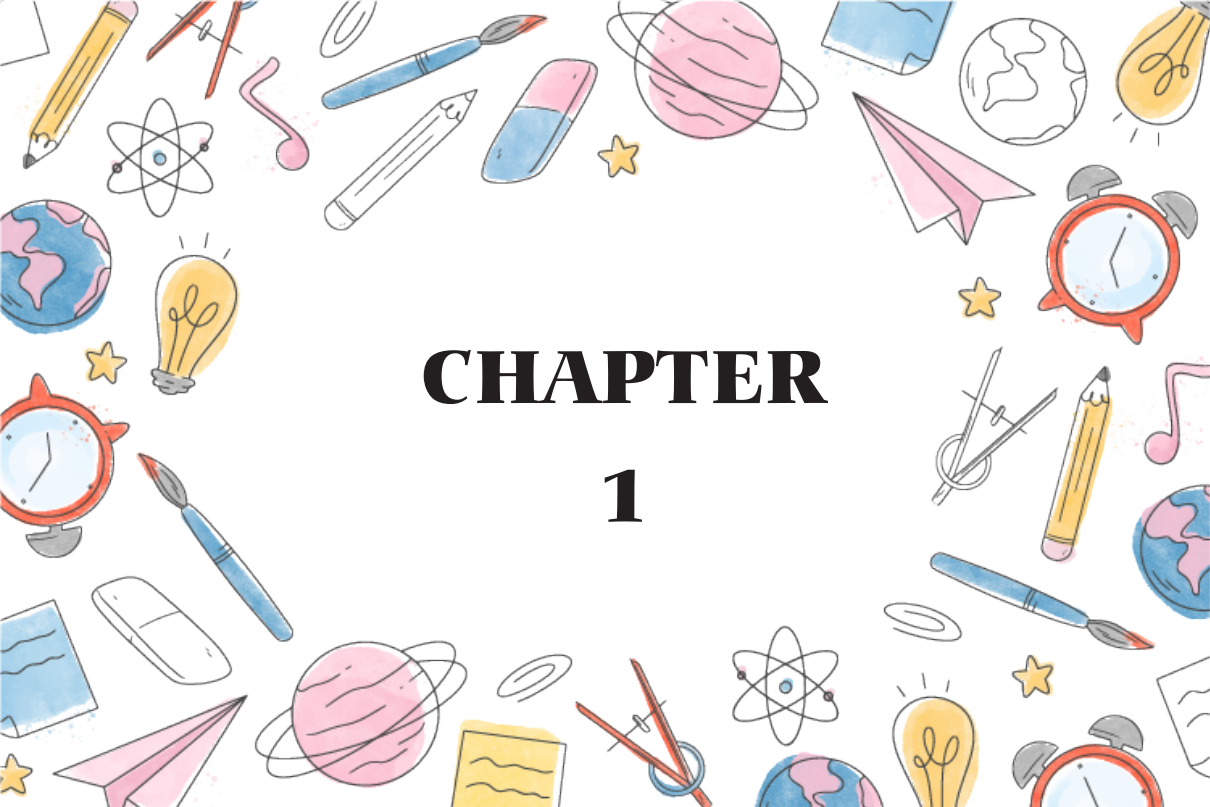




CHAPTER 1

EXPLORATION OF SECONDARY METABOLITE BIOSYNTHETIC GENE CLUSTERS IN *HUMULUS* *LUPULUS* L.: PHYLOGENETIC AND STRUCTURAL INSIGHTS INTO KEY ENZYMES

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1. Introduction

Secondary metabolites produced by plants are compounds synthesized under stressful conditions and found in the composition of important drugs for human health. (Lv et al., 2024). Active ingredients derived from medicinal plants are directly or indirectly present in the composition of many drugs produced, with approximately 80% of the world's population relying on herbal medicines and plant-based formulations for treating their illnesses (Jeyasri et al., 2023). These metabolites possess various effects such as antiviral, anti-inflammatory, antioxidant, antiestrogenic, anticancer, cardioprotective, antimalarial, etc. (Yeshe et al., 2022). Secondary metabolites containing groups such as terpenoids, alkaloids, flavonoids, and lignans have various uses beyond pharmacological applications, including fragrance, food additives, agrochemicals, and biopesticides (Anjali et al., 2023).

Since the yield of secondary metabolites depends on numerous factors, the biosynthesis of secondary metabolites is a complex process, and in this process, there are pathways such as the terpene pathway, phenylalanine pathway, alkaloid pathway, shikimate pathway (Li et al., 2024). These amino acids, such as tyrosine, phenylalanine, and tryptophan, which are the building blocks of protein synthesis, are produced in the shikimate pathway. Tyrosine serves as a precursor for compounds like pigment betalains, quinones, and isoquinoline alkaloids, phenylalanine for flavonoids, lignans, and phenylpropanoid/benzenoid volatile compounds, and tryptophan for phytoalexins, alkaloids, auxins, and indole glucosinolates (Jan et al., 2021). Terpenes are categorized into groups such as monoterpenes, diterpenes, triterpenes, sesquiterpenes, while flavonoids are examined in six groups: flavanones, flavonols, flavanols, flavones, isoflavonoids, and anthocyanidins (Li et al., 2024). Biochemical and genetic research indicates that the genes involved in the biosynthesis of these important compounds are not randomly distributed but are located adjacent to each other in metabolic gene clusters (Li et al., 2021). Thus, pathways are co-regulated, and the production of localized metabolites is facilitated (Kerwin et al., 2024). The identification of secondary metabolite biosynthetic gene clusters is important for drug discovery efforts, as it enables the understanding of the biosynthesis of natural compounds with anticancer, antibiotic, and antifungal properties (Thokchom et al., 2024). Tools such as 2metDB, antiSMASH, BAGEL, CLUSEAN, ClusterFinder, eSNaPD, EvoMining, GNP/Genome Search, GNP/PRISM, MIDDAS-M, MIPS-CG, NaPDoS, and SMURF have been developed to reveal secondary metabolite gene clusters (Weber and Kim, 2016). The plant-specific version of the antiSMASH program, known as plantiSMASH, is software that utilizes Hidden Markov Models (pHMMs) and the CD-HIT clustering method to predict

enzyme families involved in secondary metabolite biosynthetic pathways and to identify genomic regions encoding specialized metabolic enzymes from multiple different subfamilies (Kautsar et al., 2017).

Humulus lupulus L., a member of the Cannabaceae family, is a perennial plant cultivated in countries such as Germany, the United States, England, and the Czech Republic (Lyu et al., 2022). The vast majority of cultivated *H. lupulus* worldwide (approximately 98%) is used in beer production (to provide flavor, aroma, and bitterness), while a very small portion is utilized for pharmaceutical purposes such as cosmetics production, dietary supplements, and phytotherapy (Contin et al., 2023). Lupulin is secreted from the glandular hairs of mature unfertilized cones of female plants, containing α - and β -acids (bitter acids), essential oils, and prenylf-lavonoids (Tegopoulos et al., 2023). The main flavor compounds derived from *H. lupulus* are reported as 4-methyl-4-sulfanylpentan-2-one, 3-sulfanyl-4-methylpentyl acetate, 3-sulfanyl-4-methylpentan-1-ol, 3-sulfanylhhexan-1-ol, 3-sulfanylpentan-1-ol, hexanal, hexanol, 2-Nonanol, ethyl butanoate, ethyl 2-methyl butanoate, 2-Undecanone, γ -Octalactone, Eugenol, β -Caryophyllene, α -pinene, Camphene, D,L-limonene, β -Selinene, 2-Methylbutanal, 2,3-Butanedione, β -pinene, α -humulene, (E)-4-Hep-tenal, β -Myrcene, Nonanal, Nerol, α -Copaene, 3-methylthiopropenal, 2-sulfanylethyl acetate and 2-sulfanylethan-1-ol (Sun et al., 2022). The most important components in determining the quality of *H. lupulus* are α -acids, which contribute to the bitter taste of beer. In addition, volatile oils containing volatile components such as monoterpenes, sesquiterpenes, and oxidized alkanes also make significant contributions to the beer aroma (Contin et al., 2023). α ve β caryophyllenes and β -myrcene are significant components found in the volatile oil of this species, with α -caryophyllene referred to as α -humulene. Additionally, the components found in *H. lupus* have important medical significance beyond imparting beer flavor (López et al., 2023). *H. lupulus* possesses antiviral, antimicrobial, antifungal, antioxidant, anti-inflammatory, anticancer, antidiabetic, neuroprotective, cardioprotective, and various other effects (Astray et al., 2020). The effects of some compounds found in *H. lupulus* are as follows: xanthohumol exhibits antioxidant and antimicrobial effects, while humulones such as adhumulone, humulone, and cohumulone, as well as lupulones like adlupulone, lupulone, and colupulone, also possess antimicrobial properties. Additionally, iso- α -acids are particularly effective against Gram-positive bacteria (Flieger et al., 2024). Furthermore, in another research (Tamia et al., 2024), it is mentioned that there is a potential for xanthohumol and tetrahydroxanthohumol to play a role in preventing human colorectal cancer. In another article (Herzog et al., 2024), it was reported that compounds such as xanthohumol and isoxanthohumol inhibit the replication of various

