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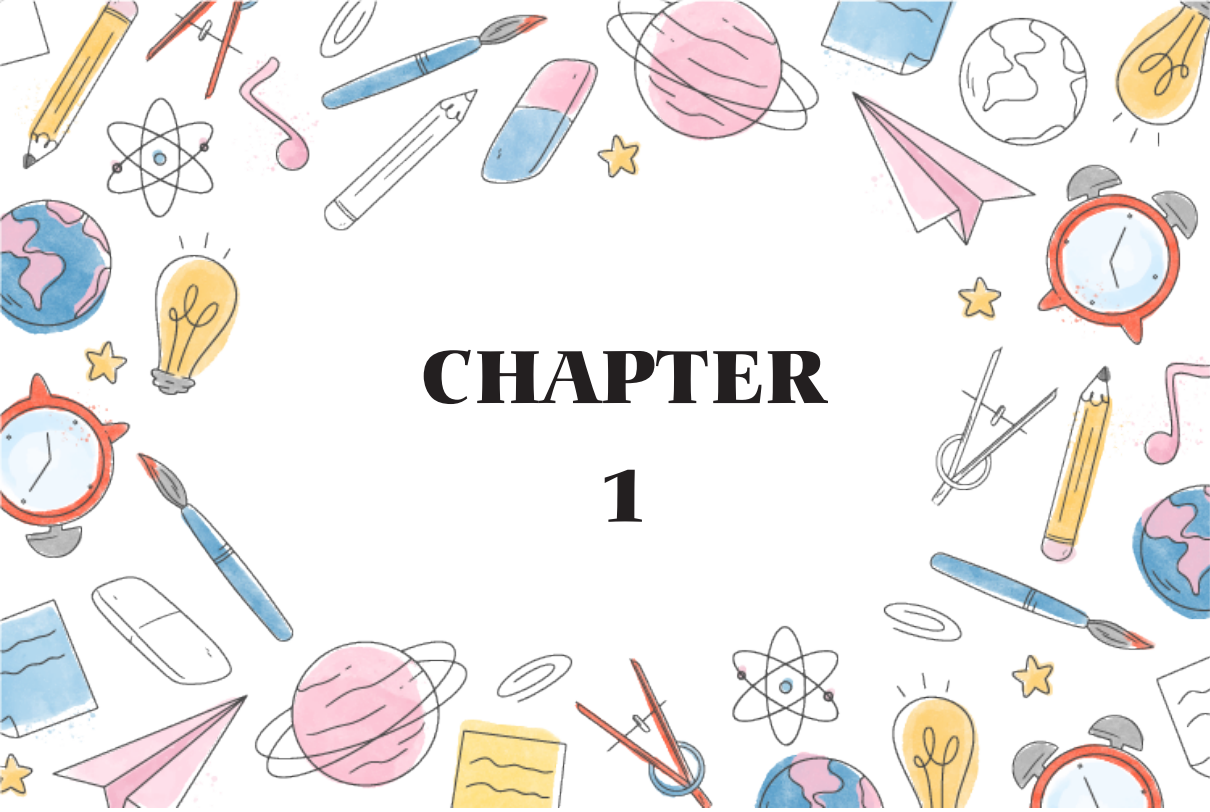

CHAPTER 1

EXPLORATION OF SECONDARY METABOLITE BIOSYNTHETIC GENE CLUSTERS IN *HUMULUS LUPUS* 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. (Yeshi 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 prenylflavonoids (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-sulfanylhexan−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-Heptenal, β -Myrcene, Nonanal, Nerol, a-Copaene, 3-methylthiopropanal, 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. drHumLupu1.1, 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 Lipoxygenase, while Cluster 2 includes CoA-ligase and Strictosidine synthase-like enzymes.

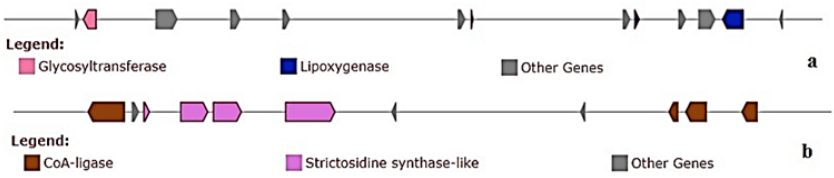

Fig. 1 *a) Cluster 1 – Saccharide, b) Cluster 2 – Alkaloid*

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.

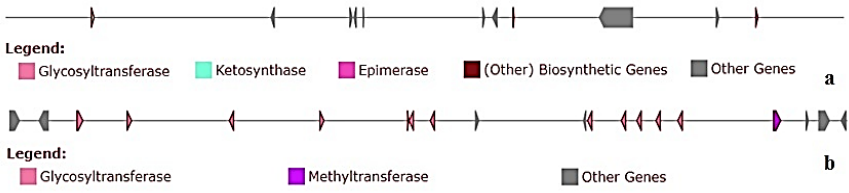

Fig. 2 *a) Cluster 1- Saccharide-Polyketide, b) Cluster 2- Saccharide*

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.

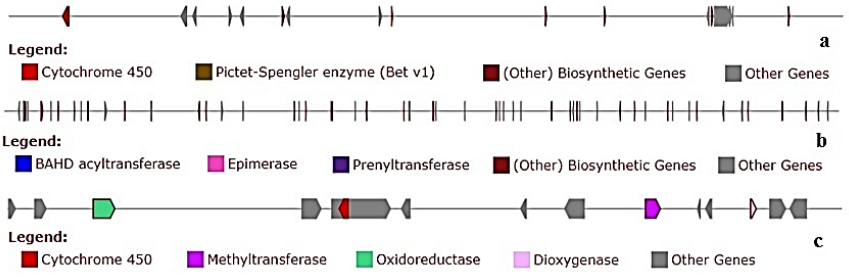

Fig. 3 *a) Cluster 1- Alkaloid, b) Cluster 2- Terpene, c) Cluster 3- Putative*

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.

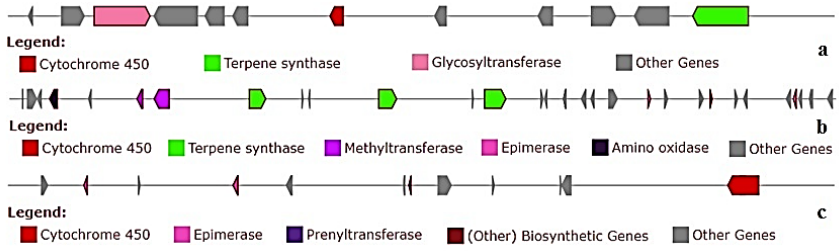

Fig. 4 *a) Cluster 1- Saccharide-Terpene, b) Cluster 2- Terpene, c) Cluster 3-Terpene*

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.

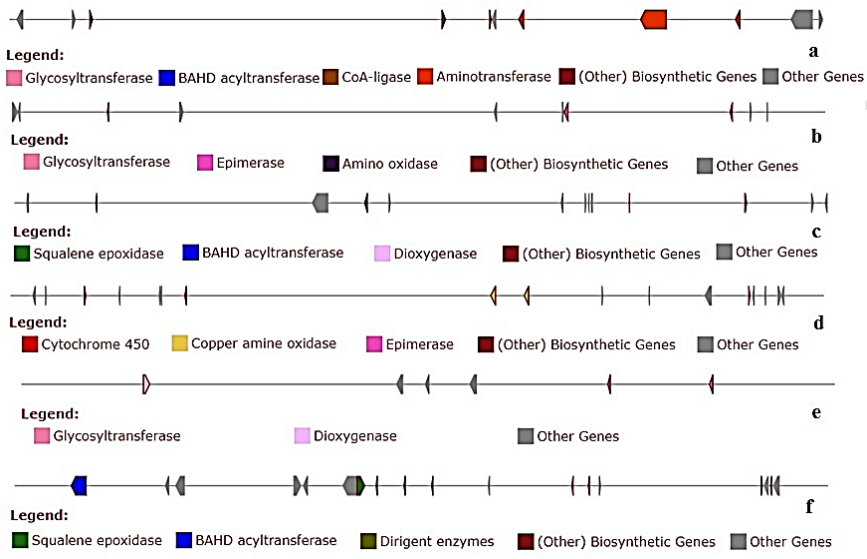

Fig. 5 *a) Cluster 1- Saccharide, b) Cluster 2- Saccharide, c) Cluster 3- Putative, d) Cluster 4- Alkaloid, e) Cluster 5- Saccharide, f) Cluster 6- Lignan*

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 Dioxygenase; in Cluster 2, only Glycosyltransferase; in Cluster 3, Epimerase, Strictosidine synthase-like, and Terpene synthase; in Cluster 4, Terpene synthase, Dioxygenase, and Oxidoreductase; and in Cluster 5, Cytochrome 450 and Terpene synthase.

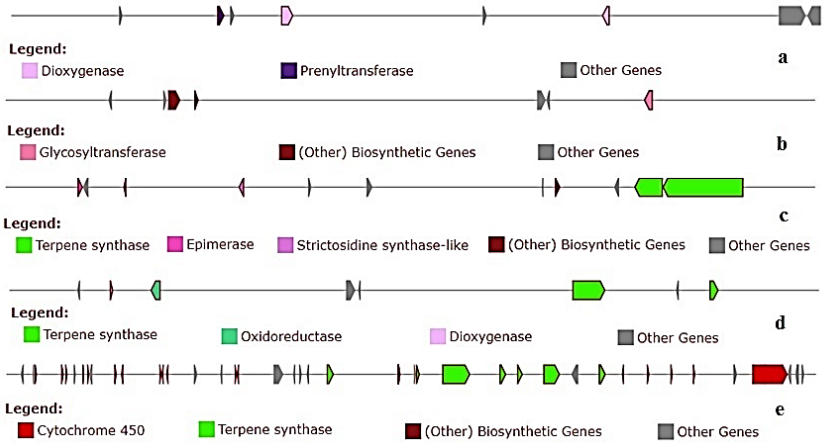

Fig. 6 *a) Cluster 1- Terpene, b) Cluster 2- Saccharide, c) Cluster 3- Terpene-Alkaloid, d) Cluster 4- Terpene, e) Cluster 5- Terpene*

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 Lipoxygenase, 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.

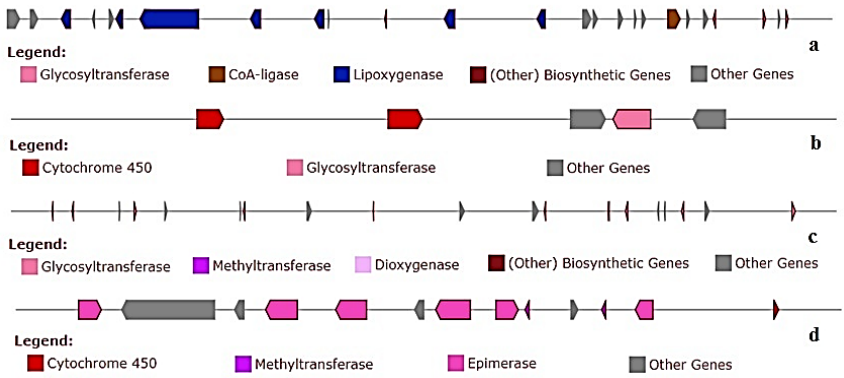

Fig. 7 *a) Cluster 1- Saccharide, b) Cluster 2- Saccharide, c) Cluster 3- Saccharide, d) Cluster 4- Putative*

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.

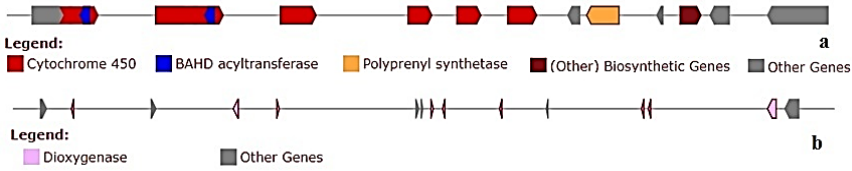

Fig. 8 *a) Cluster 1- Putative, b) Cluster 2- Putative*

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.

