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RESEARCH ARTICLE

Sequences analysis of the candidate genes involved in artemisinin biosynthetic pathway in *Artemisia annua* plant

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Abstract

Artemisia annua is a medicinal plant that produces artemisinin, which has antimalarial activity. Artemisinin biosynthesis pathway depends on number of genes such as ADS, CPR and CYP71AV1. We studied gene structure of three genes which exhibit ADS gene possess 4 introns and 5 Exons with length 1794bp, CPR has to analyze the sequences of these genes, the CPR gene contains 6 introns and 7 exons with length 1633bp and CYP71AV1gene has 1 intron and 2 Exons with length 1554bp. Cis-acting elements detection in ADS, CPR and CYP71AV1 promoters revealed several of cis-acting elements such W-box , WRKY , TATA-BOX,CAAT-BOX ,IBOX MYC ,EBOX,GATA-BOX, which could be involved in gene expression regulation of artemisinin production genes. Our genes could be influenced by different external affects as light, cold, drought and elicitors which in turn may be due to an effect on the amount of produced artemisinin. ADS, CPR and CYP71AV1 proteins evolution shows *Artemisia annua*, and *Tanacetum cinerariifolium* always are closed in one cluster which refers to a common ancestor of those two genotypes.

Key words: *Artemisia annua; Cis*-acting elements; Artemisinin,; Evolution.

Introduction

Artemisia annua is an ancient herb used in Chinese medicine for diseases treatment (Abdin et al., 2003). Artemisinin is produced in *Artemisia annua* plant during development as anti-malaria drug against strains of *Plasmodium falciparum* (Newton and White, 1999, Weathers et al., 2011, and Navrátilová and Patočka, 2012). Artemisinin is producing in cytosol and plastid through a biosynthetic precursor, isopentenyl diphosphate (IPP) which is produced in cytosol and plastid by two independent pathways (Croteau et al., 2000). The first pathway of IPP biosynthesis is in cytosol, the mevalonate pathway from acetyl CoA as a biosynthetic precursor, and another

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pathway is in plastid and the biosynthetic precursor in this pathway is pyruvate (Weathers et al., 2006). The fundamental pathways regulation for artemisinin production (Dai et al., 2010), and artemisinin synthesis regulation have been studied (Maes et al., 2011). The genes artemisinic aldehyde reductase, amorpha-4,11-diene synthase (ADS), and aldehyde dehydrogenase amorphadiene12-hydroxylase, of the artemisinin biosynthetic pathway exhibit over expression mainly in trichomes of young leaves and flower buds comparing with mature leaves, other vegetative parts in plant (Zhang et al., 2008; Olofsson et al., 2011). The genes involved in artemisinin pathway are expressed in every secretory trichome cells (Olofsson et al., 2012). Cytochrome P450 monooxygenase (CYP71AV1) is a principal enzyme in the biosynthesis pathway in artemisinin production, while cytochrome p450 reductase (CPR) is fused to redox partner enzyme for CYP71AV1 (Shen et al., 2012). Transcription factors and motifs play necessary role in gene expression and regulation in plant. The presence of cis-acting elements in plant genome is controlling gene expression level through interactions between regulatory proteins. The function of genes can be predicted and specified by action and expression of specialized motifs or cis-acting elements. The regulatory elements involved in regulation process which are classified according to their sequences. All motifs are binding to certain sequences which are called as cis-acting elements or DNA binding domains (Mubeen et al., 2018). Consequently, this study aims to have the knowledge about the structure of genes involved in artemisinin biosynthesis pathway, and Cis-acting elements which could be necessary for gene regulation and how does they controlled?. that in addition to know the evolution of these genes Which could indicate other Genus close to the artemisia plant, which may contain artemisinin.

Materials and methods

Gene structure

The nucleotide (Genomic DNA and mRNA); amino acid and promoter sequences of amorpha-4,11-diene synthase (ADS), cytochrome p450 reductase (CPR) and Cytochrome P450 monooxygenase (CYP71AV1) genes were obtained from the NCBI (http://www.ncbi.nlm.nih.gov) for the purpose of performing bioinformatics analyzes. To study gene structure the https://www.ebi.ac.uk/Tools/msa/clustalw2/ site was used to determine the exons and introns, and their lengths and location within the gene

Evolution analysis

To study the evolution of ADS, CPR and CYP71AV1 proteins which are involved in artemisinin synthesis pathway, the software MEGA7 (Kumar et al., 2016) was used to create the phylogenetic tree by using the Neighbor-Joining method.