viruses including cytomegalovirus, influenza virus, and human immunodeficiency virus. In the biosynthesis of secondary metabolites in *H. lupulus*, critical enzymes such as branched-chain amino transferase 1, branched-chain amino transferase 2, valerophenone synthase, and chalcone synthase, along with various prenyltransferases and other oxidases, function within organelles like the chloroplast and mitochondria to direct the production of different secondary metabolites, contributing significantly to the plant's chemical profile (Eriksen et al., 2021). Volatile terpenoids and isoprenoids in *H. lupulus* are synthesized through enzymatic pathways involving several key enzymes. Geranyl diphosphate (GPP) is produced by GPP synthase and then converted to beta-myrcene by monoterpene synthase or to farnesyl diphosphate (FPP) by squalene/phytoene synthase. FPP is further transformed into sesquiterpenes such as humulene and caryophyllene by sesquiterpene synthase 1. Additionally, linalool is synthesized from GPP by S-linalool synthase (Eriksen et al., 2022).

There are signature and tailoring enzymes involved in secondary metabolite biosynthesis (Chavali and Rhee, 2018). BAHD acyltransferases play significant roles in aroma formation, plant growth and development, and responses to biotic and abiotic stress conditions; CYPs are crucial for plant growth and development and biotic and abiotic stress tolerance; and terpene synthases are key in terpenoid formation, while the secondary metabolites produced by these enzymes play an important role in the treatment and prevention of diseases (Chakraborty et al., 2023; Chen et al., 2021; Qiao et al., 2024). Due to their significant roles, the current study has focused on these categories of enzymes. The aim of this study is to identify the secondary metabolite biosynthetic gene clusters in *H. lupulus* and their distribution on chromosomes, to determine the enzymes present in these gene clusters, and subsequently to elucidate the phylogeny and protein structures of the important gene categories, including BAHD acyltransferases, CYPs, and TPSs, using bioinformatics tools. The data from this study could contribute to identifying effective targets for anticancer, antimicrobial, and antiviral agents in drug discovery and development, and also provide insights into developing innovative approaches to enhance plant productivity and improve plant protection strategies in agricultural practices.

2. Materials and methods

Humulus lupulus was searched for in the NCBI database. drHumLupul.1, whose Reference Sequence was provided, has been selected. Afterwards, amino acid sequences belonging to the chromosomes of this species were downloaded in FASTA format. The obtained data was uploaded to the plantiSMASH program (Kautsar et al. 2017) for secondary metabolite

biosynthetic gene cluster analysis, and the resulting outcomes were downloaded and separately documented. The enzyme categories within these gene clusters were saved in Microsoft Excel, and the gene clusters present in each chromosome were illustrated as figures after necessary adjustments. Subsequently, the locations of secondary metabolite gene clusters from plantiSMASH and the chromosome lengths of *H. lupulus* chromosomes obtained from NCBI were uploaded to MG2C (MapGene2Chromosome) v2.1 software (Chao et al., 2021). The positions of the gene clusters on the chromosomes were then plotted using this software. After the analysis of secondary metabolite biosynthetic gene clusters, enzymes belonging to the BAHD acyltransferase, Cytochrome 450, and Terpene synthase categories were copied into separate Microsoft Excel files, each with individual pages. For each enzyme, an “Enzyme ID” was defined according to the chromosome order. Subsequently, amino acid sequences corresponding to these enzymes were downloaded from NCBI, and they were transferred to the MEGA11 program (Tamura et al., 2021) to construct phylogenetic trees. After importing the sequences into the MEGA11 program, multiple sequences were aligned using MUSCLE. The aligned file was then used to construct a phylogenetic tree employing the Maximum Likelihood Tree method with bootstrap analysis for 1000 replicates. The phylogeny was reconstructed utilizing the Jones-Taylor-Thornton substitution model. After obtaining the results, they were exported in Newick format. Subsequently, these data were uploaded to the Interactive Tree Of Life (iTOL) v6.7.3 software (Letunic and Bork, 2021). After coloring the groups in the phylogenetic tree differently and making necessary adjustments, the tree was downloaded for use in the article. As the final analysis, protein homology modeling was performed. As the final analysis step, protein homology modeling was conducted using the Phyre2 (Protein Homology/analogy Recognition Engine V 2.0) software (Kelley et al., 2016). Amino acid sequences were uploaded into the software, and the resulting outputs were downloaded as individual files. Subsequently, figures were created by assembling these results, grouping together the proteins belonging to the enzyme categories focused on in this study within three distinct figures.

3. Results

Humulus lupulus is a dioecious medicinal plant with a chromosome count of $2n = 18 + XX/XY$ (Olatoye et al., 2023). The analysis of secondary metabolite biosynthetic gene clusters has revealed gene clusters on nine chromosomes. These clusters' positions are illustrated in fig. 1-9. Detailed features are indicated in Table 1. In addition, the characteristics of enzyme categories found in each cluster are provided in Supplementary Table 1.

The analysis revealed two types of secondary metabolite biosynthetic gene clusters on chromosome 1, including saccharide and alkaloid clusters (Fig. 1). Cluster 1 contains the enzyme categories Glycosyltransferase and Lipoxigenase, while Cluster 2 includes CoA-ligase and Strictosidine synthase-like enzymes.

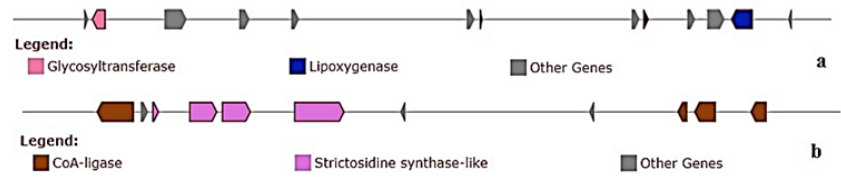


Fig. 1 a) Cluster 1 – Saccharide, b) Cluster 2 – Alkaloid

On chromosome 2, saccharide-polyketide and saccharide-type gene clusters were found (Fig. 2). Cluster 1 encompasses enzymes categorized as Ketosynthase, Glycosyltransferase, and Epimerase, while Cluster 2 features Glycosyltransferase and Methyltransferase enzymes.