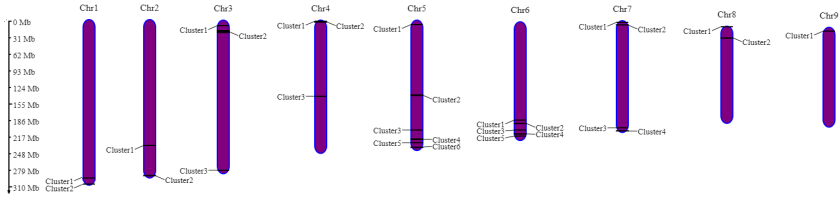

Fig. 10 *The chromosomal locations of secondary metabolite biosynthetic gene clusters*

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, deacetylvindoline 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, HlTPS7, and HlTPS8 were identified in Group III (Fig. 11). Upon examining Group I, it was determined that HlTPS6 and HlTPS13 diverge from the other TPSs within the same group. In Group II, HlBAHDAT25 and HlBAHDAT26 were found to be on a different branch from the other HlBAHDATs. In Group III, HlTPS7 and HlTPS8 also stood apart from the others. Additionally, when examining the HlCYPs, it was observed that HlCYP3 and HlCYP21 are on a separate branch from the other HlCYPs.

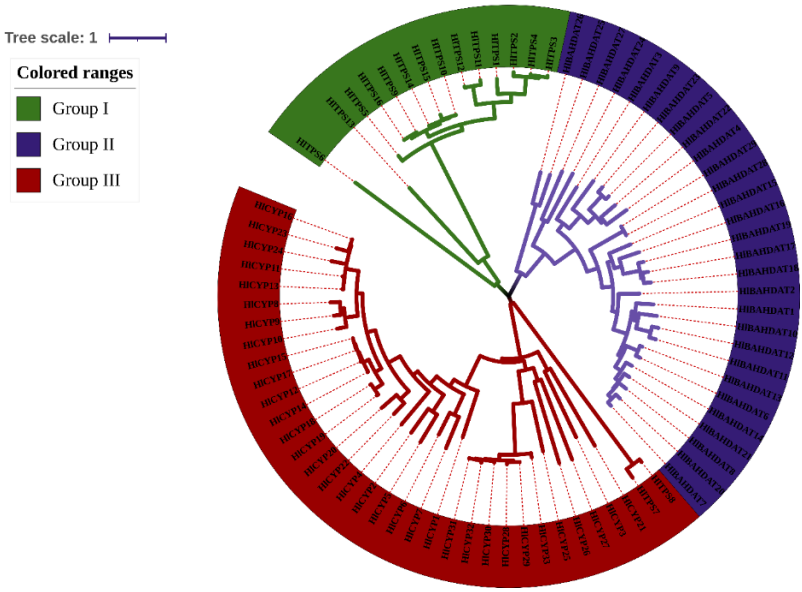

Fig. 11 *Phylogenetic trees of the BAHD acyltransferase (BAHDAT), Cytochrome 450 (CYP), and Terpene synthase (TPS) enzyme categories*

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 HlBAHDAT enzymes, with α-helices, antiparallel β-sheets, β-turns, and long loops identified in all of them (Fig. 12). Additionally, in terms of arrangement, HlBAHDAT1, HlBAHDAT3,

HlBAHDAT5, HlBAHDAT22 and HlBAHDAT23 were found to be similar to each other, while HlBAHDAT2, HlBAHDAT4, HlBAHDAT6, HlBAHDAT7, HlBAHDAT8, HlBAHDAT9 and HlBAHDAT21 were observed to resemble one another. Furthermore, HlBAHDAT10, HlBAHDAT11, HlBAHDAT12, HlBAHDAT14, and HlBAHDAT20 were distinct from the others, with antiparallel β-sheets being particularly prominent in their structures. In addition, HlBAHDAT17, HlBAHDAT24, HlBAHDAT25, HlBAHDAT26, and HlBAHDAT27 were observed to be more distinct from the other enzymes.

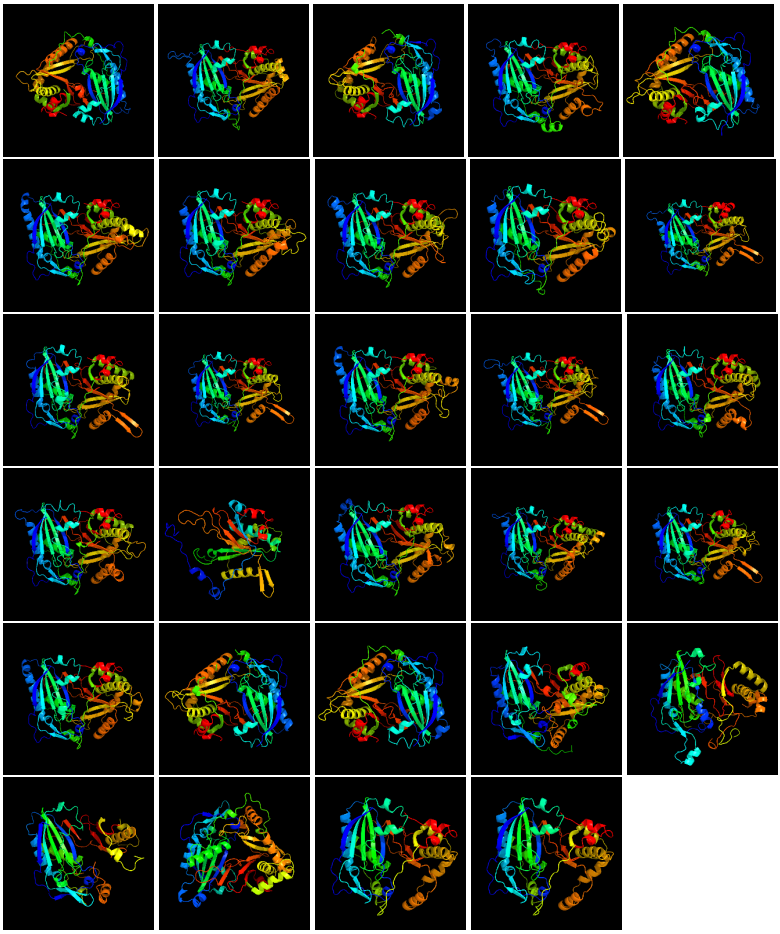

Fig. 12 *Predicted 3D structures of HlBAHDATs, arranged by protein number*

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 HlCYP2 to HlCYP20, as well as from HlCYP24 and HlCYP26 to HlCYP33, 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 HlCYP1, HlCYP21, and HlCYP25. Besides these, HlCYP22 includes α-helices, β-turns, and long loops, while HlCYP23 includes α-helices, antiparallel β-sheets, and short loops. Upon examining the structural arrangement, it has been observed that HlCYP2, HlCYP4, HlCYP6, HlCYP8, HlCYP9, HlCYP10, HlCYP11, HlCYP12, HlCYP13, HlCYP14, HlCYP15, HlCYP16, HlCYP17, HlCYP18, and HlCYP19 exhibit similar structures, whereas HlCYP25, HlCYP28, HlCYP29, HlCYP30, HlCYP31, HlCYP32, and HlCYP33 also display structural similarities. Additionally, a large number of antiparallel β-sheets are prominent in HlCYP26 and HlCYP27.

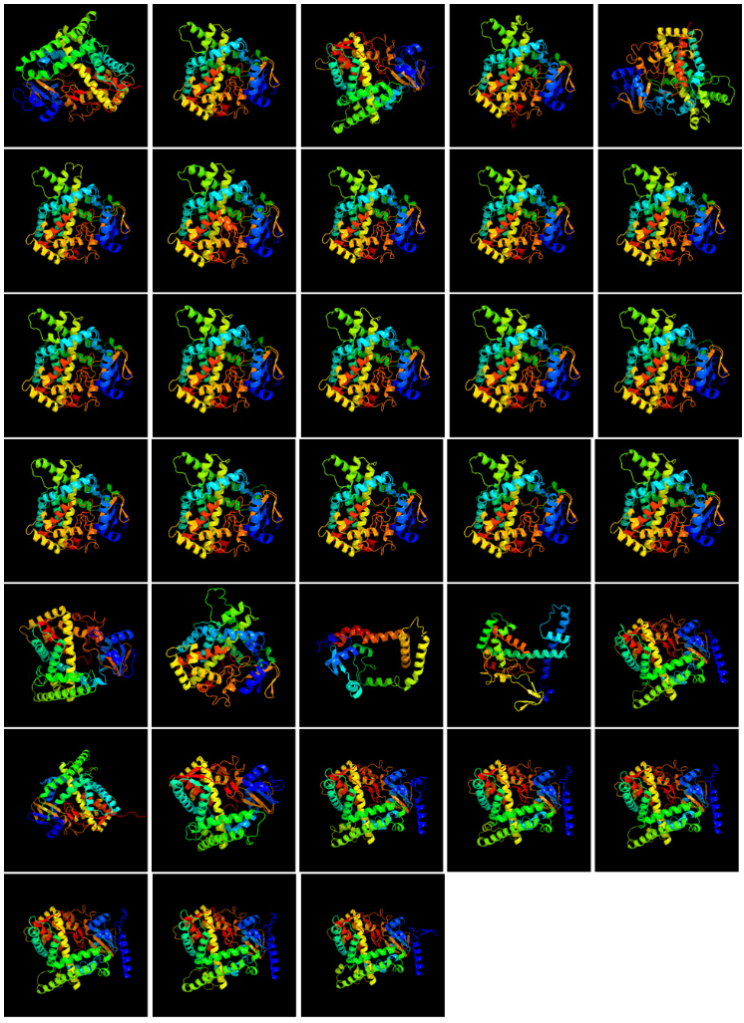

Fig. 13 *Predicted 3D structures of HlCYPs, arranged by protein number*

Based on the homology modeling results of HlTPS 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 HlTPS7 and HlTPS8. These enzymes contain only α-helices and long loops. Structurally, HlTPS1, HlTPS2, HlTPS3, HlTPS4, HlTPS9, HlTPS10, HlTPS11, HlTPS14, and HlTPS16 were found to be similar, featuring numerous α-helices, antiparallel β-sheets, β-turns, and long loops. On the other hand, HlTPS5 and HlTPS15 were observed to have a similar arrangement, containing α-helices, β-turns, and long loops.