Cis-acting elements

Promoter analysis for cis-acting regulatory DNA elements prediction was carried out by using the Signal

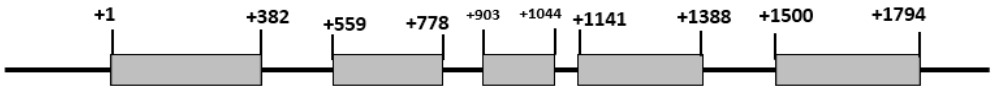
Scan program at
http://www.dna.affrc.go.jp/PLACE/

Results and Discussion

Analysis of open reading frame (ORF) genes involved in the artemisinin biosynthetic pathway.

To study the structure gene, we had sequence from NCBI site for ADS, CPR, CYP71AV1 genes. The ADS gene contains 4 introns and 5 Exons as shown in Fig. 1. the introns lengths are (177bp, 125bp, 97bp and 112bp) with positions (+382 to +559, +778 to +903, +1044 to +1141 and +1388 to +1500) respectively as shown in the (table1). Moreover, the Exon lengths are (382bp, 219bp, 141bp, 247bp and 293bp) with position (+1 to 382, +559 to +778, +903 to +1044, +1141 to +1388, and +1500 to +1794) respectively. However, the length of this gene is 1794bp which is transcript to 1283bp for mRNA CYP71AV1 gene contains 1 intron and 2 Exons (Figure 3 and table 3), the intron length is (177bp) with position (+721 to +988). The Exons lengths are (721bp and 566bp) with positions (+1 to 721 and +988 to +1554) respectively Moreover the length of this is 1554bp which is transcript to 1256bp for mRNA

Figure 1: The artemisinin ADS gene structure of Open Reading Frame. The gray boxes refer to exons and the black lines between boxes refer to introns.



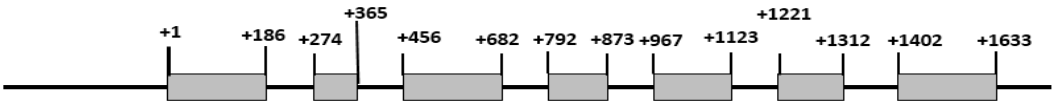


Figure 2: The artemisinin CPR gene structure of Open Reading Frame. The gray boxes refer to exons and the black lines between boxes refer to introns.

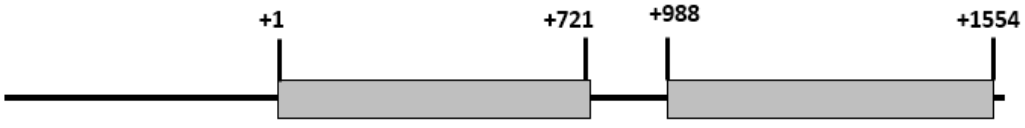


Figure 3: The artemisinin CYP71AV1 gene structure of Open Reading Frame. The gray boxes refer to exons and the black line between the two boxes refer to intron.

Table 1: analysis of open reading frame (ORF) from ADS gene.

Genes	Total Introns	Intron No.	Intron length (bp)	Position intron	Total exons	Exon No.	Exon length (bp)	Position exon	Gene length (bp)	mRNA length (bp)
ADS	4	1	177	+382 to +559	5	1	382	+1 to +382	1794	1283
		2	125	+778 to +903		2	219	+559 to +778		
		3	97	+1044 to +1141		3	141	+903 to +1044		
		4	112	+1388 to +1500		4	247	+1141to +1388		
		-	-	-		5	294	+1500 to +1794		

Table 2: analysis of open reading frame (ORF) from CPR gene.

Gene	Total Introns	Intron No.	Intron length (bp)	Position intron	Total exons	Exon No.	Exon length (bp)	Position exon	Gene length (bp)	mRNA length (bp)
CPR	6	1	88	+186 +274	7	1	186	+1 to +186	1633	1067
		2	91	+365+456		2	91	+274 +365		
		3	110	+682+792		3	226	+456 +682		
		4	94	+873+967		4	81	+792 +873		
		5	98	+1123 +1221		5	156	+967 +1123		
		6	90	+1312+1402		6	91	+1221 +1312		
						7	231	+1402 +1633		

Table 3: analysis of open reading frame (ORF) from CYP71AV1gene.