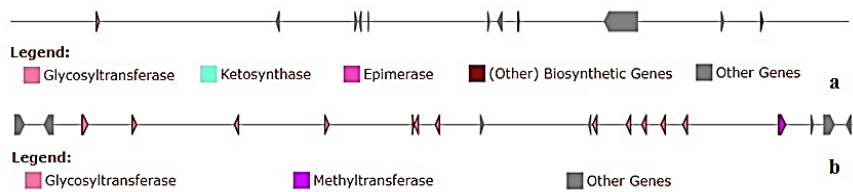


Fig. 2 a) Cluster 1- Saccharide-Polyketide, b) Cluster 2- Saccharide

The secondary metabolite gene clusters identified on chromosome 3 are as follows: Cluster 1, alkaloid; Cluster 2, terpene; and Cluster 3 is putative (Fig. 3). When examined in terms of enzymes, Cluster 1 contains Cytochrome P450 and Pictet-Spengler enzyme (Bet v1), while Cluster 2 features BAHD acyltransferase, Epimerase, and Prenyltransferase enzymes. Additionally, in Cluster 3, enzymes categorized as Oxidoreductase, Cytochrome P450, Methyltransferase, and Dioxygenase have been observed.

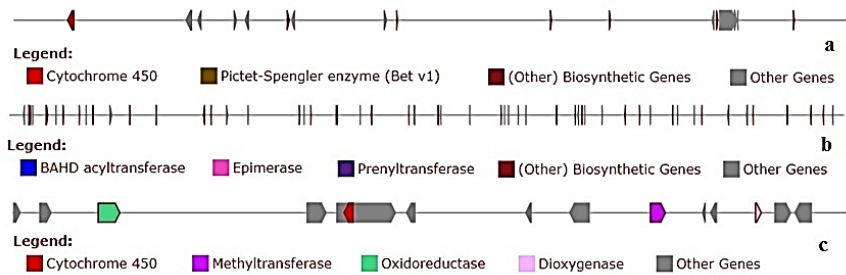


Fig. 3 a) Cluster 1- Alkaloid, b) Cluster 2- Terpene, c) Cluster 3- Putative

Three secondary metabolite biosynthetic gene clusters have been identified on chromosome 4, as depicted in Fig. 4. In Cluster 1, the enzyme categories Glycosyltransferase, Cytochrome 450, and Terpene synthase have been observed. The enzyme categories Amino oxidase, Methyltransferase, Terpene synthase, Epimerase, and Cytochrome 450 have been detected in Cluster 2. Additionally, Cluster 3 contains enzymes belonging to the Epimerase, Prenyltransferase, and Cytochrome 450 categories.

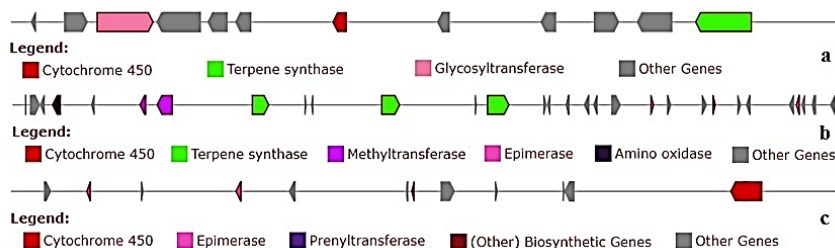


Fig. 4 a) Cluster 1- Saccharide-Terpene, b) Cluster 2- Terpene, c) Cluster 3- Terpene

Fig. 5 displays six secondary metabolite biosynthetic gene clusters located on chromosome 5. Cluster 1 contains enzymes belonging to the categories of BAHD acyltransferase, CoA-ligase, Glycosyltransferase, and Aminotransferase, while Cluster 2 has enzymes categorized as Glycosyltransferase, Epimerase, and Amino oxidase. Additionally, in Cluster 3, BAHD acyltransferase, Dioxygenase, and Squalene epoxidase enzymes are observed, while Cluster 4 contains Cytochrome 450, Copper amine oxidase, and Epimerase enzymes. Furthermore, Cluster 5 exhibits Dioxygenase and Glycosyltransferase enzymes. Moreover, in Cluster 6, Glycosyltransferase, BAHD acyltransferase, Squalene epoxidase, and Dirigent enzymes have been identified.

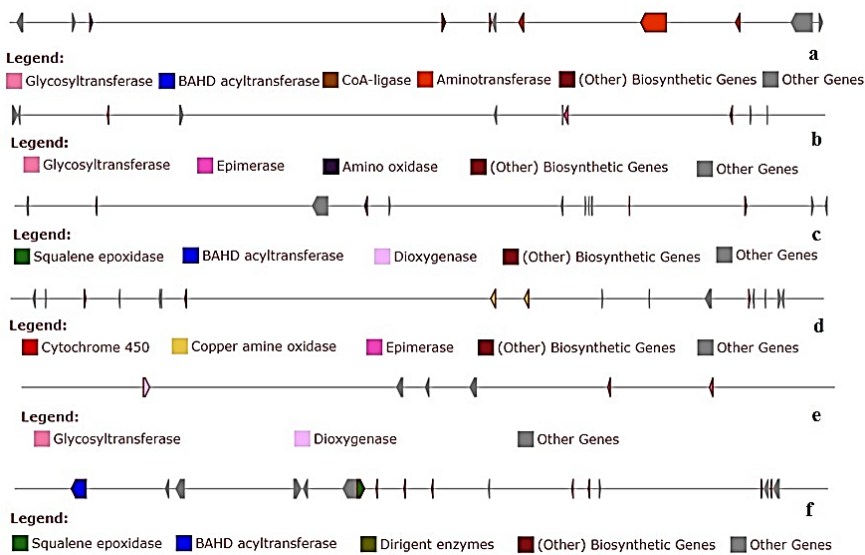


Fig. 5 a) Cluster 1- Saccharide, b) Cluster 2- Saccharide, c) Cluster 3- Putative, d) Cluster 4- Alkaloid, e) Cluster 5- Saccharide, f) Cluster 6- Lignan

On chromosome 6, there are five types of secondary metabolite gene clusters: Cluster 1, terpene; Cluster 2, saccharide; Cluster 3, terpene-alkaloid; Cluster 4, terpene; and Cluster 5, terpene, as shown in Fig. 6. The enzyme categories are as follows: In Cluster 1, Prenyltransferase and Dioxxygenase; in Cluster 2, only Glycosyltransferase; in Cluster 3, Epimerase, Strictosidine synthase-like, and Terpene synthase; in Cluster 4, Terpene synthase, Dioxxygenase, and Oxidoreductase; and in Cluster 5, Cytochrome 450 and Terpene synthase.

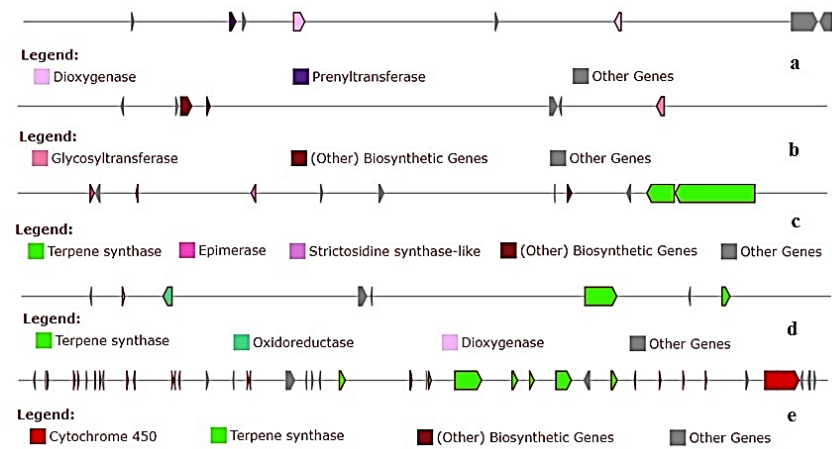


Fig. 6 a) Cluster 1- Terpene, b) Cluster 2- Saccharide, c) Cluster 3- Terpene-Alkaloid, d) Cluster 4- Terpene, e) Cluster 5- Terpene

Gene clusters in saccharide and putative types have been observed on Chromosome 7 (Fig. 7). The enzyme categories are as follows: In Cluster 1, there are Lipoxxygenase, CoA-ligase, and Glycosyltransferase. In Cluster 2, there are Cytochrome P450 and Glycosyltransferase. In Cluster 3, there are Dioxygenase, Methyltransferase, and Glycosyltransferase. In Cluster 4, there are Epimerase, Methyltransferase, and Cytochrome P450.