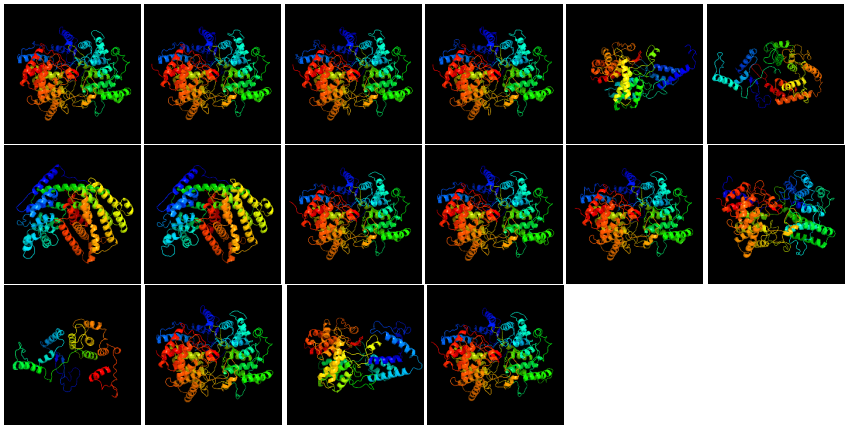

Fig. 14 *Predicted 3D structures of HlTPSs, arranged by protein number*

4.Discussion

A set of non-homologous genes, located in close physical proximity on chromosomes to produce a specific metabolite, forms gene clusters (Bharadwaj 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 plantiSMASH 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, HlBAHD acyltransferases were observed on chromosomes 3, 5, and 8, with the highest number found on chromosome 3. HlCYPs were identified on all chromo-

somes except chromosomes 1, 2, and 9, with the highest presence on chromosome 6. HlTPSs 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 Simonsen, 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 CYP-714C2-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 dammarenediol synthase, lupeol synthase, and β-amyrin synthase catalyze the formation of dammarenediol-II, lupeol, and β-amyrin, contributing to the triterpenoid biosynthesis pathway, while cycloartenol synthase produces cycloartenol, entering the sterol pathway in *Betula platyphylla* Sukaczev(-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 (Ngenge 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.Spooner, 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 HlTPS7 and HlTPS8 are located in a different group from the other HlTPS 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. HlTPS7 and HlTPS8 lack β-sheets or β-turns, while the other HlTPS enzymes contain these structures. Upon detailed examination of the phylogenetic tree, it is observed that HlTPS6 and HlTPS13 are located in different branches within Group I compared to the others. Similarly, in terms of protein structure arrangement, it was found that HlTPS6 and HlTPS13 differ from the others. Except for HlTPS5 and HlTPS15 within the same group, the other enzymes possess numerous α-helices, antiparallel β-sheets, β-turns, and long loops, while HlTPS6 and HlTPS13 have fewer α-helices, only one antiparallel β-sheet, and long loops. It is also observed that HlTPS5 is located in a distinct branch from the others. In the second group of the phylogenetic tree, only HlBAHDAT enzymes are present. Upon examining the phylogenetic tree, it has been determined that HlBAHDAT24, HlBAHDAT25, HlBAHDAT26, and HlBAHDAT27 are located in a distinct branch. Similarly, an analysis of their protein structures reveals that these enzymes differ significantly from the other HlBAHDAT enzymes. Additionally, it was observed that HlBAHDAT28 and HlBAHDAT29, 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 HlBAHDAT enzymes demonstrated that their protein structures were consistent with their distribution in the phylogenetic tree. In the phylogenetic tree, Group III contains HlCYP enzymes, as well as HlTPS7 and HlTPS8. The helix-loop-helix structure is predominant in HlCYP enzymes, while HlTPS7 and HlTPS8, unlike other HlTPS 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 HlCYP1, HlCYP21, HlCYP22, HlCYP23, HlCYP24, HlCYP25, HlCYP26, and HlCYP27 differ from other HlCYP enzymes in some distinct characteristics. For example, HlCYP1, HlCYP21, and HlCYP25 have parallel β-sheets; HlCYP22 lacks β-sheets; HlCYP23 contains short loops, unlike the others; HlCYP24 has fewer α-helices and β-sheets compared to most others; and HlCYP26 and HlCYP27 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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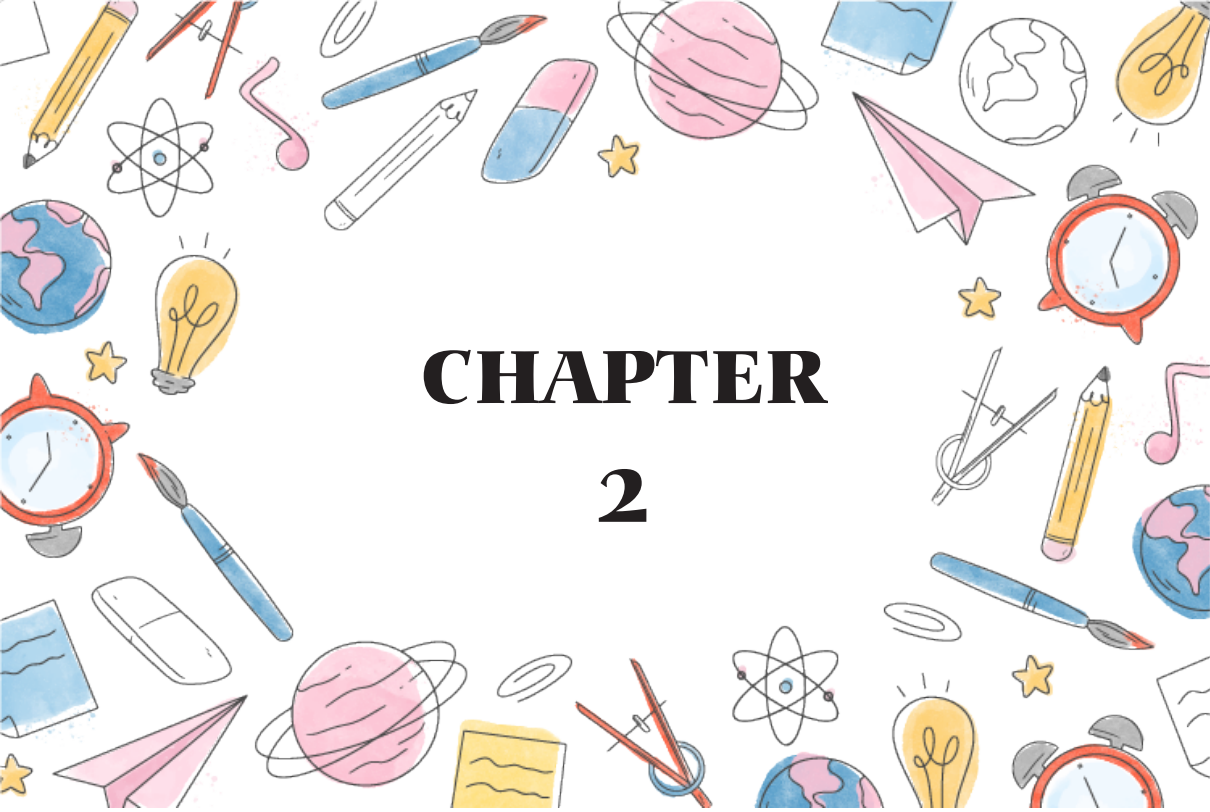
Supplementary Table 1

Supplementary Table 2



CHAPTER 2

IN SILICO IDENTIFICATION AND CHARACTERIZATION OF SECONDARY METABOLITE BIOSYNTHETIC GENE CLUSTERS IN *POPULUS NIGRA L.*

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1. INTRODUCTION

Populus nigra L., belonging to the Salicaceae family, is rich in a wide variety of phytochemicals found in its aerial parts, including chrysin, α-curcumene/ar-curcumene, caffeic acid phenethyl ester, salicin, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, isoquercetin, quercetin, luteolin, kaempferol, apigenin, cinnamic acid, rosmarinic acid, ellagic acid, hemiterpenes, monoterpenes, sesquiterpene hydrocarbons, 1,8-cineole, eugenol, pinostrobin, pinocembrin chalcone, and pinobanksin-3-acetate (Tebbi and Debbache-Benaida 2022). Owing to its phytochemical constituents, *P. nigra* demonstrates antioxidant, anti-inflammatory, antibacterial, antifungal, antidiabetic, melanin production-enhancing, antitumor, hepatoprotective, vasorelaxant, and hypouricemic activities (Kis et al. 2020). Furthermore, this plant is also utilized in the treatment of various diseases, including influenza, bronchitis, asthma, laryngitis, cough, sore throat, pulmonary hemorrhage, hemorrhoids, rheumatic inflammation, anal pain, and ulcerations (Zaidi et al. 2023).

Plants produce secondary metabolites such as phenolic compounds, alkaloids, flavonoids, and terpenoids to enhance their resistance to biotic and abiotic stress conditions, and it has been reported that approximately two million secondary metabolites have been identified to date (Upadhyay et al. 2024; Jahan et al. 2025). Secondary metabolites not only play a role in alleviating oxidative stress induced by stress factors such as extreme temperatures, drought, and salinity, but also function in the development of key characteristic traits in plants, including taste, aroma, and color (Muthusamy and Lee 2023). The vast diversity of secondary metabolites arises not only from biochemical modifications such as hydroxylation, glycosylation, oxidation, methylation, phosphorylation, acylation, and prenylation, but also from the pivotal catalytic functions of specific enzymes involved in these processes (Jan et al. 2021). There are several pathways involved in the production of secondary metabolites, including the shikimic acid pathway, the methylerythritol phosphate (MEP) pathway, the mevalonic acid (MVA) pathway, and the malonic acid pathway (Divekar et al. 2022). Plant biosynthetic gene clusters are often characterized by the close genomic proximity of multiple non-homologous genes, each contributing to the same biosynthetic process (Zhan et al. 2022). Biosynthetic gene clusters in plants, due to the coordinated regulation and physical proximity of genes, play a crucial role in the functional characterization of specialized metabolite biosynthetic pathways and the identification of new, evolutionarily conserved gene clusters (Polturak and Osbourni 2021). The identification of gene clusters involved in the production of secondary metabolites is carried out by utilizing sequence similarities in DNA sequences, and comparative analyses are used to definitively determine the functions of the

genes in the pathways and the products obtained at the end of the process (Udwary et al. 2025). Biosynthetic gene clusters can be identified using bioinformatics tools, and this process involves recognizing genes that encode enzymes or protein domains known to play a role in secondary metabolite production, as well as applying specific biosynthetic rules to predict the types of natural products synthesized by the identified genes (Weber and Kim 2016). A wide range of bioinformatics tools, such as 2metDB, antiSMASH, BAGEL, CLUSEAN, ClusterFinder, ClustScan, eSNaPD, EvoMining, GNP/PRISM, NaPDos, and SMURF, have been developed to facilitate the identification, annotation, and comparative analysis of secondary metabolites and their biosynthetic gene clusters (Weber and Kim 2016). PlantiSMASH, a plant-specific version of antiSMASH, is a comprehensive bioinformatics tool that facilitates the discovery of complex metabolic pathways across diverse species by enabling the automatic identification of biosynthetic gene clusters in plants and prioritizing them through coexpression and comparative genomic analyses (Kautsar et al. 2017).

The aim of this study is to identify the secondary metabolite biosynthetic gene clusters in *Populus nigra* and to investigate the functional roles of the enzymes within these clusters in biosynthesis using bioinformatics tools. In this field, there are no studies identifying secondary metabolite biosynthetic gene clusters in *P. nigra*. The data obtained will contribute to a deeper understanding of the genetic foundation of *P. nigra*'s secondary metabolite production and may facilitate a more detailed examination of the genetic factors influencing secondary metabolite production in plant biotechnology. Furthermore, this study could provide significant guidance for future scientific research on secondary metabolite gene clusters in different plant species.

2. MATERIAL AND METHOD

To access genome information for the *Populus nigra*, a search was conducted in the NCBI database using the keyword "*Populus nigra*." Among the results, the one with a provided reference sequence and the assembly named "ddPopNigr1.1" was selected. Subsequently, amino acid sequences of all chromosomes belonging to *P. nigra* were downloaded in .fasta format. These data were then uploaded to the plantiSMASH tool (Kautsar et al. 2017) for secondary metabolite biosynthetic gene cluster analysis. After the analysis was completed, the results for each chromosome were downloaded and saved as separate files based on chromosome number. The secondary metabolite biosynthetic gene clusters identified in each chromosome were then visualized after the necessary arrangements. Additionally, the enzyme categories associated with these gene clusters

were recorded in Microsoft Excel format and organized according to chromosome number.

3. RESULTS

As a result of the analysis conducted in *P. nigra*, no secondary metabolite biosynthetic gene clusters were observed on chromosomes 6, 12, and 18. The gene clusters identified on the other chromosomes are presented as figures between Figure 1 and Figure 16. Furthermore, the properties of the secondary metabolite gene clusters identified on each chromosome are presented in detail in Table 1. Additionally, the enzyme categories within the secondary metabolite gene clusters located on each chromosome are provided in detail in Supplementary Table 1.