Gene	Total Introns	Intron No.	Intron length (bp)	Position intron	Total exons	Exon No.	Exon length (bp)	Position exon	Gene length (bp)	mRNA length (bp)
CYP 71AV1	1	1	177	+721 to +988	2	1	721	+1 to +721	1554	1256
						2	566	+988 to +1554		

Cis-acting elements analysis of genes involved in artemisinin pathway:

Further sequence analysis of the putative *cis* acting Elements was performed using The site "PLACE Web Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalscan .html) promoter databases, and manually search were used for the identification of Cis-acting elements in 1 kb upstream sequence from the starting ATG of the ADS,CYP71AV1 and CPR genes The analysis reveals at least 8 cis-acting elements , The elements are w-box , WRKY , TATA BOX,CAAT BOX ,IBOX MYC ,EBOX,GATA BOX, These cis acting elements detected in ADS, CPR and CYP71AV1 promoter sequences as shown in table 4, 5 and 6).

Table 4: Cis-regulatory elements prediction in the 1-kb ADS promoter fragment.

ID	Position	Sequence	Function
TATABOX	413 (+) 932 (+)	TATATAA	TATA BOX a part of promoter element around -30 to starting transcription (Wang et al., 2013b)
W-box ATNPR1	161 (+) 169 (+) 174 (+) 774 (+) 992 (+)	TGACT/ TGACY	W-box is a binding site of (WRKY) transcription factor (Nishiuchi et al., 2004)
WRKY71OS	161 (+) 169 (+) 174 (+) 774 (+) 993 (+)	TGAC	The WRKY are transcription factors which is binding to the W-box of the ADS promoter to regulate artemisinin biosynthesis (Schluttenhofer and Yuan, 2015; Phukan et al., 2016; Zhang et al., 2016)
MYCCONSENSUSAT or E-BOX	239 (+) 487 (+) 500 (+) 705 (+) 808 (+) 833 (+) 921 (+)	CANNTG	cis-acting regulatory element involved in light responsiveness, cold, drought and freezing induction (Hartmann et al., 2005; Wang et al., 2013b).
CAAT BOX	68 (+) 98 (+) 210 (+) 514 (+) 659 (+)	CAAT	CAAT-box is a common promoter element that controlling of temporal and spatial gene expression (Chowdhary et al., 2010)
GATA BOX	42 (+) 193 (+) 244 (+) 475 (+)	GATA	Light responsive element **(**Zhang et al., 2015)
IBOX	193 (+)	GATAA	Light responsive element (William et al.,1995)

Table 5: Cis-regulatory elements prediction in the 1-kb CPR promoter fragment.

ID	Position	Sequence	Function
w-box WBOXNTERF3	708 (+) 708 (+)	TGACT	W-box is a binding site of (WRKY) transcription factor (Nishiuchi et al., 2004)
WRKY	708 (+)	TGAC	The WRKY are transcription factors which is binding to the W-box of the ADS promoter to regulate artemisinin biosynthesis (Schluttenhofer and Yuan, 2015; Phukan et al., 2016; Zhang et al., 2016)
GATABOX	903 (+) 226 (+) 250 (+) 305 (+) 660 (+) 903 (+)	GATA	Part of a light responsive element (Zhang et al., 2015)
IBOXCORE	11 (+) 226 (+) 250 (+) 903 (+)	GATAA	Light responsive element (William et al.,1995)
MYCCONSENSUSAT or E-BOX	445 (+) 451 (+) 691 (+) 824 (+)	CANNTG	cis-acting regulatory element involved in light responsiveness, cold, drought and freezing induction (Hartmann et al., 2005; Wang et al., 2013b).
CAATBOX	76 (+) 169 (+) 542 (+) 756 (+) 756 (+) 868 (+) 948 (+)	CAAT	CAAT-box is a common promoter element that controlling of temporal and spatial gene expression (Chowdhary et al., 2010)
TATABOX	29 (+)	TATAAAT	TATA BOX a part of promoter element around -30 to starting transcription (Wang et al., 2013b)

Table 6: Cis-regulatory elements prediction in the 1-kb Cyp71AV1 promoter fragment.