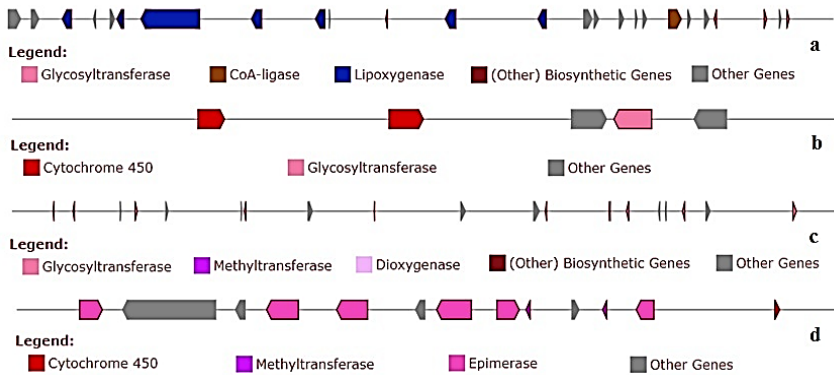


Fig. 7 a) Cluster 1- Saccharide, b) Cluster 2- Saccharide, c) Cluster 3- Saccharide, d) Cluster 4- Putative

Two gene clusters have been identified on Chromosome 8, and both are of putative type (Fig. 8). In Cluster 1, enzyme categories such as Cytochrome P450, BAHD acyltransferase, and Polyprenyl synthetase are observed, while in Cluster 2, enzymes belonging to the Dioxygenase category have been identified.

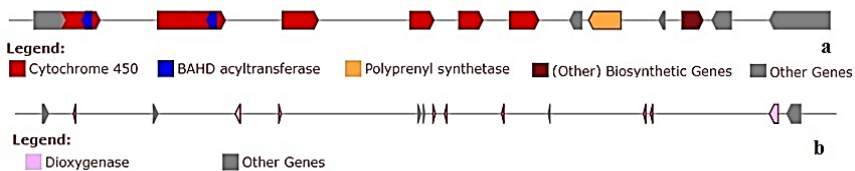


Fig. 8 a) Cluster 1- Putative, b) Cluster 2- Putative

Only one gene cluster has been detected on Chromosome 9 (Fig. 9). The enzyme categories identified are Glycosyltransferase and Methyltransferase.

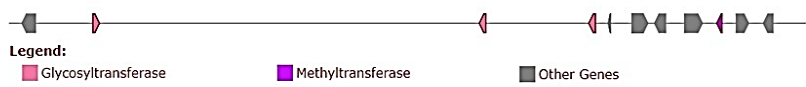


Fig. 9 The saccharide-type secondary metabolite biosynthetic gene cluster on chromosome 9.

Table 1 *The properties of secondary metabolite biosynthetic gene clusters*

Chromosome number	Cluster number	Cluster type	From	To	Size (kb)
1	1	Saccharide	306223098	306371992	148.89
1	2	Alkaloid	310322454	310489570	167.12
2	1	Saccharide -Polyketide	237255984	238052048	796.06
2	2	Saccharide	294351751	294625417	273.67
3	1	Alkaloid	10020514	10499825	479.31
3	2	Terpene	19837312	23296817	3459.51
3	3	Putative	283280608	283461369	180.76
4	1	Saccharide- Terpene	1555745	1651048	95.30
4	2	Terpene	3179103	3758502	579.40
4	3	Terpene	143788104	144710096	921.99
5	1	Saccharide	7918898	8287352	368.45
5	2	Saccharide	141134819	142132108	997.29
5	3	Putative	206414953	207385555	970.60
5	4	Alkaloid	224280354	225092565	812.21
5	5	Saccharide	231162743	231455560	292.82
5	6	Lignan	239914975	240307842	392.87
6	1	Terpene	185875075	186054953	179.88
6	2	Saccharide	191813667	192120658	306.99
6	3	Terpene-Alkaloid	204370108	204918004	547.90
6	4	Terpene	211455392	211855630	400.24
6	5	Terpene	215262367	216254669	992.30
7	1	Saccharide	3324747	3828357	503.61

7	2	Saccharide	7924538	8024402	99.86
7	3	Saccharide	202209864	203082651	872.79
7	4	Putative	207836495	208077060	240.56
8	1	Putative	1453948	1546383	92.44
8	2	Putative	22154396	22558363	403.97
9	1	Saccharide	7169930	7340567	170.64

The distribution of secondary metabolite gene clusters in *H. lupulus* is illustrated in Fig. 10. Analysis revealed that the highest number of gene clusters was observed on chromosome 5, while the lowest was on chromosome 9.

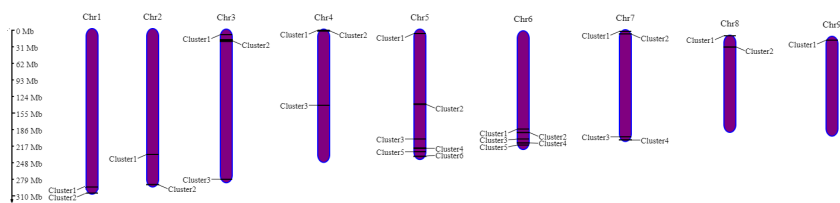


Fig. 10 The chromosomal locations of secondary metabolite biosynthetic gene clusters

Based on the analyses conducted, enzymes were identified in *H. lupulus* across the following categories: Amino oxidase, Aminotransferase, BAHD acyltransferase, CoA-ligase, Copper amine oxidase, Cytochrome P450, Dioxygenase, Dirigent enzymes, Epimerase, Glycosyltransferase, Ketosynthase, Lipoxygenase, Methyltransferase, Oxidoreductase, Pictet-Spengler enzyme (Bet v1), Polyprenyl synthetase, Squalene epoxidase, Strictosidine synthase-like, and Terpene synthase (Supplementary Table 1). The current study focuses on BAHD acyltransferase, Cytochrome P450, and Terpene synthase. In the BAHD acyltransferase category, the following enzymes were observed: stemmadenine O-acetyltransferase-like, vinorine synthase-like, BAHD acyltransferase BIA1-like, deacetyl-vindoline O-acetyltransferase-like, spermidine hydroxycinnamoyl transferase-like, and shikimate O-hydroxycinnamoyltransferase-like. In the Cytochrome 450 category, enzymes such as cytochrome P450 98A2-like, cytochrome P450 71AU50-like, beta-amyrin 28-monooxygenase-like, phenylacetaldehyde oxime monooxygenase CYP71AN24-like, cytochrome P450 CYP736A12-like, flavonoid 3'-monooxygenase-like, cytochrome P450 71D9-like, cytochrome P450 71D10-like, cytochrome P450 71D11-like, desmethyl-deoxy-podophyllotoxin synthase-like, cytochrome P450 87A3-like, cytochrome P450 714C2-like, protein LUTEIN DEFI-

CIENT 5, chloroplastic, cytochrome P450 94C1-like, cytochrome P450 72A397-like isoform X2, and cytochrome P450 CYP72A219-like were observed. In the Terpene synthase category, the enzymes cycloartenol synthase 2, cycloartenol synthase-like, beta-amyrin synthase-like, alpha-humulene synthase-like, and lupeol synthase-like were identified (Supplementary Table 2).

After identifying the mentioned enzymes in *H. lupulus*, a phylogenetic analysis was conducted, and a phylogenetic tree was constructed. As a result of the phylogenetic analysis, while only terpene synthases were observed in Group I and only BAHD acyltransferases in Group II, Cytochrome P450s, HITPS7, and HITPS8 were identified in Group III (Fig. 11). Upon examining Group I, it was determined that HITPS6 and HITPS13 diverge from the other TPSs within the same group. In Group II, HIBAHDAT25 and HIBAHDAT26 were found to be on a different branch from the other HIBAHDATs. In Group III, HITPS7 and HITPS8 also stood apart from the others. Additionally, when examining the HICYPs, it was observed that HICYP3 and HICYP21 are on a separate branch from the other HICYPs.