Secondary metabolite biosynthetic gene clusters of the Saccharide and Putative types were identified on Chromosome 1. In Cluster 1, enzyme categories including Glycosyltransferase, Scl acyltransferase, and Prenyltransferase were determined, whereas Aminotransferase and Dioxygenase categories were observed in Cluster 2. Additionally, Epimerase and Glycosyltransferase categories were found in Cluster 3, while Cluster 4 contained Glycosyltransferase, BAHD acyltransferase, and Aminotransferase categories (Figure 1).

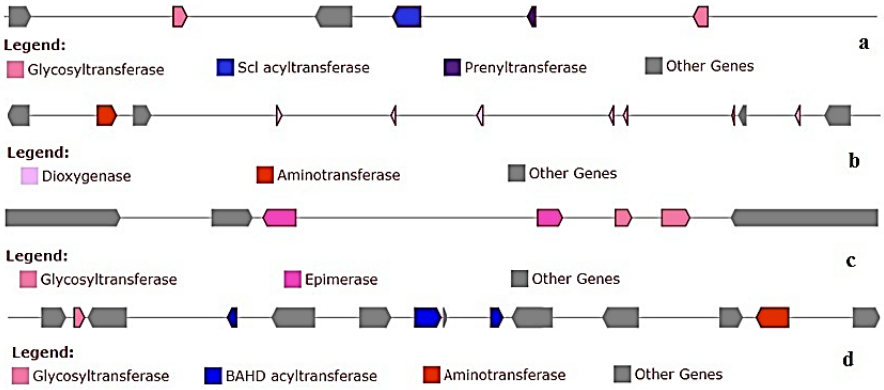

Figure 1. *Gene clusters on Chromosome 1: a) Cluster 1- Saccharide; b) Cluster 2- Putative; c) Cluster 3- Saccharide; d) Cluster 4- Saccharide*

Gene clusters classified as Terpene and Saccharide types were identified on Chromosome 2. The associated enzyme categories were determined as follows: Methyltransferase, Cytochrome P450, and Terpene synthase in Cluster 1; Methyltransferase, Glycosyltransferase, and Fatty acid desaturase in Cluster 2; and Oxidoreductase, Epimerase, and Glycosyltransferase in Cluster 3 (Figure 2).

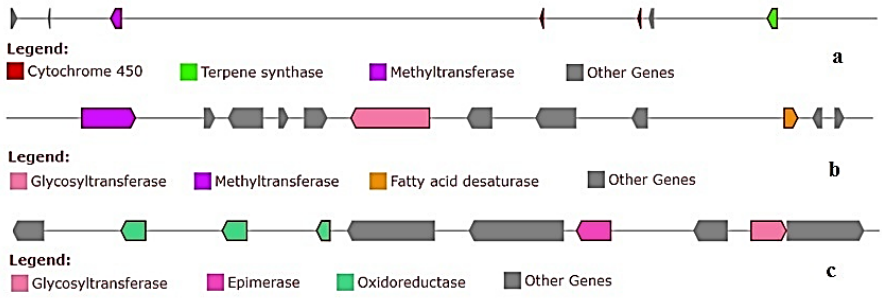

Figure 2. *Gene clusters on Chromosome 2: a) Cluster 1- Terpene; b) Cluster 2- Saccharide; c) Cluster 3- Saccharide*

On chromosome 3, three different types of secondary metabolite biosynthetic gene clusters, namely Polyketide, Saccharide, and Putative, were identified. In Cluster 1, the enzyme categories Oxidoreductase, Epimerase, and Ketosynthase were observed. Cluster 2 contained Glycosyltransferase, Methyltransferase, Cytochrome 450, and Scl acyltransferase. In Cluster 3, Cytochrome 450 and Glycosyltransferase were detected. Cluster 4 included four types of enzyme categories: Oxidoreductase, Scl acyltransferase, CoA-ligase, and BAHD acyltransferase. In Cluster 5, Glycosyltransferase, Cytochrome 450, and Epimerase categories were identified, whereas in Cluster 6, Glycosyltransferase and Cytochrome 450 were observed (Figure 3).

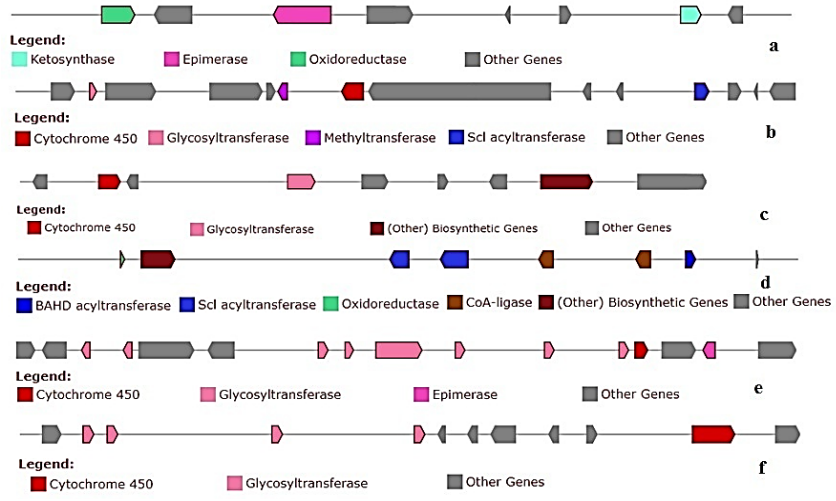

Figure 3. *Gene clusters on Chromosome 3: a) Cluster 1- Polyketide; b) Cluster 2- Saccharide; c) Cluster 3- Saccharide; d) Cluster 4- Putative; e) Cluster 5- Saccharide; f) Cluster 6- Saccharide*

Chromosome 4 contains gene clusters belonging to the saccharide, polyketide, and terpene types. Cluster 1 comprises the enzyme categories Glycosyltransferase, CoA-ligase, and BAHD acyltransferase. The Ketosynthase enzyme category was identified in Cluster 2, whereas the Terpene synthase enzyme category was determined in Cluster 3. Besides these, although Aminotransferase, Glycosyltransferase, and Squalene epoxidase categories were observed in Cluster 4, PRISE enzymes and Cytochrome 450 were identified in Cluster 5 (Figure 4).

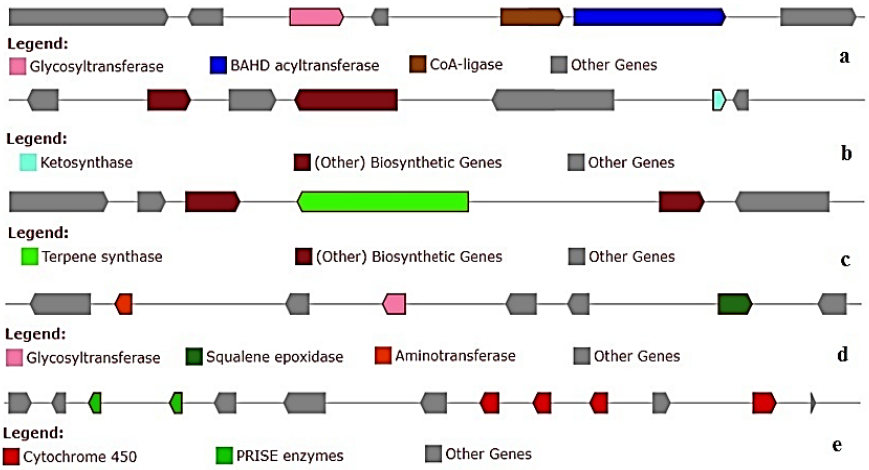

Figure 4. *Gene clusters on Chromosome 4: a) Cluster 1- Saccharide; b) Cluster 2- Polyketide; c) Cluster 3- Terpene; d) Cluster 4- Saccharide; e) Cluster 5- Terpene*

Chromosome 5 contains three secondary metabolite gene clusters, each belonging to a different type, identified respectively as saccharide, polyketide, and putative types. The enzyme categories present in these gene clusters are as follows: Glycosyltransferase, CoA-ligase, and BAHD acyltransferase in Cluster 1; Ketosynthase in Cluster 2; and Cytochrome 450, BAHD acyltransferase, and Cellulose synthase-like in Cluster 3 (Figure 5).

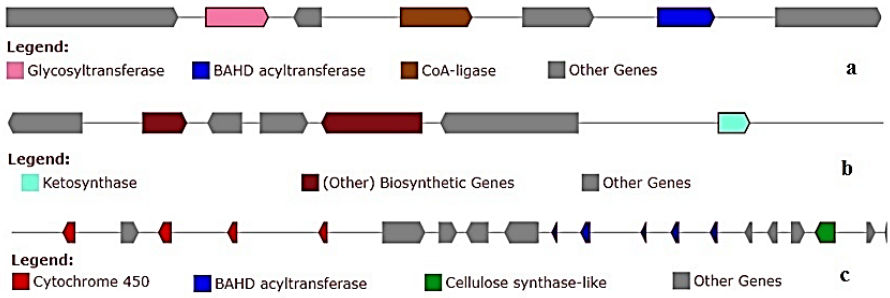

Figure 5. *Gene clusters on Chromosome 5: a) Cluster 1- Saccharide; b) Cluster 2- Polyketide; c) Cluster 3- Putative*

On Chromosome 7, two different types of secondary metabolite biosynthetic gene clusters were identified, classified as saccharide and putative types. The enzyme categories identified in Cluster 1 include BAHD acyltransferase, Scl acyltransferase, and Glycosyltransferase, while Cluster 2 contains CoA-ligase, COesterase, Oxidoreductase, and Cytochrome 450. Additionally, Methyltransferase, Glycosyltransferase, and Dioxygenase were identified in Cluster 3 (Figure 6).

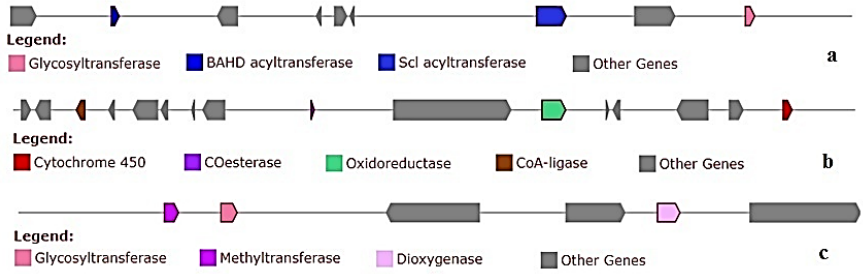

Figure 6. *Gene clusters on Chromosome 7: a) Cluster 1- Saccharide; b) Cluster 2- Putative; c) Cluster 3- Saccharide*

A secondary metabolite biosynthetic gene cluster belonging to the saccharide type was identified on chromosome 8, comprising enzymes categorized as CoA-ligase, epimerase, and glycosyltransferase (Figure 7).

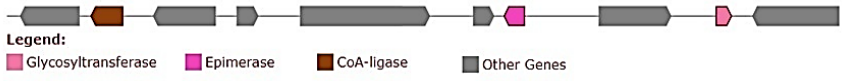

Figure 7. *Saccharide gene cluster on Chromosome 8*

Two types of secondary metabolite biosynthetic gene clusters, classified as terpene and alkaloid types, were identified on chromosome 9.

The enzyme categories present in these clusters are as follows: terpene synthase and epimerase in Cluster 1; BAHD acyltransferase, methyltransferase, Pictet-Spengler enzyme (Bet v1), and amino oxidase in Cluster 2; and methyltransferase, cytochrome P450, and terpene synthase in Cluster 3 (Figure 8).