ID	Position	Sequence	Function
w-box WBOXATNPR1	59 (+) 60 (+) 141 (+) 512 (+) 553 (+)	TTGAC TGACT TTGAC	W-box is a binding site of (WRKY) transcription factor (Nishiuchi et al., 2004)
WRKY	60 (+) 142 (+) 225 (+) 513 (+) 554 (+)	TGAC	The WRKY are transcription factors which is binding to the W-box of the ADS promoter to regulate artemisinin biosynthesis (Schluttenhofer and Yuan, 2015; Phukan et al., 2016 and Zhang et al., 2016)
MYCCONSENSUSAT or E-BOX	443 (+) 509 (+) 582 (+) 666 (+) 675 (+) 814 (+)	CANNTG	cis-acting regulatory element involved in light responsiveness, cold, drought and freezing induction (Hartmann et al., 2005; Wang et al., 2013b).
GATABOX	796 (+)	GATA	Part of a light responsive element (Zhang et al., 2015)
I-BOX	796 (+)	GATAA	Light responsive element (William et al.,1995)
TATA-BOX	28 (+) 73 (+)	TATAAAT	TATA BOX a part of promoter element around -30 to starting transcription (Wang et al., 2013b)
CAAT-BOX	52 (+) 311 (+) 352 (+) 420 (+) 463 (+) 487 (+) 539 (+)	CAAT	CAAT-box is a common promoter element that controlling of temporal and spatial gene expression (Chowdhary et al., 2010)

These elements which are found in our genes promoter under study could play a role in gene regulation in response as a response to external and internal influences. ADS gene is one of the most important genes for artemisinin production. Different studies have suggested that the artemisinin biosynthesis is regulated by the transcription factors, which bind on their promoters specially at the cis-acting elements (Ptashne and Gann

1997; Hong et al. 2009). These elements include W-box, CAAT-box, 5_-UTR py- rich stretch, light responsive elements and TATA-box sequences. The promoter boxes also resulted in the discovery of several cis-elements including light responsive elements (Zhang et al., 2015) such as I-BOX and GATA-BOX these are light responsive elements and the I-box Conserved sequence upstream of light-regulated genes of both monocots and dicots. But E-box is regulatory element involved in light responsiveness, cold and freezing induction (Wang et al., 2013b). TATA-box is a well-conserved core promoter element usually located about 25–32 bp upstream of the Transcription Start Site (TSS) in eukaryotes. Moreover, a putative TATA-box was predicted at the positions of -36 to -28 (ATTATAATA) of the ALDH1 promoter, (Liu et al., 2016). The predicted transcription start site (TSS, +1), located at 30 bp upstream of the ATG start codon (Zhang et al., 2015), so the TATA BOX cis acting elements a part of promoter element around -30 to starting transcription. On the other hand, CAAT-boxis another important cis-acting element, which is considered to be the binding site for the RNA polymerase. (Liu et al., 2016) CAAT-box (CAAT) was also found at +52 to +539 upstream of the putative Transcription Start Site (TSS) of CYP71AV1 promoter so CAAT-BOX includes the starting point of transcription start site is indicated (Wang et al., 2013a). Moreover, W-box is having 2 types (HVISO1/NTERF3) (Wang et al., 2011, 2012; Yang et al., 2015) which are the binding sites of (WRKY) transcription factor, WRKY was binding to the W-box of the ADS promoter to regulate artemisinin biosynthesis (Nishiuchi et al., 2004) MYB recognition site -binding site and W-box recognizing transcription factors such as WRKY to binding with w-box to starting transcription (Liu et al., 2016). Our genes could be enhanced by different external influences as light, cold, drought and elicitors which in turn may be due to an effect on the amount of artemisinin produced.

Evolution of genes involved in Artemisinin pathway

The ADS evolution: To study evolution relationship of ADS genes from *A. annua*, and *Tanacetum cinerariifolium* and synthetic construct (sequences found in NCBI site). The phylogenetic tree for ADS amino acid sequences by Neighbor-Joining method revealed of two clusters (figure 4). The first cluster contains *A. annua*, and *Tanacetum* cinerariifolium but the synthetic construct ADS is found in a separated cluster.

