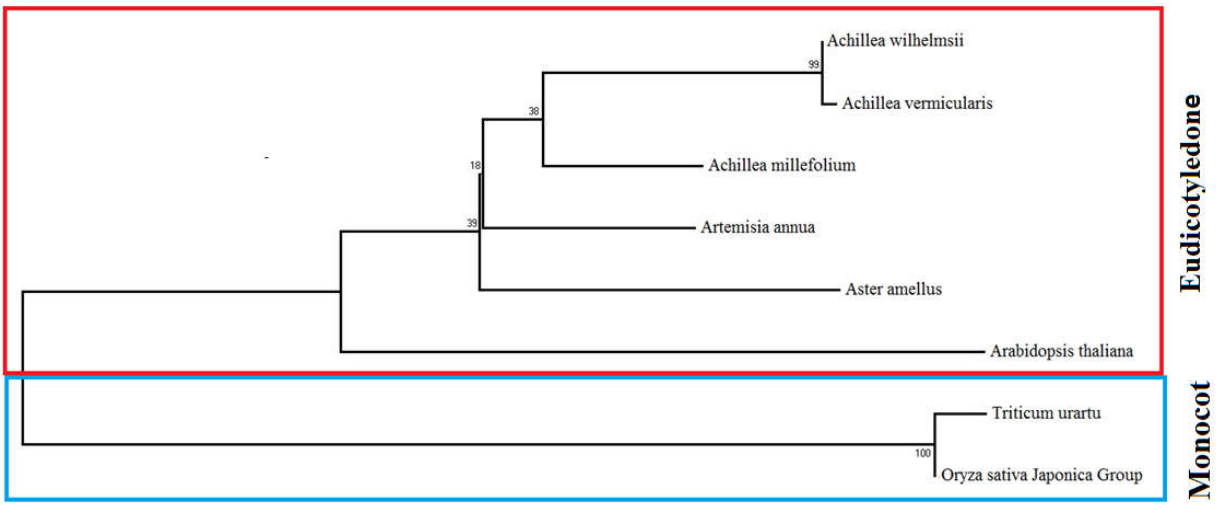

Fig. 4. Phylogenetic tree created based on the similarity of the partial nucleotide sequences of *SQS* genes using the neighbor-joining method.

Table 2. Homology matrix of the amino acid sequences of the SQS in seven individual organisms and studied species of *Achillea*.

	S. cerevisiae	***H. sapiens***	***O. sativa***	***T. urartu***	***A. thaliana***	***A. amellus***	***A. annua***	***A. millefolium***	***A. wilhelmsii***	***A. vermicularis***
S. cerevisiae	100									
H. sapiens	51.11	100								
O. sativa	44.83	58.62	100							
T. urartu	48.28	60.92	83.33	100						
A. thaliana	47.19	56.18	76.67	78.89	100					
A. amellus	45.98	59.77	78.89	85.56	82.22	100				
A. annua	45.98	60.92	80.00	85.56	81.11	93.33	100			
A. millefolium	47.13	58.62	76.67	83.33	77.78	90.00	95.56	100		
A. wilhelmsii	47.13	58.62	74.44	82.22	76.67	88.89	94.44	96.67	100	
A. vermicularis	47.13	56.32	72.22	81.11	75.56	91.11	91.11	93.33	96.67	100

SQS polypeptides were compared among *Saccharomyces cerevisiae* (yeast, P29704), *Homo sapiens* (human, P37268), *Triticum urartu* (M8B0M6), *Oryza sativa* subsp. *japonica* (Q6Z368), *Arabidopsis thaliana* (P53799), *Aster amellus* (E5KHT2), *Artemisia annua* (Q6SYC8), and predicted amino acid sequences of *Achillea millefolium*, *A. wilhelmsii,* and *A. vermicularis*.