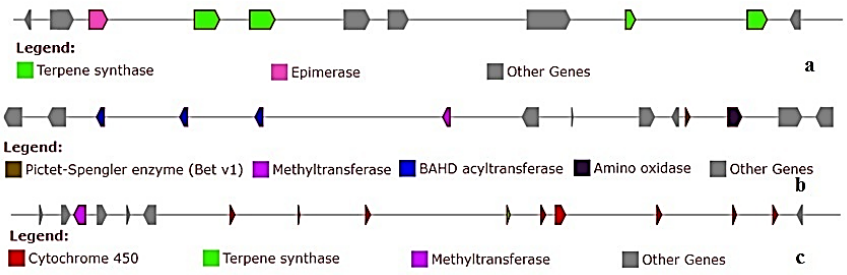

Figure 8. *Gene clusters on Chromosome 9: a) Cluster 1- Terpene; b) Cluster 2- Alkaloid; c) Cluster 3- Terpene*

On chromosome 10, a secondary metabolite biosynthetic gene cluster classified as putative type was identified, including amino oxidase, Scl acyltransferase, and BAHD acyltransferase enzyme categories (Figure 9).

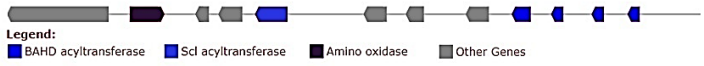

Figure 9. *Putative gene cluster on Chromosome 10*

Two secondary metabolite biosynthetic gene clusters of the terpene type were detected on chromosome 11. The first cluster contains enzymes belonging to the terpene synthase, methyltransferase, and cytochrome P450 categories, while the second cluster includes enzymes from the BAHD acyltransferase and terpene synthase categories (Figure 10).

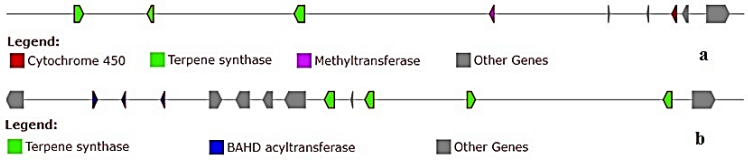

Figure 10. *Gene clusters on Chromosome 11: a) Cluster 1- Terpene; b) Cluster 2- Terpene*

Two types of secondary metabolite biosynthetic gene clusters, classified as alkaloid and saccharide types, were identified on chromosome 13. Cluster 1 contains enzymes belonging to the terpene synthase, strictosidine

synthase-like, and cytochrome P450 categories, while Cluster 2 includes enzymes from the CoA-ligase and glycosyltransferase categories (Figure 11).

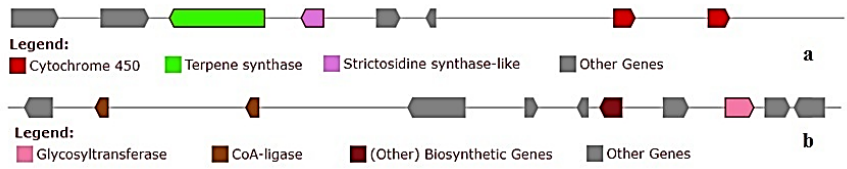

Figure 11. *Gene clusters on Chromosome 13: a) Cluster 1- Alkaloid; b) Cluster 2- Saccharide*

On chromosome 14, a putative-type secondary metabolite biosynthetic gene cluster was observed, comprising enzymes from the methyltransferase, dioxygenase, and cytochrome P450 categories (Figure 12).

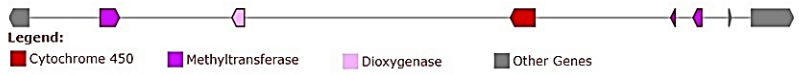

Figure 12. *Putative gene cluster on Chromosome 14*

Two secondary metabolite biosynthetic gene clusters, classified as polyketide and saccharide-terpene types, were identified on chromosome 15. The enzyme categories present in these clusters are as follows: Cluster 1 contains ketosynthase and BAHD acyltransferase, while Cluster 2 includes terpene synthase, cytochrome P450, and glycosyltransferase (Figure 13).

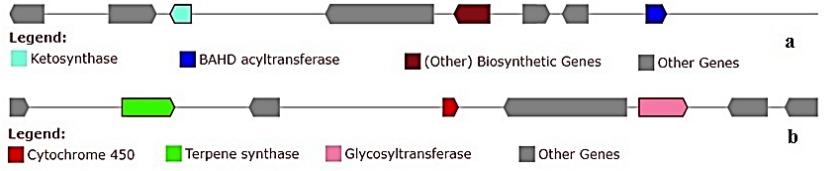

Figure 13. Gene clusters on Chromosome 15: a) Cluster 1- Polyketide; b) Cluster 2- Saccharide-Terpene

A secondary metabolite biosynthetic gene cluster of the terpene type was observed on chromosome 16, containing the enzyme categories terpene synthase and cytochrome P450 (Figure 14).

Figure 14. *Terpene gene cluster on Chromosome 16*

Two types of gene clusters, alkaloid and saccharide, were observed on chromosome 17. In Cluster 1, oxidoreductase, Pictet-Spengler enzyme (Bet v1), and fatty acid desaturase were identified; in Cluster 2, methyltransferase, aminotransferase, and strictosidine synthase-like enzymes were detected; and in Cluster 3, aminotransferase and glycosyltransferase enzyme categories were observed (Figure 15).

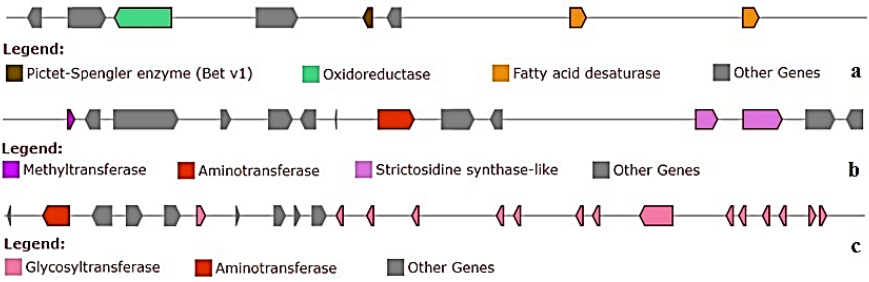

Figure 15. *Gene clusters on Chromosome 17: a) Cluster 1- Alkaloid; b) Cluster 2- Alkaloid; c) Cluster 3- Saccharide*

A putative-type secondary metabolite biosynthetic gene cluster was identified on chromosome 19, containing enzymes belonging to the methyltransferase, Scl acyltransferase, epimerase, and amino oxidase categories (Figure 16).

Figure 16. *Putative gene cluster on Chromosome 19*

Among the 41 identified secondary metabolite gene clusters, chromosome 3 contained the most (six clusters), whereas chromosomes 8, 10, 14, 16, and 19 had the least. Regarding cluster types, saccharide was the most frequently observed secondary metabolite gene cluster, whereas saccharide-terpene was the least common. These clusters were located within the genomic region spanning nucleotide positions 559,831 to 43,950,142, with lengths ranging from 28.16 kb to 470.56 kb (Table 1). The enzyme categories associated with the secondary metabolite gene clusters include amino oxidases, aminotransferases, BAHD acyltransferases, cellulose synthase-like enzymes, CoA ligases, carboxylesterases (COesterases), cytochrome P450s, dioxygenases, epimerases, fatty acid desaturases, glycosyltransferases, ketosynthases, methyltransferases, oxido-

reductases, Pictet-Spengler enzymes (Bet v1), prenyltransferases, PRISE enzymes, Scl acyltransferases, squalene epoxidases, strictosidine synthase-like enzymes, and terpene synthases. Among all chromosomes, chromosomes 3, 4, and 7 exhibited the greatest enzyme category diversity (nine categories each), in contrast to chromosome 16, which possessed the fewest enzyme categories (two). Another noteworthy observation regarding chromosome 16 is that it contains only cytochrome P450 and terpene synthases, which are functionally related enzymes. In addition, cellulose synthase-like enzymes were identified exclusively on chromosome 5, COesterase solely on chromosome 7, prenyltransferase only on chromosome 1, and both PRISE enzymes and squalene epoxidases were uniquely found on chromosome 4 (Supplementary Table 1).

Table 1. *Chromosomal Positions and Types of Secondary Metabolite Biosynthetic Gene Clusters*

Chromosome number	Cluster number	Cluster type	From	To	Size (kb)
1	1	Saccharide	18119317	18214708	95.39
1	2	Putative	34462667	34713391	250.72
1	3	Saccharide	39001196	39042119	40.92
1	4	Saccharide	39350041	39468232	118.19
2	1	Terpene	851860	1322424	470.56
2	2	Saccharide	42996769	43082065	85.30
2	3	Saccharide	43888440	43950142	61.70
3	1	Polyketide	4930302	5027026	96.72
3	2	Saccharide	17480271	17641484	161.21
3	3	Saccharide	17823665	17921425	97.76
3	4	Putative	24675802	24773683	97.88
3	5	Saccharide	25331585	25471293	139.71
3	6	Saccharide	26759837	26864578	104.74
4	1	Saccharide	607525	637466	29.94
4	2	Polyketide	2977429	3033632	56.20
4	3	Terpene	3522333	3566640	44.31
4	4	Saccharide	8606568	8705095	98.53
4	5	Terpene	9124852	9213513	88.66
5	1	Saccharide	559831	587990	28.16
5	2	Polyketide	2890124	2947039	56.91
5	3	Putative	21882240	22114367	232.13
7	1	Saccharide	11720579	11863488	142.91
7	2	Putative	13948930	14214487	265.56
7	3	Saccharide	15969230	16040616	71.39
8	1	Saccharide	12690554	12775408	84.85
9	1	Terpene	2404679	2501831	97.15
9	2	Alkaloid	14029666	14164656	134.99
9	3	Terpene	14838489	15128723	290.23
10	1	Putative	14687266	14760463	73.20
11	1	Terpene	13298189	13614255	316.07
11	2	Terpene	15004255	15257717	253.46
13	1	Alkaloid	1782757	1848370	65.61
13	2	Saccharide	14376982	14495766	118.78
14	1	Putative	2872089	3045452	173.36
15	1	Polyketide	2311744	2369724	57.98
15	2	Saccharide-Terpene	14881920	14978576	96.66
16	1	Terpene	10419149	10587017	167.87
17	1	Alkaloid	11228957	11289957	61.00
17	2	Alkaloid	11816790	11920970	104.18
17	3	Saccharide	13185642	13365718	180.08
19	1	Putative	6640999	6855850	214.85

DISCUSSION

In this study, 41 secondary metabolite biosynthetic gene clusters were identified within the *Populus nigra*, with these clusters distributed across multiple chromosomes. The distribution of these clusters across multiple chromosomes indicates that secondary metabolite production is broadly regulated throughout the genome. Notably, six gene clusters located on chromosome 3 suggest that this chromosome is enriched in biosynthetic genes and may play a critical role in metabolite production. This observation provides valuable insight into the influence of chromosomal organization of gene clusters on metabolic activity and diversity in the *P. nigra*. Secondary metabolite biosynthetic gene clusters have been reported in *Elaeis guineensis* Jacq. (18), *Malus domestica* (Suckow) Borkh. (51), *Musa acuminata* subsp. *malaccensis* (Ridley) Simmonds (33), *Populus trichocarpa* Torr. & A.Gray ex Hook. (48), *Prunus mume* (Siebold) Siebold & Zucc. (33), *Prunus persica* (L.) Batsch (33), *Salix purpurea* L. (33), *Theobroma cacao* L. (48), and *Vitis vinifera* L. (46) (Kautsar et al. 2017). A total of 40 and 43 secondary metabolite biosynthetic gene clusters were previously identified in *Citrus sinensis* (L.) Osbeck and *Punica granatum* L., respectively (Öz 2024a; Öz 2024b). The number of gene clusters identified in *P. nigra* is similar to the values reported in other plant species. The similar number of gene clusters in *P. nigra*, especially compared to woody and perennial plants such as *P. trichocarpa* and *V. vinifera*, suggests the presence of an evolutionary strategy to conserve complex biosynthetic pathways that support adaptation and survival. The gene clusters identified in *P. nigra* present new opportunities for functional characterization and biotechnological applications. Metabolites derived from these clusters may possess antimicrobial, antioxidant, or signaling properties, making them valuable in pharmaceutical, agricultural, or industrial fields. Furthermore, targeted activation or modification of selected gene clusters using synthetic biology tools could enable enhanced production of specific metabolites.