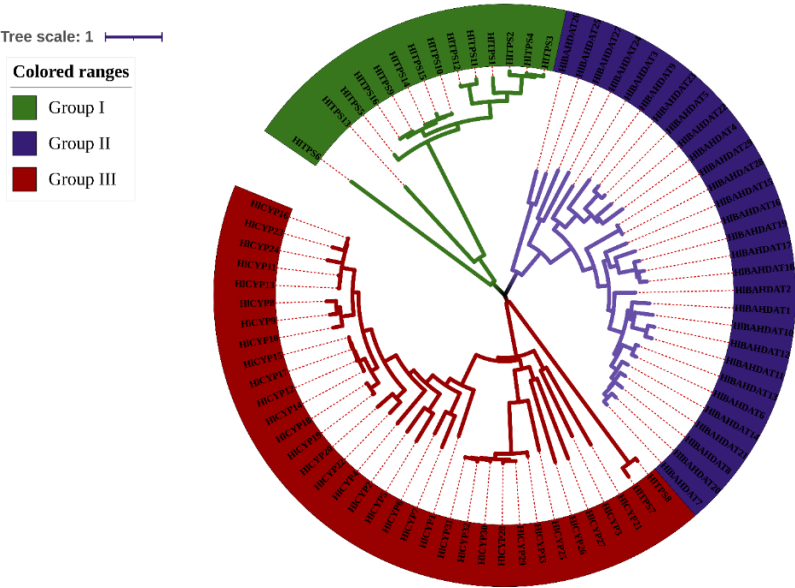


Fig. 11 Phylogenetic trees of the BAHD acyltransferase (BAHDAT), Cytochrome 450 (CYP), and Terpene synthase (TPS) enzyme categories

After the phylogenetic analysis, protein structure homology modeling of the mentioned enzymes was performed. It was observed that β -sheets structures were predominant in all HIBAHDAT enzymes, with α -helices, antiparallel β -sheets, β -turns, and long loops identified in all of them (Fig. 12). Additionally, in terms of arrangement, HIBAHDAT1, HIBAHDAT3,

HIBAHDAT5, HIBAHDAT22 and HIBAHDAT23 were found to be similar to each other, while HIBAHDAT2, HIBAHDAT4, HIBAHDAT6, HIBAHDAT7, HIBAHDAT8, HIBAHDAT9 and HIBAHDAT21 were observed to resemble one another. Furthermore, HIBAHDAT10, HIBAHDAT11, HIBAHDAT12, HIBAHDAT14, and HIBAHDAT20 were distinct from the others, with antiparallel β -sheets being particularly prominent in their structures. In addition, HIBAHDAT17, HIBAHDAT24, HIBAHDAT25, HIBAHDAT26, and HIBAHDAT27 were observed to be more distinct from the other enzymes.

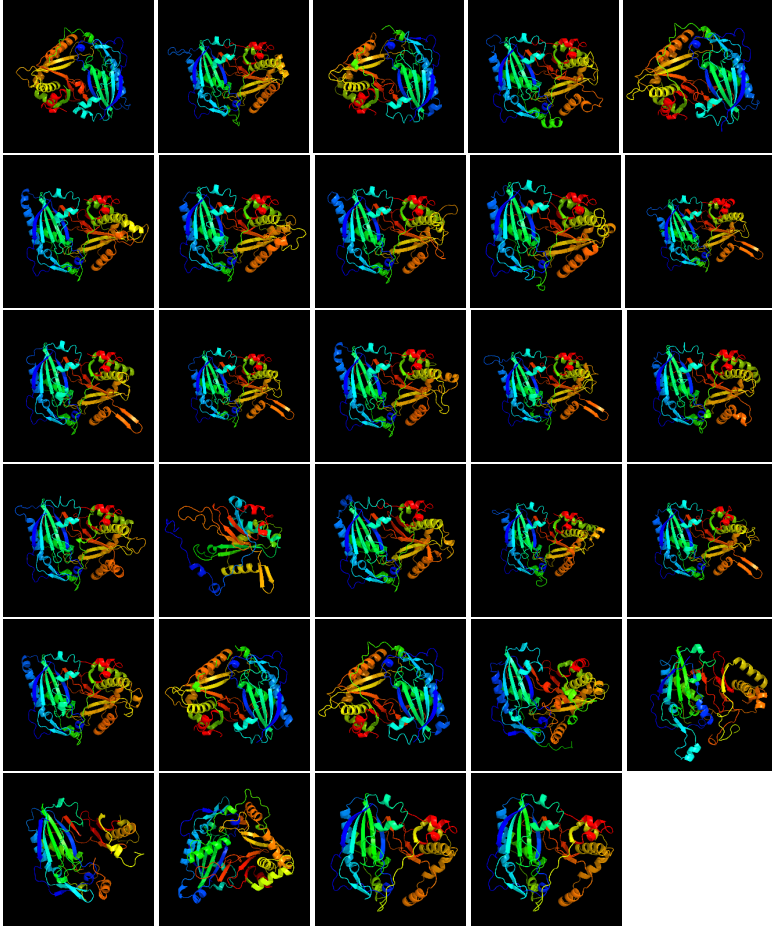


Fig. 12 Predicted 3D structures of HIBAHDATs, arranged by protein number

In the protein structures of CYP enzymes, the dominance of helix-loop-helix structures is particularly noticeable (Fig. 13). It has been determined that all proteins from HICYP2 to HICYP20, as well as from HICYP24 and HICYP26 to HICYP33, contain α -helices, antiparallel β -sheets, β -tur-

ns, and long loops. Additionally, α -helices, parallel β -sheets, antiparallel β -sheets, β -turns, and long loops have been identified in HICYP1, HICYP21, and HICYP25. Besides these, HICYP22 includes α -helices, β -turns, and long loops, while HICYP23 includes α -helices, antiparallel β -sheets, and short loops. Upon examining the structural arrangement, it has been observed that HICYP2, HICYP4, HICYP6, HICYP8, HICYP9, HICYP10, HICYP11, HICYP12, HICYP13, HICYP14, HICYP15, HICYP16, HICYP17, HICYP18, and HICYP19 exhibit similar structures, whereas HICYP25, HICYP28, HICYP29, HICYP30, HICYP31, HICYP32, and HICYP33 also display structural similarities. Additionally, a large number of antiparallel β -sheets are prominent in HICYP26 and HICYP27.