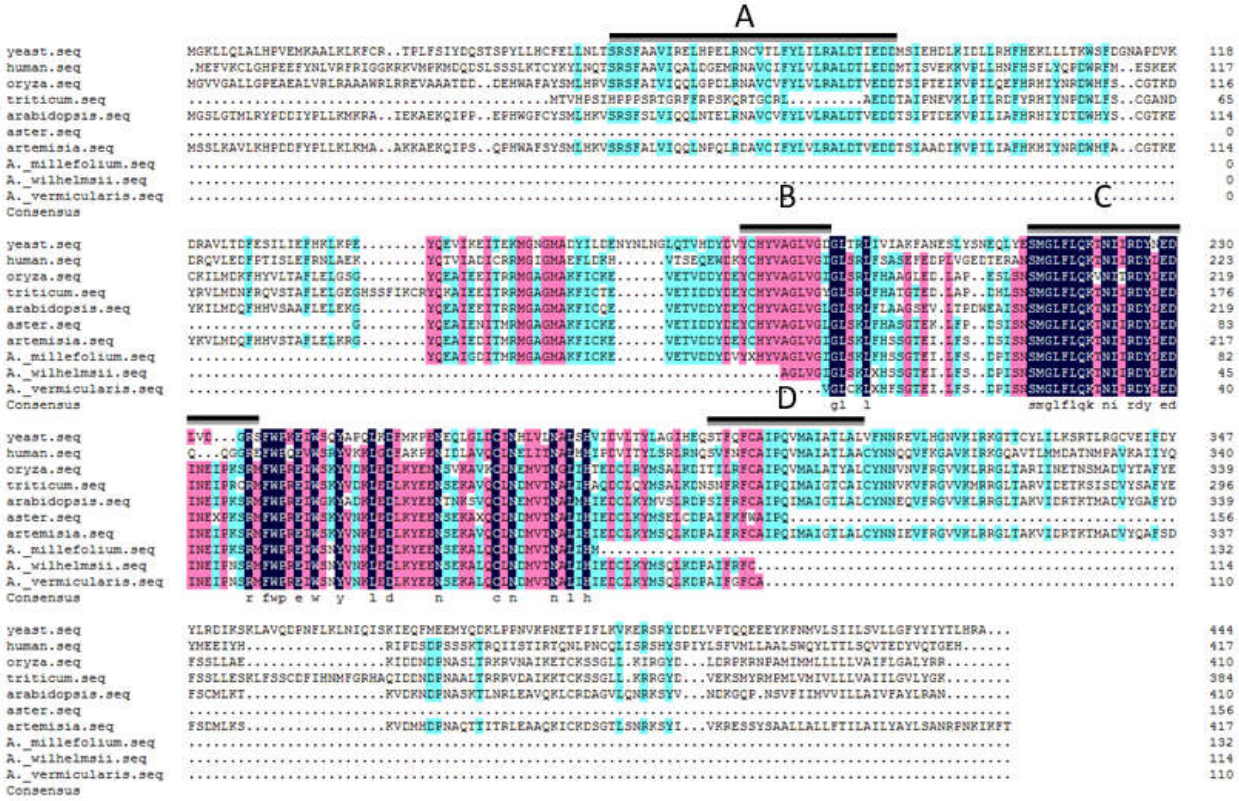

Fig. 5. Comparison of SQS amino acid sequences between *Achillea* species and several organisms: Data for other organisms were obtained from nucleotide databases NCBI GenBank: *Saccharomyces cerevisiae* (yeast, P29704), *Homo sapien*s (human, P37268), *Triticum urartu* (M8B0M6), *Oryza sativa* subsp. *japonica* (Q6Z368), *Arabidopsis thaliana* (P53799), *Aster amellus* (E5KHT2), *Artemisia annua* (Q6SYC8), and deduced amino acid sequences of *A. millefolium*, *A. wilhelmsii*, *A. vermicularis*. The DNAMAN software was used to generate the alignment. Dots indicate gaps in aligned sequences. The shading colors of the residues corresponding to the level of similarity. The conserved regions are indicated by A, B, C, and D letters.