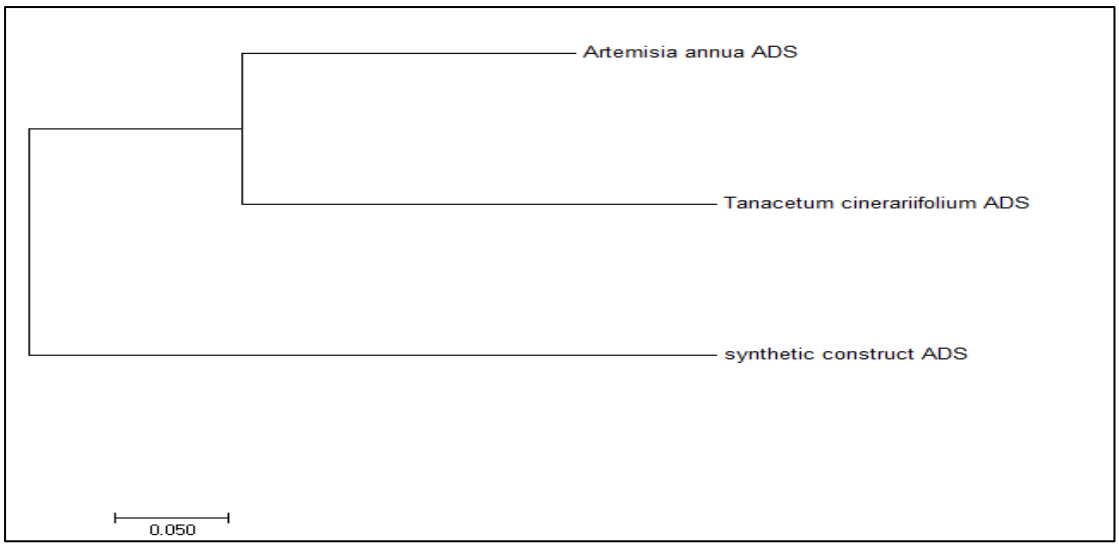


Figure 4: Phylogenetic tree of amino acid sequences for ADS gene from *Artemisia annua*, *Tanacetum cinerariifolium* and synthetic construct.

The CPR evolution

The phylogenetic tree for CPR amino acid sequences of 6 deferent genotypes, *Artemisia annua, Tanacetum cinerariifolium, Matricaria chamomilla var recutita, Salvia miltiorrhiza, Sesamum radiatum* and *Marrubium vulgare* appeared into two separated clusters (figure 5). In the first cluster includes *A. annua* and *Tanacetum cinerariifolium* were included *Matricaria chamomilla* var recutita, while the second cluster composed of synthetic construct.

The CYP71AV1 evolution

The phylogenetic tree for CYP71AV1 amino acid sequences of three deferent genotypes, *Artemisia annua, Tanacetum cinerariifolium* and *lactuca sativa* were included in two clusters (figure 6). *Artemisia annua* and *Tanacetum cinerariifolium* are in a cluster While, *lactuca sativa* is in another cluster. The presence of *Artemisia annua,* and *Tanacetum cinerariifolium* in one cluster for ADS, CPR and CYP71AV1 that means these genotypes belongs to one ancestor. The sequences of our proteins are used for evolution relationship analysis and for phylogenetic trees construction. Sequences of DNA and RNA exposing to change over evolutionary time resulting through mutations, deletions or insertions, the genes translated products will also have evolution changes (Opperdoes and Lemey, 2018). phylogenetic clustering could be helpful into research questions concerning the identification of common ancestor (Han et al., 2019). *Artemisia annua,* and *Tanacetum cinerariifolium* always are present in the same cluster which refers to a common ancestor of those two genotypes.