Among the types of secondary metabolite gene clusters, saccharide biosynthetic gene clusters were the most frequently observed, while saccharide-terpene clusters appeared only once. The dominance of saccharide-associated clusters implies that carbohydrate-derived secondary metabolites may play pivotal roles in *P. nigra*, potentially contributing to structural functions such as cell wall formation,

as well as mediating responses to biotic and abiotic stress factors. In addition, analysis of the secondary metabolite biosynthetic gene clusters in *P. nigra* revealed eight terpene clusters, seven putative clusters, and four clusters each belonging to the alkaloid and polyketide types. However, the terpene gene clusters identified in *P. nigra* indicate the presence of a genetic capacity for terpenoid biosynthesis in this species. Terpenes are diverse biochemical compounds that play a critical role in plants' defense and adaptation to environmental stresses (Ninkuu et al. 2021). Accordingly, the presence of these gene clusters suggests that *P. nigra* may possess effective chemical defense mechanisms against biotic and abiotic stress factors. Alkaloids are specialized metabolites that play an important role in plant defense against environmental stresses, act as reservoirs for nitrogen storage, and provide protection against various pests (Bhambhani et al. 2021). Considering the multifunctional roles of alkaloids in defense, nitrogen management, and stress tolerance, the presence of these gene clusters in *P. nigra* indicates that the species is genetically equipped to cope with challenging environmental conditions. Indeed, a study (Yıldırım and Kaya 2017) has shown that *P. nigra* genotypes develop different adaptation strategies to drought stress, such as drought escape, avoidance, and tolerance. This suggests that the gene clusters related to alkaloid biosynthesis may form the molecular basis of the species' multifaceted defense mechanisms against such stress conditions. Moreover, the diversity observed in secondary metabolite gene clusters in *P. nigra* reveals the richness of the species' chemical defense strategies against environmental pressures and provides important insights into its potential for secondary metabolite production.

It has been observed that enzyme categories associated with secondary metabolite gene clusters vary across chromosomes. The presence of nine enzyme categories on chromosomes 3, 4, and 7 indicates that these chromosomes possess rich genetic diversity in terms of secondary metabolite biosynthesis. In contrast, chromosome 16 contains only two enzyme categories, suggesting that it may have a more specific function. The enzyme categories on chromosome 16 are cytochrome P450 and terpene synthases. Among these, terpene synthases are involved in the synthesis of terpenes and terpenoids, while cytochrome P450 enzymes play a role in defense mechanisms (Ling et al. 2023) . This indicates that chromosome 16

may be associated with the production of metabolites involved in more specialized biological processes such as defense and responses to environmental stresses. The co-occurrence of terpene synthases and cytochrome P450 enzymes suggests the possibility of a coordinated mechanism in the biosynthesis of terpene-derived defense compounds. In addition to the overall diversity of enzyme categories across chromosomes, the confinement of certain enzymes to specific chromosomes further supports the notion of functional specialization within the genome of P. nigra. For instance, the identification of cellulose synthase-like enzymes solely on chromosome 5, carboxylesterases only on chromosome 7, prenyltransferases exclusively on chromosome 1, and both PRISE enzymes and squalene epoxidases solely on chromosome 4 suggests that the biological roles of these enzymes may be genetically segregated and specifically regulated at the chromosomal level. This chromosome-specific enzyme distribution implies that different chromosomes may have specialized in distinct metabolic functions.

In conclusion, the identification of secondary metabolite biosynthetic gene clusters in the *Populus nigra* genome represents a valuable step toward understanding the metabolic capacity of the plant and exploring potential biotechnological applications. Comparative genomic studies across different *Populus* species can provide broader insights into the evolution and functional diversity of these gene clusters. This will enable a better understanding of the diversity in metabolite production and adaptive mechanisms among species. Furthermore, the use of bioinformatics approaches not only facilitates the identification of gene clusters but also offers significant advantages in modeling biosynthetic pathways and predicting metabolite profiles. This information allows for the development of targeted strategies in biotechnology and plant breeding. In particular, metabolic engineering and genetic modification can enable the efficient production of high-value natural products. Additionally, research focusing on the relationship between gene clusters involved in secondary metabolite biosynthesis and environmental stress response mechanisms may contribute to the development of plant varieties resistant to abiotic and biotic stresses, addressing challenges posed by climate change. This study provides important references not only for *P. nigra* but also more broadly for tree genomes and plant secondary metabolite biosynthesis, emphasizing once again the sig-

nificance of bioinformatics-based gene cluster analyses in plant biology. In the future, the integration of multi-omics approaches will enable more comprehensive functional profiling of gene clusters, allowing for a more holistic understanding of plant metabolite biosynthesis mechanisms. Thus, it will contribute significantly to both fundamental scientific knowledge and applied biotechnology fields.

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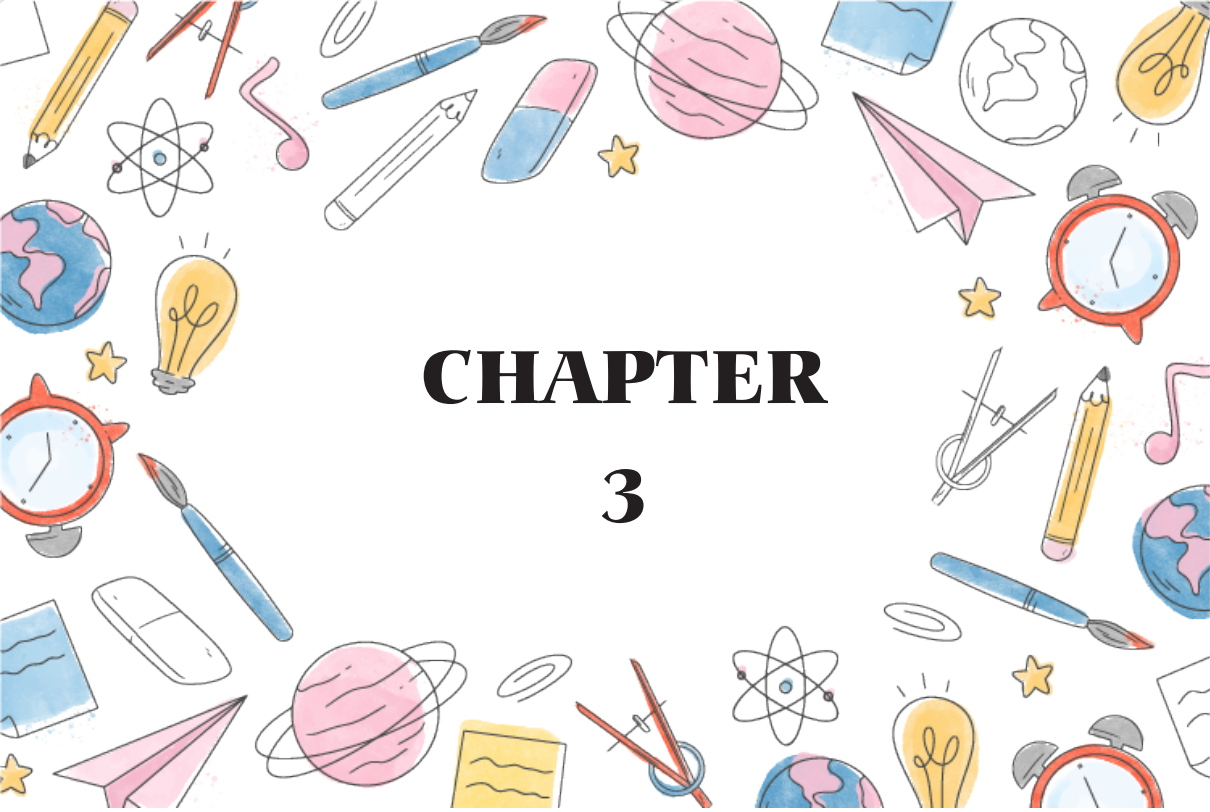
Supplementary Table 1



CHAPTER 3

IMPORTANCE OF ARTIFICIAL INTELLIGENCE FOR INSECT PEST MANAGEMENT

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Abstract: Artificial intelligence Or AI is gradually extending its presence in various fields of Science and Technology. Crop Protection is also one of the areas, where there are high possibilities of advancement in the structure of Integrated Pest Management with particular reference to Insect Pest Management (IPM), where the usage of harmful chemical pesticides for crop protection is highly discouraged. These synthetic pesticides can induce deleterious effects, such as, degradation of land and water resources, killing non-target beneficial insects, and inducing pesticide-resistance in the pests. AI in IPM enhances efficiency by using machine language, computer vision and sensors to detect, monitor, simulate and predict the devastating impact of insect pest infestations. This will enable the reduction in the pesticide use, minimizing environmental negative impacts, and improving the crop productivity. AI enables targeted interventions and autonomous systems, offering sustainable, cost-effective pest management solutions for farmers. Furthermore, AI enhances decision-making in pest management strategies, supporting precision agriculture by providing real-time, actionable insights with continued advancement, improving crop yields, and promoting eco-friendly practices in agriculture. However, AI can have some possible negative impacts and implications in the form of rejecting the human commands, and ignoring human interventions in decision making which could lead to full machine autonomy. Nevertheless, owing to the numerous benefits of AI in IPM we strongly welcome this technology for enhancing pest less crop productivity.

Keywords: Artificial Intelligence, Machine Learning, IPM,

INTRODUCTION

Artificial intelligence (AI) has modernized several fields of science and technology, one of the most potentially impactful areas of its application is in insect pest management (IPM) (Pervez et al., 2020). IPM is a safer method of managing herbivorous insect pests by utilizing semiochemicals. The importance of AI in IPM lies in its ability to mechanize, optimize, and enhance the precision of pest management, thereby improving crop productivity, introducing eco-friendly strategies for reducing environmental impact, and minimizing the reliance on chemical pesticides. AI refers to the miniature of human intelligence in machines programmed to think, learn, and perform tasks typically requiring human intelligence. AI technologies include machine learning (ML), deep learning (DL), natural language processing (NLP), computer vision, and robotics. Remote sensing (RS) technologies, such as drones, satellites, and ground-based sensors, decision support system (DSS), internet of things (IOT), provide valuable information about weather patterns, detecting pest activity and pest infestations. Insect pests are a major threat to agriculture, causing crop damage and

reducing productivity. Traditional methods of IPM often rely on chemical pesticides. Still these methods can have harmful effects on the environment such as ecosystem disruption, biodiversity loss, killing non-target beneficial insect and human health. With the growing challenges of insect pest control and maximize crop productivity, AI and RS offer more efficient and precise solutions for pest management.

AI TECHNOLOGIES IN INSECT PEST MANAGEMENT

1. The Role of AI in plant disease research caused by insect pests:

Artificial intelligence (AI) has increasingly become an essential element in the realm of plant pathology, particularly in comprehending and managing diseases caused by insect pests. These diseases can be challenging to monitor manually due to the complexity of their symptoms, the variety of insect vectors involved, and the swift rate at which infections can propagate. Kariyanna and Sowjanya (2024) reviewed the use of AI in Insect Pest Management and emphasized how AI is helpful in the Recognition of images and classification, Automated pest identification, Predictive modelling and pest forecasting, Deep learning, Precision monitoring and Automation.