Discussion

In the present study, we isolated and characterized partial squalene synthase (*SQS*) genes from the leaves of three *Achillea* species for the first time. *SQS* catalyzed the assembly of the isoprenoids to produce triterpenoids, in a two-step reaction. The first step is head-to-head condensation of two FPPs to form an intermediate presqualene diphosphate (PSPP), and the second is the rearrangement of the PSPP and its reduction to squalene in the presence of NADPH and Mg^{2+} (Poulter and Rilling, 1981; Poulter, 1990; Kalariya *et al.,* 2021). The sequence analysis of SQS genes in Cucurbitaceae plants revealed highly conserved domains involved in the generation of squalene, e.g., in *Panax ginseng*, three conserved domains (168~183, 202~224, and 280~298) is essential for the two half-reactions catalyzed by SQS (Kim *et al.*, 2011). In eukaryotic organisms, SQSs have 4 conserved domains (A-D) in which some amino acids play a crucial role in the proper function of the enzyme (Gu *et al.*, 1998). Domains B, C, and D are highly conserved while domain A is less conserved (Yan *et al.*, 2003). Domains A, B, and C are responsible for the condensation of two FPPs, tyrosine residue (Y) in domain B is likely to play an important role in this step. Domains A and C presumably interact with Mg^{2+} to build a bridge between diphosphate units in FPP. Several studies have reported that aspartate-rich motifs (DYLED) located in domain C are important for binding the substrate via the Mg^{2+}-bridge and regulating the rate of enzyme activity (Gu *et al.*, 1998; Pandit *et al.*, 2000; Nguyen *et al.*, 2013; Zhang *et al.*, 2018). The sequence of Cucurbitaceae *SQS* genes

showed antiparallel α-helices with two aspartate-rich regions (DXXXD) that formed a catalytic center. The aspartate-rich motifs in Cucurbitaceae SQS were located at residues 77 to 82 (DTVEDD) and 213 to 217 (DYLED) (Qian *et al.*, 2019). Protein blast of the amino acid SQS sequences in three *Achillea* species revealed two regions overlapping with domains B and C.

Phylogenetic analysis of three isolated sequences demonstrated that *A. wilhelmsii* and *A. vermicularis* were much similar to each other and then to *A. millefolium*. These results were following the taxonomic classification of *the Achillea* genus. Considering the Orientalist Classification, species of *A. wilhelmsii* and *A. vermicularis* are located in *the Filipendulinae* section, while *A. millefolium* is classified in a different section called *Millefolium* (APG III, 2009). The results also revealed that *SQS* gene sequences in *Achillea* species were significantly similar to *Artemisia annua* (belong to the same family), which all, along with other dicotyledons were placed in a separate clade from monocotyledons. The same result was obtained through a comparison of the predicted peptides encoded by partial *SQS* genes in the *Achillea* and equivalent SQSs in other organisms. Nguyen *et al.* (2013) reported a similar result on *Glycine max*. Moreover, previous findings recommended that due to different structures of SQS enzymes between mono- and dicot- plants, they are arranged in distinct subgroups (Hata *et al.*, 1997). Moreover, Dhar *et al*. (2013) have shown that the phylogenetic tree of the SQS sequence of different species matched the taxonomic classifications.

Previously evidence demonstrated that the functional mechanism and configuration of SQS enzyme are significantly conserved among eukaryotes and have a close relationship with taxonomic distance (Nakashima *et al.*, 1995). Recently, Zhang *et al.* (2018) identified a gene sequence of the *SQS* which interferes with biosynthesis pathway of celastrol in *Tripterygium wilfordii*. Their result showed that the position of *T. wilfordii* in the Eudicotidea cluster was consistent with the genetic distance in phylogenetic analyses. In addition, low-copy nuclear genes have been recently used as taxonomic markers. In this regard, *Krak et al.* (2012; 2013) used three of these genes including squalene synthase, gamma-glutamylcysteine synthase, and glycine hydroxyl methyltransferase to classify the Asteraceae family at the low taxonomic level. The importance of these kinds of markers is due to the high rate of gene evolution and lack of homogenization at high taxonomic levels (Sang, 2002). In the same direction, the results of the present study showed that the *SQS* sequences can be useful to reveal the taxonomical distance of the *Achillea* species at the genus level. Hence, we suggest further research in this area.

Conclusion

Achillea species are medicinal plants used for their potential health benefits due to the presence of certain compounds, such as phenolic acids, cumarins, and terpenoids. Results of our research showed a fragment of genomic DNA with strong similarity to *the SQS* gene in *Artemisia annua,* encoding the *SQS* gene in *Achillea*. SQS is an enzyme responsible for the flow of carbon from the isoprenoid pathway to the production of terpenoids. Sequence predicting of the transcript and protein of the enzyme based on sequence analysis of the fragments could be used to design primers to study the SQS gene expression of *Achillea* species at different stages of the plant growth or to investigate the effect of environmental stress conditions on the production of terpenoid compounds in this plant in future.

Conflict of interests

The authors declared no conflicts of interest.

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