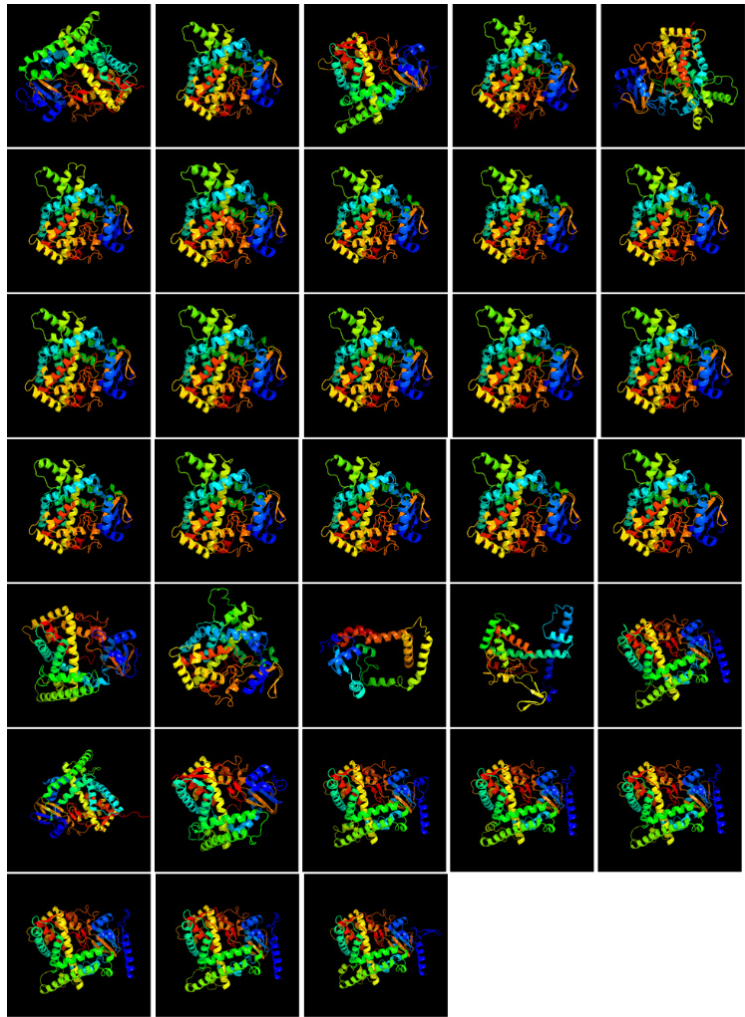


Fig. 13 Predicted 3D structures of HICYPs, arranged by protein number

Based on the homology modeling results of HITPS enzymes, the helix-loop-helix structure is predominant in all enzymes (Fig. 14). Antiparallel β -sheets or β -turns were identified in all enzymes except for HITPS7 and HITPS8. These enzymes contain only α -helices and long loops. Structurally, HITPS1, HITPS2, HITPS3, HITPS4, HITPS9, HITPS10, HITPS11, HITPS14, and HITPS16 were found to be similar, featuring numerous α -helices, antiparallel β -sheets, β -turns, and long loops. On the other hand, HITPS5 and HITPS15 were observed to have a similar arrangement, containing α -helices, β -turns, and long loops.

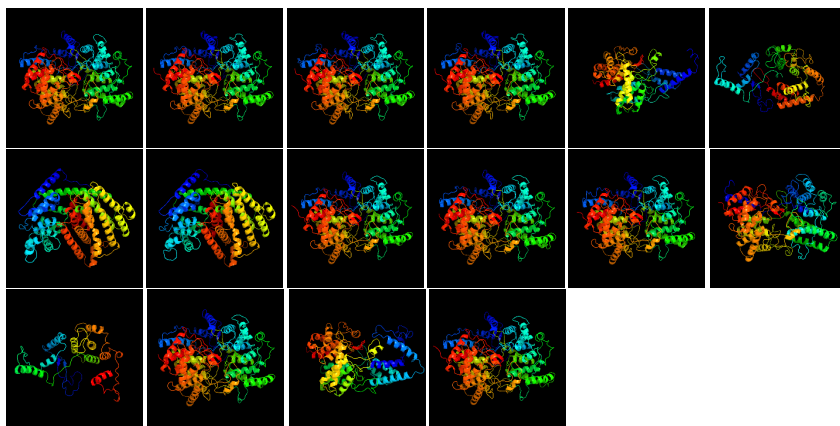


Fig. 14 Predicted 3D structures of HITPSs, arranged by protein number

4.Discussion

A set of non-homologous genes, located in close physical proximity on chromosomes to produce a specific metabolite, forms gene clusters (Bhadraraj et al., 2021). 28 gene clusters involved in secondary metabolite biosynthesis have been identified in *H. lupulus*. The majority of these clusters are of the saccharide type (10), followed by the terpene type (6), with the least common being saccharide-polyketide (1), saccharide-terpene (1), lignan (1), and terpene-alkaloid (1). Moreover, chromosome 5 contains the highest number of secondary metabolite biosynthetic gene clusters, while chromosome 9 contains the fewest. Research on gene clusters involved in the biosynthesis of secondary metabolites, which have significant functions in plants, is relatively scarce, with most studies focusing on microorganisms. In a study (Öz, 2024) on *Citrus sinensis* (L.) Osbeck, 40 secondary metabolite gene clusters were identified, with the highest number being saccharide type and the fewest being terpene-alkaloid, saccharide-terpene, and terpene-saccharide-polyketide types. Another study (Siddiqui et al., 2024) reported that *Camelina sativa* (L.) Crantz has 89 secondary meta-

bolite biosynthetic gene clusters. In the study where the software plantSMASH was used and 48 plants were analyzed (Kautsar et al., 2017), the number of gene clusters identified is as follows: *Arabidopsis thaliana* (L.) Heynh. (45), *Arabis alpina* L. (35), *Arachis duranensis* Krapov. & W.C.Greg. (34), *A. ipaensis* Krapov. & W.C.Greg. (34), *Beta vulgaris* L. (34), *Brachypodium distachyon* (L.) P.Beauv. (29), *B. stacei* Catalán, Joch. Müll., L.A.J.Mur & T.Langdon (43), *Brassica napus* L. (68), *B. oleracea* L. (34), *B. rapa* L. (51), *Cajanus cajan* (L.) Huth (23), *Cicer arietinum* L. (28), *Cucumis sativus* L. (30), *Elaeis guineensis* Jacq. (18), *Fragaria vesca* L. (35), *Glycine max* (L.) Merr. (76), *Gossypium raimondii* Ulbr. (47), *Kalanchoe marnieriana* H.Jacobsen (62), *Malus domestica* (Suckow) Borkh. (51), *Manihot esculenta* Crantz (36), *Medicago truncatula* Gaertn. (54), *Musa acuminata* subsp. *malaccensis* (Ridl.) N.W.Simmonds (33), *Oryza brachyantha* A.Chev. & Roehr. (37), *O. sativa* subsp. *indica* Shig. Kato (54), *O. sativa* subsp. *japonica* Shig.Kato (46), *Panicum virgatum* L. (53), *Phaseolus vulgaris* L. (36), *Physcomitrella patens* (Hedw.) Bruch & Schimp. (9), *Populus trichocarpa* Torr. & A.Gray ex Hook. (48), *Prunus mume* Siebold & Zucc. (33), *P. persica* (L.) Batsch (33), *Salix purpurea* L. (33), *Sesamum indicum* L. (41), *Setaria italica* (L.) P.Beauv. (50), *S. viridis* (L.) P.Beauv. (51), *Solanum lycopersicum* L. (45), *S. pennellii* Correll (45), *S. tuberosum* L. (51), *Sorghum bicolor* (L.) Moench (54), *Theobroma cacao* L. (48), *Vigna angularis* (Willd.) Ohwi & H.Ohashi (40), *V. radiata* (L.) R.Wilczek (42), *Vitis vinifera* L. (46), *Zea mays* L. (34) and *Ziziphus jujuba* Mill.(49). Compared to the plants studied, the number of secondary metabolite biosynthetic gene clusters in *H. lupulus* is found to be lower than in most other plants, except for *Cajanus cajan*, *Elaeis guineensis*, and *Physcomitrella patens*.

The analysis of secondary metabolite biosynthetic gene clusters in plants is very rare, and in the studies that have been conducted, the gene clusters on individual chromosomes have not been identified. Instead, the total number of these gene clusters and their types are reported. Detailed information on gene clusters on chromosomes was provided solely in the research conducted on *C. sinensis* (Öz, 2024). In *C. sinensis*, nine chromosomes are present, with the highest number of secondary metabolite gene clusters found on chromosome 3 and the lowest on chromosome 4. In the current study, however, *H. lupulus* shows the highest number of gene clusters on chromosome 5 and the lowest on chromosome 9. Furthermore, both studies reveal that the most common type of secondary metabolite gene cluster is Saccharide. In addition to these, when evaluating the BAHD acyltransferases, Cytochrome P450, and Terpene synthases, HIBAHD acyltransferases were observed on chromosomes 3, 5, and 8, with the highest number found on chromosome 3. HICYPs were identified on all chromo-

somes except chromosomes 1, 2, and 9, with the highest presence on chromosome 6. HITPSs were found on chromosomes 4 and 6, with the highest observation again on chromosome 6. Since CYP450s are a large enzyme superfamily (Rasool & Mohamed, 2016), the presence of these enzymes on most chromosomes of *H. lupulus* is an expected situation. Additionally, the fact that these enzyme categories, such as TPS and CYP, which play key roles in stress resistance, plant hormone production, and the mechanisms of drug biosynthesis, are predominantly identified on chromosome 6 suggests that this chromosome could play a significant role in the medicinal effects of *H. lupulus* and in the plant's defense mechanisms.