AI technologies, especially those utilizing deep learning and image processing, provide robust tools for automating the early detection and diagnosis of these diseases. Through the examination of high-resolution photographs of leaves, stems, and fruit, AI models—particularly convolutional neural networks (CNNs)—can identify fine patterns and visual indicators linked to insect-borne diseases that are usually skipped by the human eyes. "CNNs have shown remarkable performance in crop disease detection, even in harsh conditions (Ai et al., 2020)." A significant application of AI in this field is demonstrated by the research conducted by Selvaraj et al. (2019), who created AI-based systems for detecting diseases in banana plants, including Banana Bunchy Top Virus (BBTV), black Sigatoka, and Xanthomonas wilt. These illnesses can be transmitted directly or worsened by insect vectors such as aphids, thrips, and beetles. Utilizing mobile phone imaging and drone technology, the team developed a practical and affordable method for diagnosing issues in the field. This advancement allows farmers, particularly those in isolated areas, to receive immediate updates about the health of their crops and take action before the disease spreads. Early detection not only protects crops but also minimizes financial losses and reliance on excessive pesticide application. Recent progress has been achieved in crops like cotton, which is especially susceptible to insect pests including whiteflies and bollworms. These pests are infamous not only for the harm they inflict on the plants but also for spreading viral diseases like

Cotton Leaf Curl Virus (CLCuV), a significant threat to cotton yield globally. Toscano-Miranda et al. (2022) showed how artificial intelligence can be utilized alongside hyperspectral, thermal, and multispectral imaging to effectively monitor cotton fields with great accuracy. Imaging systems, deployed on drones or land vehicles, gather extensive amounts of data that AI algorithms then process to identify pest outbreaks and signs of disease in real-time. The capability to visualize the spatial patterns and advancement of the disease assists researchers and extension agents in creating targeted interventions that are more effective and environmentally friendly. In addition, AI technologies extend beyond mere visual recognition. As noted by Jafar et al. (2024), combining AI with Internet of Things (IoT) devices and environmental sensors enables the forecasting of insect population trends and potential disease outbreaks. Environmental elements like temperature, humidity, and rainfall play a crucial role in the development cycles of insect vectors. By gathering this information and inputting it into machine learning algorithms, scientists can predict times of increased risk for the transmission of pest-related diseases. These predictions can aid in creating early warning systems that notify farmers and agricultural officials to implement preventive measures like prompt pesticide use, introducing biological controls, or adjusting irrigation methods. Besides its practical uses in various fields, AI is also utilized in academic studies to model the spread of insect-borne diseases across different areas and under a range of climate change conditions. These models assist researchers in comprehending how changing weather trends might impact the future spread of insect carriers and the illnesses they transmit. Gaining this type of understanding is essential for developing sustainable pest and disease management approaches that are profitable and environmentally sound.

2. The Role of AI in Detecting Insect Movement and Migration:

Insect populations are undergoing significant alterations in their distribution and numbers as a result of environmental stressors. Keeping track of these changes is essential for the conservation of biodiversity and the management of agricultural pests. Conventional monitoring techniques are frequently time-consuming and have limited coverage. Recent technological developments, especially in artificial intelligence and image recognition, offer scalable approaches for the real-time, precise, and automated monitoring of insects (Van Klink et al., 2022).

Emerging Technologies in Insect Ecology

Van Klink et al. (2022) emphasized how technologies like eDNA, autonomous sensors, and machine learning have revolutionized the field of

Insect Ecology. These innovations enable extensive, long-term monitoring and provide fresh perspectives on insect behavior, movement patterns, and ecosystem interactions. AI-driven interpretations of acoustic and visual information permit non-invasive, ongoing data collection, greatly enhancing the temporal and spatial precision of monitoring initiatives. Deep learning and computer vision have the potential to revolutionize the field of entomology (Høye et al. 2021). AI systems are now capable of identifying insect species from photos and videos with a level of accuracy that rivals that of experienced entomologists. Furthermore, these models can be trained to recognize specific behaviors, such as mating, foraging, and flight patterns, which allows for a comprehensive examination of insect movements. The combination of drone imagery and camera traps with AI technologies further improves our capacity to analyze insect migration across extensive areas (Høye et al., 2021).

AI in Pest Monitoring and Prediction

Batz et al. (2023) investigated the use of image analysis and AI for monitoring aphid pests and discussed how AI algorithms can detect aphid species from images captured in the field and forecast infestations by analyzing temporal and spatial data. This predictive ability facilitates prompt actions in agricultural environments, decreasing dependency on chemical pesticides and enhancing integrated pest management (IPM) approaches (Batz et al., 2023).

Monitoring Insect Movement and Migration

The use of AI is becoming crucial for identifying and analyzing how insects move and migrate. Conventional techniques, like mark-recapture methods or manual observation, have limitations in their range and can be labor-intensive. In contrast, AI facilitates ongoing and automated observation of insect behaviors through video recordings and image sequences. Deep learning techniques, especially convolutional neural networks (CNNs), are capable of processing vast amounts of visual data to identify individual insects and track their movements over time (Høye et al., 2021). By examining movement patterns, researchers can ascertain the direction, speed, and behavior of different insect species. Alongside CNNs, recurrent neural networks (RNNs) and long short-term memory (LSTM) models are utilized for examining the temporal dynamics in the movement patterns of insects. These models excel at uncovering patterns in time series data, allowing for predictions regarding the timing and paths of migration. By combining these models with environmental variables such as temperature, humidity, and wind patterns, the precision of migration forecasts is

significantly improved (Van Klink et al., 2022). Such predictive modeling is crucial for comprehending how climate change and alterations in habitat are affecting the distribution and dispersal of insects.

Camera traps and drones with AI-driven image recognition capabilities can observe insects across vast geographic regions and for long durations. For example, when combined with AI analysis, time-lapse photography can uncover trends in daily and seasonal movements. In agricultural environments, these technologies are essential for early detection of pest species and monitoring their dispersion throughout crop fields (Batz et al., 2023). This facilitates more precise pest management strategies and minimizes reliance on broad-spectrum insecticides.

The capacity of AI to recognize subtle behavioral signals and track long-distance migration patterns has significant implications for ecology and conservation. For species that migrate, AI can assist in charting migration routes and pinpointing essential stopover areas, leading to more effective conservation efforts. Additionally, this knowledge is progressively being incorporated into global biodiversity monitoring systems, promoting a unified and data-informed approach to managing insect populations in an increasingly changing environment (Van Klink et al., 2022).

3. The Role of AI in Insect Pest Classification:

AI has introduced significant changes to farming methods, especially in the areas of detecting, identifying, and categorizing insect pests. Conventional techniques for identifying pests are resource-intensive, lengthy, and frequently need specialized taxonomic knowledge. AI, particularly when combined with computer vision and machine learning, has developed into a significant alternative that can deliver real-time, precise, and scalable pest management solutions. Kasinathan et al. (2021) investigated different machine learning methods used for classifying pests in agricultural crops and highlighted the effectiveness of supervised learning algorithms, including Support Vector Machines (SVM), Random Forest (RF), and Convolutional Neural Networks (CNNs), which leverage features derived from images of pests taken in real agricultural settings. These approaches have demonstrated impressive accuracy in identifying pests like aphids, beetles, and caterpillars, providing significant support for field scouting and integrated pest management strategies. Barbedo (2020) emphasized the application of proximal imagery, in which high-resolution photos are taken in close proximity to the subject with smartphones or specialized cameras. This enabled accurate identification of insects, even in diverse field settings. The growing significance of deep learning, especially CNNs, in

the automation of feature extraction, which is essential for dependable pest classification amidst varying lighting, orientation, and occlusion scenarios.

Valan et al. (2019) showed that it is possible to achieve expert-level accuracy in identifying insects by applying transfer learning from pre-trained convolutional neural network models, such as Inception and ResNet. These models can be adjusted to accurately classify insect species by utilizing features extracted from extensive datasets like ImageNet, even when there is a limited amount of labeled data available. This approach greatly minimizes the reliance on large annotated datasets, which are often difficult to find in the field of entomology. Furthermore, employing deep feature representations enhances performance across a variety of taxonomic groups. Combination of morphological characteristics with high-dimensional image features, demonstrating that AI can outperform human expertise in Taxonomy. Mekha and Parthasarathy (2022) emphasized the fusion of AI with Internet of Things (IoT) devices, such as sensor-equipped traps and drones, enabling real-time monitoring and highlighted the critical role of AI in decision support systems, where pest data is gathered, analyzed, and interpreted for prompt agricultural actions. The data may be integrated by merging images, environmental information, and pest life cycles to enhance the precision of classification.

4. The Role of AI in Pest Monitoring, Forecasting, and Early Warning Systems:

AI tools are more frequently used for pest tracking, prediction, and alert systems, improving the capability of farmers and researchers to anticipate and handle pest invasions with enhanced accuracy. A significant advancement in pest monitoring involves the implementation of image recognition and machine learning algorithms that can autonomously detect pest species from images captured in the field. Li and Wang (2024) discussed the application of AI-based systems for more efficient monitoring and management of insect pest outbreaks. Utilizing machine learning algorithms alongside image recognition technologies, pests can be identified, classified, and tracked in real-time. These innovations facilitate early detection of infestations, allowing for quick and targeted interventions that minimize the need for excessive pesticide use. Additionally, AI-driven models are being developed to forecast pest life cycles and the likelihood of outbreaks based on environmental data, aiding in the alignment of management practices with the developmental stages of pests.

Batz et al. (2023) highlighted the progress AI is making in the surveillance of aphid populations, especially through systems that rely on image-based recognition. These tools facilitate the processing of large vo-

lumes of data in real-time, a task that would be tedious and prone to mistakes if performed manually. Monitoring powered by AI not only guarantees precise identification of pests but also aids in gathering data on a scale that enhances our comprehension of pest dynamics in both space and time. Predicting pest outbreaks is yet another domain where AI demonstrates its significant value. Machine learning algorithms, which have been developed using past pest behavior and climate information, can foresee upcoming infestations. As noted by Wu et al. (2022), these systems are extensively utilized in China, where AI models enhance national early warning systems by anticipating the emergence and proliferation of pests in relation to environmental factors. These forecasting tools facilitate prompt decision-making, allowing for proactive instead of reactive pest management strategies. Furthermore, integrating AI with remote sensing technologies enables the establishment of a thorough early warning system. Aziz et al. (2025) emphasized that by analyzing remote sensing data with AI algorithms, it is possible to identify minor changes in crop health that could suggest pest presence before any visible signs emerge. This capacity for early detection allows for timely interventions, minimizing both crop loss and the need for pesticides. Kariyanna and Sowjanya (2024) further emphasized the adaptability of AI technologies in pest management, ranging from automated traps and drones used for data gathering to decision support systems that suggest specific treatment approaches. They claim that AI not only improves pest monitoring but also promotes sustainable farming methods by reducing human mistakes and resource inefficiencies.

5. Role of Artificial Intelligence in Pest damage and Yield Forecasting:

The integration of AI with emerging technologies such as the Internet of Things (IoT) and remote sensing is aiding researchers and practitioners in addressing crop damage caused by pests while enhancing the precision of yield predictions. Malhotra and Firdaus (2022) focused on how sensor networks gather environmental and entomological information that informs AI algorithms for predicting pest occurrences. These advanced systems are capable of examining trends in insect activity, environmental signals, and crop health to anticipate potential damage caused by pests. The authors also highlight the significance of safeguarding data within these networks, ensuring the trustworthiness and accuracy of information utilized in decision-making. AI models are capable of combining pest occurrence data with climatic conditions, soil characteristics, and crop growth factors to predict how pest damage will affect yield (Javaid et al. 2023). This combination allows for more strategic pest management approaches that not only minimize crop losses but also enhance overall productivity. Such studies

collectively demonstrated that AI serves as a valuable partner in managing insect pests—enabling prompt identification, targeted actions, and reliable predictions of crop yields. Implementing AI can significantly reduce crop damage caused by pests, lessen the ecological harm resulting from excessive pesticide use, and support the development of more resilient and sustainable agricultural practices. Machine learning and deep learning techniques-based AI models evaluate environmental data, images, and sensor inputs to forecast pest invasions and determine possible damage (Li and Wang 2024). This allows for timely identification and focused actions. In forecasting yields, AI utilizes information from satellites, meteorological data, and soil conditions, employing models such as LSTMs to accurately project crop results. These innovations improve pest control and refine yield forecasts, leading to more effective agricultural methods.