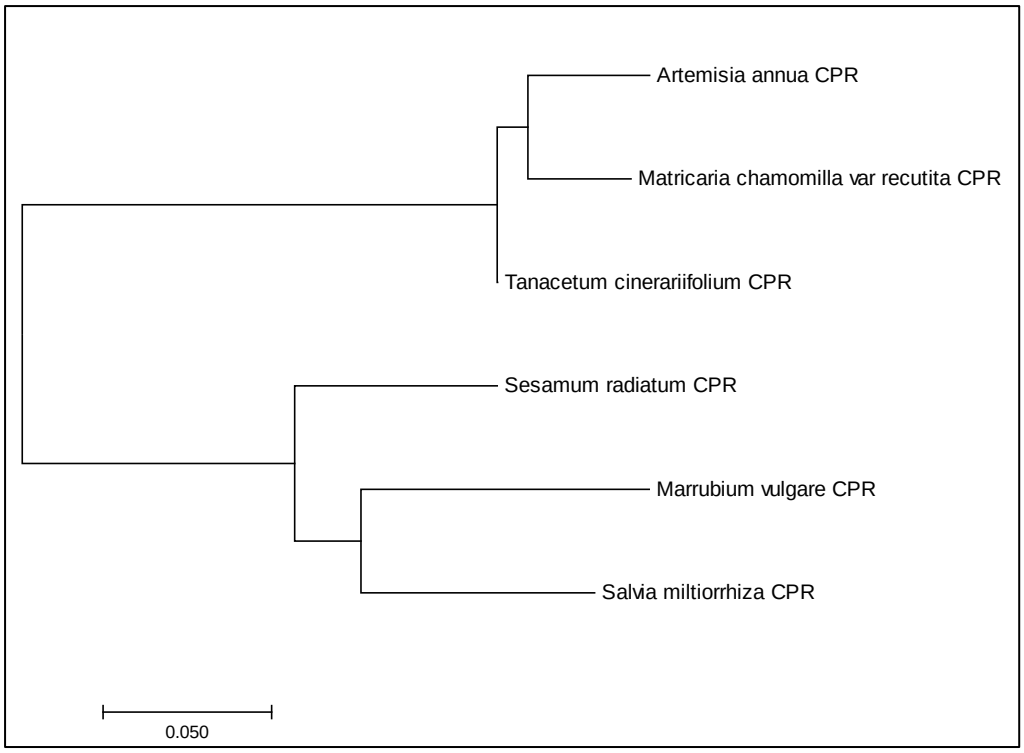


Figure 5: Phylogenetic tree of amino acid sequences for ADS gene from *Artemisia annua*, *Tanacetum cinerariifolium*, *Matricaria chamomilla var recutita*, *Salvia miltiorrhiza*, *Sesamum radiatum* and *Marrubium vulgare*.

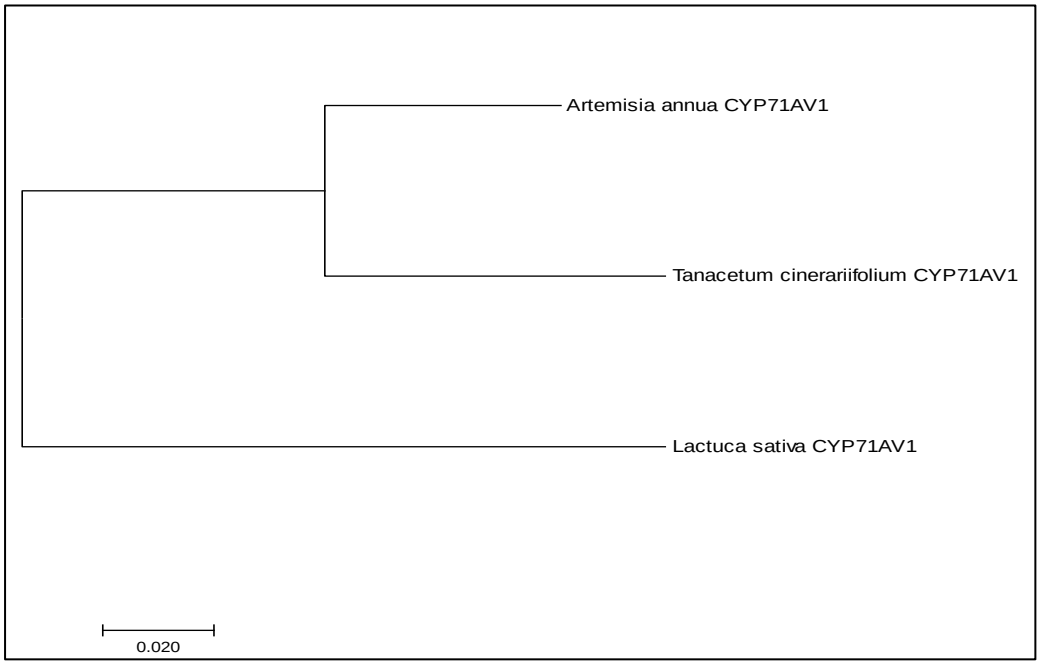


Figure 6. Phylogenetic tree of amino acid sequences for CYP71AV1gene from *Artemisia annua*, *Tanacetum cinerariifolium* and *lactuca sativa*

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