BAHD acyltransferases are an important enzyme family with pharmacological effects such as analgesic, antibacterial, antiviral, and antitumor activities (Liu et al., 2024). In the current study, stemmadenine O-acetyltransferase-like, vinorine synthase-like, shikimate O-hydroxycinnamoyltransferase-like, BAHD acyltransferase BIA1-like, deacetylvindoline O-acetyltransferase-like, and spermidine hydroxycinnamoyl transferase-like enzymes, which belong to this enzyme category, were identified in *H. lupulus*. The enzyme that catalyzes the formation of O-acetylstemmadenine from stemmadenine is stemmadenine O-acetyltransferase (Shahsavarani et al., 2023). O-acetylstemmadenine is involved in the synthesis of vindoline, a precursor to the anticancer drugs vincristine and vinblastine, while deacetylvindoline O-acetyltransferase catalyzes the final acetylation in the production of vindoline (Carqueijeiro et al., 2018; Qu et al., 2019). In addition, vinorine is an alkaloid with antitumor, anti-inflammatory, and antibacterial effects, and vinorine synthase plays a role in the production of vinorine (Guo et al., 2018). The presence of stemmadenine O-acetyltransferase-like, vinorine synthase-like, and deacetylvindoline O-acetyltransferase-like enzymes in *H. lupulus* suggests that the mentioned pharmacological activities may also occur in this plant. Furthermore, the presence of all three enzymes on chromosome 3 implies that it may be more appropriate for cancer-related studies to focus on this chromosome.

CYPs in plants play a role in the formation of defense compounds, plant hormones, fatty acid conjugates, and the production processes of important drugs (Darabi et al., 2017). In plants, these CYP450s are divided into two types: the A-type and the non-A type. The A-type is grouped into the CYP71 clan, while the non-A type is classified into the CYP51, CYP72, CYP74, CYP85, CYP86, CYP97, CYP710, CYP711, CYP727, and CYP746 clans (Guttikonda et al., 2010). The majority of the enzyme categories identified in *H. lupulus* were observed to be of type A (Supplementary Table 2). It is already reported that (Nelson and Werck-Reichhart, 2011) more than half of plant CYPs belong to the CYP71 clan, supporting the result obtained in the current study. It is stated that A-type enzymes are

mostly involved in secondary metabolite biosynthesis, while non-A-type enzymes play a role in hormone or lipid production (Rasool & Mohamed, 2016). CYP71A and CYP71Ds are predominantly associated with mono- and sesquiterpene oxidation, whereas CYP72s catalyze the synthesis of di- and triterpenoid phytohormones, and CYP72As, in particular, are involved in triterpene synthesis, as shown in various studies (Weitzel and Simonson, 2015). One of the enzymes identified in *H. lupulus*, CYP71D9-like, is involved in catabolism, transport, and secondary metabolite production (Xu et al., 2021). In *Celastrus angulatus* Maxim., the genes *CYP71D9*, *CYP71D10*, and *CYP71D11*, which are thought to play a role in the biosynthesis of the insecticidal compound Celangulin V, have been identified (Li et al., 2019). Similarly, in *H. lupulus*, CYP71D9-like, CYP71D10-like, and CYP71D11-like enzymes are present in high amounts. Whether these enzymes have a similar function can be determined through comprehensive experimental studies. Another enzyme found in *H. lupulus* is CYP714C2-like. Research has shown that (Zhang et al., 2024) CYP714C2 is involved in the gibberellin biosynthesis pathway in strawberries. In addition, its overexpression in *Pyrus bretschneideri* Rehder. has been reported to alter plant hormone signaling, the MAPK signaling pathway, starch and sucrose metabolism, plant-pathogen interactions, and certain pathways related to resistance (Zhang et al., 2024). Another enzyme thought to be associated with defense compounds in *H. lupulus* is CYP94C1-like. A study (Widemann et al., 2015) concluded that CYP94B3 and CYP94C1 attenuated defense responses during infection caused by *Botrytis cinerea*. It has been reported that the ethanolic extract of *H. lupulus* exhibits moderate antifungal activity against *Botrytis cinerea*, while isoxanthohumol, derived from this plant, shows a high level of antifungal activity (Yan et al., 2021). The current study suggests that the presence of the CYP94C1-like gene on chromosome 7 may play a role in this antifungal effect. Future studies focusing on this chromosome and conducting more comprehensive research are expected to contribute to a better understanding of the plant's resistance mechanisms against diseases. Similarly, while a connection between CYP72A219 and the metabolic resistance mechanism has been reported (Guan et al., 2024), the identification of CYP72A219 on chromosome 8 in *H. lupulus* suggests that it may also play a role in a similar mechanism in this plant. Another enzyme identified in this plant within the CYP72A category is CYP72A397. This enzyme is involved in the synthesis of hederagenin by catalyzing the hydroxylation at the C-23 position of oleanolic acid (Han et al., 2018). The hederagenin compound exhibits a range of effects, including anti-depressant, anti-inflammatory, anti-diabetic, anti-viral, anti-neurodegenerative, and anti-tumor activities (Zeng et al., 2018). In *H. lupulus*, a CYP72A397-like isoform X2 has been detected, suggesting that this enzyme may contribute to the medicinal effects of this plant. Anot-

her enzyme category, CYP98A, which is involved in the phenylpropanoid pathway, plays a role in the biosynthesis of cutin and suberin, as well as in response to biotic stress conditions (Tobias et al., 2018; Zhang et al., 2023). It has been reported that one of these enzymes, CYP98A 2-like, is downregulated in flavonoid biosynthesis (Guo et al., 2023). In *H. lupulus*, CYP98A 2-like has also been identified on chromosome 3, and it is possible that this chromosome is associated with flavonoid synthesis. One of the enzymes involved in the flavonoid biosynthesis pathway is flavonoid 3'-monooxygenase, which is reported to play a role in the breakdown of kaempferol—a flavonol known for its medicinal effects, such as anticancer, anti-inflammatory, and antidiabetic properties (Kumari et al., 2023). The enzyme flavonoid 3'-monooxygenase involved in flavonoid synthesis has been identified in *Ampelopsis megalophylla* Diels & Gilg. (Yang et al., 2019) In *H. lupulus*, a flavonoid 3'-monooxygenase-like enzyme has also been detected on chromosome 5, and it is believed to contribute to the production of flavonoids in this plant. Additionally, it has been demonstrated in previous studies that kaempferol is present in *H. lupulus* (Korpelainen and Pietiläinen, 2021), and the presence of a flavonoid 3'-monooxygenase-like enzyme, which is similar to the flavonoid 3'-monooxygenase enzyme involved in kaempferol degradation, supports the findings of the present study. In a study (Rastogi et al., 2020), the high transcript levels of the beta-amyrin 28-monooxygenase enzyme in *Ocimum tenuiflorum* L. under abiotic stress conditions suggest that this enzyme may play a role in the plant's defense mechanism. Similarly, the presence of the beta-amyrin 28-monooxygenase-like enzyme in the current study indicates that *H. lupulus* may also have the potential to enhance its tolerance to abiotic stress conditions.

The enzymes in the TPS category, another enzyme group identified in *H. lupulus*, include cycloartenol synthase, beta-amyrin synthase, alpha-humulene synthase, and lupeol synthase. It has been reported that dammarediol synthase, lupeol synthase, and β -amyrin synthase catalyze the formation of dammarediol-II, lupeol, and β -amyrin, contributing to the triterpenoid biosynthesis pathway, while cycloartenol synthase produces cycloartenol, entering the sterol pathway in *Betula platyphylla* Sukaczew(-Yin et al., 2020). Cycloartenol synthase is involved in the conversion of 2,3-oxidosqualene to cycloartenol, which is one of the key structures in the synthesis of phytosterols such as stigmasterol, sitosterol, and campesterol (Wang et al., 2022). This enzyme has been shown to play a role in phytosterol biosynthesis in various plants including *Fritillaria thunbergii* Miq., *Paris polyphylla* Sm., *Nicotiana tabacum* L., *Betula platyphylla*, and *Glycyrrhiza glabra* L. (Mishra et al., 2024), and it is thought that it may also be involved in similar functions in *H. lupulus*. In addition, α -amyrin

and β -amyrin, found in plants such as *Olea europaea* L., *Solanum lycopersicum*, *Aesculus hippocastanum* L., *Liriodendron tulipifera* L., *Aloe vera* (L.) Burm.f., *Calendula officinalis* L., *Betula alba* L., *Coffea arabica* L., *Viscum album* L., *Malus domestica*, *Pisum sativum* L., *Syzygium aromaticum* (L.) Merr. & L.M.Perry, *Protium kleinii* Cuatrec., *Alstonia boonei* De Wild., *Brassica oleracea*, *Swertia longifolia* Boiss., *Byrsonima crassifolia* Kunth, and *Canarium tramdenum* C.D.Dai & Yakovlev, have demonstrated effects such as anti-inflammatory, hepatoprotective, antigout, antitumor, anxiolytic, and anti-skin hyperpigmentation (Viet et al., 2021). β -amyrin and lupeol are important representatives of the pentacyclic triterpene family, which is widely distributed in the plant kingdom and contains biologically active compounds (Ngege Tamfu et al., 2022). Lupeol's role in the prevention and treatment of cancers such as lung, liver, colorectal, osteosarcoma, and bladder cancer has been established through studies demonstrating its mechanisms, including apoptosis induction, suppression of cell proliferation, and inhibition of cancer cell migration and invasion (Liu et al., 2021). Additionally, lupeol exhibits antioxidant, anti-inflammatory, and anti-diabetic effects and provides protective benefits against cardiovascular, renal, neurological, and skin diseases (Sohag et al., 2022). α -Humulene, a compound with antifungal, anti-inflammatory, anti-biofilm, and antibacterial properties, and has been shown to be effective against Gram-positive and Gram-negative pathogens involved in the development of dental caries, persistent endodontic infections, and periodontal diseases (Becker and Holtmann, 2024; de Almeida Rossato et al., 2022). α -humulene produced by α -humulene synthase can be present in the essential oil of *H. lupulus* at concentrations of up to 40%, depending on the part of the plant used (Mendes de Lacerda Leite et al., 2021). The presence of α -humulene synthase has also been reported in plants such as *Aquilaria crassna* Pierre, *A. sinensis* (Lour.) Gilg, *Picea glauca* (Moench) Voss, *Santalum austrocaledonicum* Vieill., *Solanum habrochaites* S.Knapp & D.M.Sponner, and *Zingiber zerumbet* (L.) Sm., in addition to *H. lupulus* (Sundaraj et al., 2023). In the current study, α -humulene synthase-like structures were detected on chromosome 6, suggesting a potential role in α -humulene production. This finding indicates that focusing research efforts on this chromosome could be highly beneficial. Moreover, targeting this gene could enhance α -humulene synthesis, and modern gene editing techniques, such as CRISPR/Cas9, may offer significant opportunities for the industrial-scale production of these bioactive compounds. The biosynthetic and biological roles of the enzymes identified in *H. lupulus* not only highlight the plant's potential in health-related applications but also suggest promising avenues for biotechnological innovations. To further elucidate the diversity and evolutionary relationships of these enzymes, phylogenetic analyses have been carried out.

The phylogenetic analysis conducted on BAHDAT, CYP and TPS enzyme categories in *H. lupulus* identified three main groups. The division into three groups is an expected result; however, what is intriguing is that HITPS7 and HITPS8 are located in a different group from the other HITPS enzymes. Upon examining Supplementary Table 2, it becomes evident that while the domain of these two enzymes is Terpene_synth_C, the others have an SQHop_cyclase_C domain, indicating that their classification into a different group is expected. Another significant finding is that the results of the phylogenetic analysis and protein homology modeling are observed to support each other. HITPS7 and HITPS8 lack β -sheets or β -turns, while the other HITPS enzymes contain these structures. Upon detailed examination of the phylogenetic tree, it is observed that HITPS6 and HITPS13 are located in different branches within Group I compared to the others. Similarly, in terms of protein structure arrangement, it was found that HITPS6 and HITPS13 differ from the others. Except for HITPS5 and HITPS15 within the same group, the other enzymes possess numerous α -helices, antiparallel β -sheets, β -turns, and long loops, while HITPS6 and HITPS13 have fewer α -helices, only one antiparallel β -sheet, and long loops. It is also observed that HITPS5 is located in a distinct branch from the others. In the second group of the phylogenetic tree, only HIBAHDAT enzymes are present. Upon examining the phylogenetic tree, it has been determined that HIBAHDAT24, HIBAHDAT25, HIBAHDAT26, and HIBAHDAT27 are located in a distinct branch. Similarly, an analysis of their protein structures reveals that these enzymes differ significantly from the other HIBAHDAT enzymes. Additionally, it was observed that HIBAHDAT28 and HIBAHDAT29, which are positioned on the same branch in the phylogenetic tree, also exhibited the same protein homology modeling. Furthermore, a detailed analysis of the other HIBAHDAT enzymes demonstrated that their protein structures were consistent with their distribution in the phylogenetic tree. In the phylogenetic tree, Group III contains HICYP enzymes, as well as HITPS7 and HITPS8. The helix-loop-helix structure is predominant in HICYP enzymes, while HITPS7 and HITPS8, unlike other HITPS enzymes, show only α -helices and long loops. This finding demonstrates the complementarity between the phylogenetic analysis and protein structure analyses. It is observed that HICYP1, HICYP21, HICYP22, HICYP23, HICYP24, HICYP25, HICYP26, and HICYP27 differ from other HICYP enzymes in some distinct characteristics. For example, HICYP1, HICYP21, and HICYP25 have parallel β -sheets; HICYP22 lacks β -sheets; HICYP23 contains short loops, unlike the others; HICYP24 has fewer α -helices and β -sheets compared to most others; and HICYP26 and HICYP27 possess numerous antiparallel β -sheets. These differences have also been distinctly identified in the phylogenetic tree.

5. Conclusion

This study details the gene clusters contributing to the biosynthesis of secondary metabolites in *H. lupulus* and their chromosomal distributions. It has been determined that the majority of the identified 28 gene clusters are of the saccharide type, with the highest number located on chromosome 5. The distribution of gene clusters across chromosomes can contribute to the understanding of the biological mechanisms underlying the resistance of *H. lupulus* to plant diseases and its pharmacological effects. In particular, the CYP and TPS enzymes concentrated on chromosome 6 have significant potential to contribute to the plant's medicinal properties. Furthermore, enzymes such as flavonoid 3'-monooxygenase and β -amirin 28-monooxygenase may contribute to the plant's defense mechanisms and pharmacological potential. The presence of enzymes like stemmadine O-acetyltransferase, vinorine synthase, and deacetylvindoline O-acetyltransferase also enhances the anticancer potential of *H. lupulus*. The distinct structure and organization observed among gene clusters present significant targets for future genetic engineering efforts. Notably, the presence of enzymes involved in the production of important compounds such as α -humulene on chromosome 6 increases the feasibility of applying modern gene editing techniques, such as CRISPR/Cas9, for the industrial-scale synthesis of these compounds. Future research has the potential to optimize the necessary genetic and biochemical processes to better understand the functions of these enzymes and advance the industrial production of these valuable components.

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Supplementary Table 1

Supplementary Table 2