6. Robotics and Drones in Insect Pest Management:

The incorporation of robotics and drones into the management of insect pests is quickly changing agricultural methods. These advancements provide unmatched accuracy and effectiveness, tackling numerous problems associated with conventional pest management techniques. By utilizing advanced monitoring, gathering real-time data, and implementing targeted interventions, these technologies offer significant potential for improving sustainable farming practices and minimizing environmental effects.

Drones for Pest Surveillance and Monitoring

Drones are significantly transforming pest management by offering detailed imagery, multispectral information, and immediate monitoring options. These unmanned aerial vehicles (UAVs) come with sophisticated sensors, including infrared and multispectral cameras, which allow them to identify minor indications of pest infestations that are frequently undetectable by the naked eye (Iost Filho et al., 2020). Drones offer crucial insights into the health of crops, the spread of pests, and the environmental context. By utilizing machine learning and AI algorithms, the information gathered by drones can be processed to recognize and categorize pest types, their populations, and the level of damage to crops (Subramanian et al., 2021). The drones have revolutionized pest monitoring by allowing farmers to survey extensive agricultural areas swiftly and effectively. Timely detection of pests is essential for successful pest management, and drones enable farmers to spot infestations in their early phases, thereby lessening damage and decreasing pesticide application. By pinpointing areas with high pest concentration, drones facilitate targeted interventions solely where needed,

greatly lowering the environmental impact of pest management methods. Additionally, drones can enhance conventional ground surveys, offering a quicker and more efficient method for monitoring extensive regions. As noted by Iost Filho et al. (2020), drones' capability to map and monitor pest populations over time facilitates the development of adaptable pest management strategies that respond to pest behavior and agricultural conditions.

Robotic Systems for Precision Pest Control

Robotic systems play a crucial role in executing precise pest control strategies. These systems are developed to perform pest management activities on their own, minimizing the requirement for manual intervention and improving the overall effectiveness of pest management initiatives. Robots can be outfitted with sensors and actuators to detect pests and implement corrective measures, such as applying pesticides or introducing beneficial biological agents to the impacted regions. Basri et al. (2021) examine how autonomous robots can be utilized for various tasks in pest management, such as the selective release of natural predators, including parasitoids, or the application of small quantities of insecticides. The primary benefit of robotic systems is their capability to function independently, administering treatments solely in areas that require it, resulting in accurate pest control while reducing chemical usage and protecting the surrounding ecosystem. This level of precision is particularly crucial in agricultural settings, where excessive pesticide application can result in pest resistance and negatively impact non-target species. Moreover, these robots can move between plants or crops with little disturbance, guaranteeing that pest control actions are applied effectively and precisely. The creation of robotic systems designed for particular pest management functions provides versatility and adaptability throughout various agricultural environments (Gonzalez-de-Santos et al., 2017).

Collaboration Between Drones and Robotics in Insect Pest Control

The joint application of drones and robotic systems presents considerable benefits for integrated pest management (IPM). Drones offer up-to-date information and monitoring capabilities, while robots utilize this information to carry out targeted pest control measures. This collaboration results in a holistic, flexible pest management approach that optimizes the effectiveness of both technologies. For instance, drones can identify areas with pest problems within a field, and robots can then autonomously approach these areas to introduce beneficial insects or administer targeted treatments. Combining fleets of drones and robots for pest management

enhances both the accuracy and efficiency of pest control while decreasing dependence on chemical pesticides, resulting in more sustainable farming methods (Gonzalez-de-Santos et al. 2017). The combination of drones and robots enables a data-centric strategy for managing pests, which is essential for making well-informed choices. Immediate insights from drone information can be utilized to enhance robotic actions, ensuring that pest control efforts are executed at the most effective time and place (Iost Filho et al. 2020).

Prospective Pathways and Obstacles

Although the possibilities of utilizing drones and robots for pest management are vast, numerous challenges still need to be overcome. One major obstacle is the expense associated with these technologies, which poses a considerable barrier for small farmers. Despite the reduction in prices of drones and robots in recent years, affordability continues to be a concern in certain areas. In addition, the technical intricacies of these systems necessitate training and support to guarantee their successful implementation and use. The effectiveness of drones may be influenced by weather factors like wind and rainfall, while robots need to be able to maneuver through different terrains and types of crops (Basri et al. 2021). The ability of these systems to adapt and perform reliably under actual conditions is essential for their enduring success. Furthermore, concerns related to regulations and safety are important issues that must be considered. Utilizing drones necessitates compliance with airspace laws, while autonomous robots must be equipped with safety mechanisms to avoid accidents and ensure they do not disrupt other farming activities.

CONCLUSION

AI has become a fundamental component in contemporary pest management strategies, providing effective solutions for observation, prediction, and timely alerts. As these advancements progress, they are expected to enhance pest control by making it more anticipatory, accurate, and eco-friendly. There are limitations and **numerous obstacles while using AI, as such, (i) AI models frequently struggle when evaluated in unfamiliar settings because of differences in background, lighting conditions, or pest structure, (ii) creating extensive, well-annotated datasets remains a significant hurdle, particularly for uncommon or elusive species, and (iii)** numerous deep learning models operate as "black boxes," which hinders trust and acceptance among users who value explainable decision-making. Finally, we conclude that AI is an advanced innovative approach to transform our traditional methods for dealing with insect pests

and plant diseases. By improving early detection and diagnosis, as well as forecasting and strategic planning, AI technologies have elevated our potential to safeguard crops more precisely and with reduced environmental harm. As these technologies progress, their incorporation into regular agricultural practices will be essential for maintaining food security and promoting sustainable farming globally.

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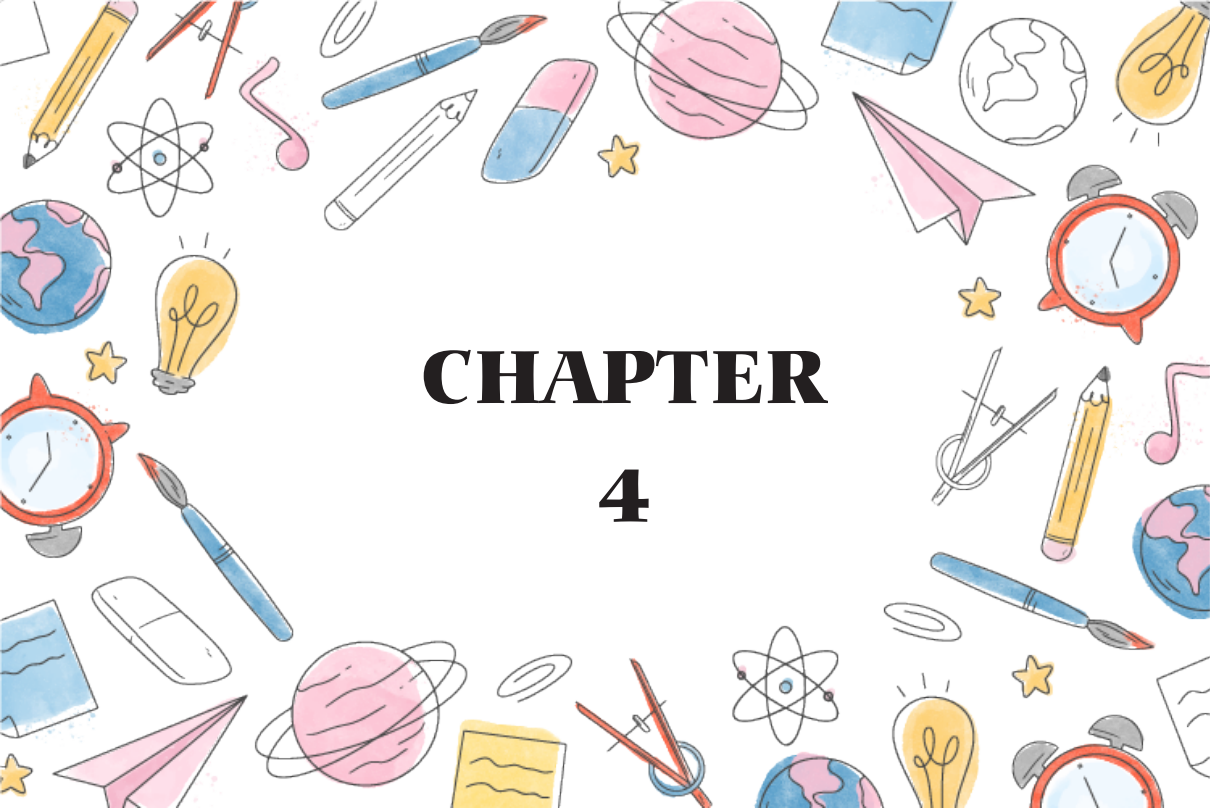

CHAPTER 4

STRUCTURE AND MORPHOLOGY OF FRUITING BODIES IN MYXOMYCETES (MYXOGASTREA) IV: COLUMELLA AND PSEUDOCOLUMELLA

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Abstract: Columella is a structural extension of the stem that develops towards the sporangium. In a sporophore, the columella is a mass of structures found in the area inside the peridium, which starts at the point where the structure touches the substrate and extends upwards in a dome-shaped shape within the sporophore. Pseudocolumella are structures that are not attached to the stem, found only in Physarida, and are found as a mass of lime within the mass of spores and capillitium. Capillitium may be attached to the columella or pseudocolumella. The columella or pseudocolumella may or may not be evident in a mature fruit body. In some species, it may not resemble a stem. The presence or absence and color or shape of the columella is an important diagnostic feature of description Myxomycetes.

Introduction:

Myxogastria; myxomycetes, true slime molds or plasmodial slime molds, have been known since at least 1650 and are considered members of Protista (Martin and Alexopoulos, 1969). Fungi have a cell wall. They decompose their food with extracellular enzymes into monomers in the external environment and they ingest them. Myxomycetes plasmodium structure does not have a cell wall, plasmodium has only a cell membrane. Spores have a very strong cell wall. Myxomycetes plasmodium is just a large amoeba that divides and grows continuously as long as conditions are suitable. It catches its prey, food, with its pseudopods by phagocytosis, swallows and feeds. In other words, slime molds swallow their food whole and then digest it with their intracellular enzymes. When the plasmodium food runs out or unsuitable conditions begin, it forms fruiting bodies in the shape and number appropriate to its genetic structure. Usually the fruiting body of slime mold is quite small, 1-2 mm, but some species can grow up to 25 cm in diameter (Baba and Sevindik, 2022a).

Taxonomic categories of Myxomycete are in Kingdom Protozoa, Phylum Amoebozoa, Divisio Mycetozoa and Subdivisio Eumycetozoa (Akgül et al., 2021; Baba and Sevindik, 2023). Amoebozoa is a group of very large and cosmopolitan organisms, the base of the tree containing eukaryotic organisms. Typical for almost all Amoebozoa is the amoeboid cell movement displayed at least at some stages of their sometimes complex life cycles. They exhibit typical eukaryotic features, such as a nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus, feeding by phagocytosis, possession of one or two flagella, a cytoskeleton containing actin and myosin, and sexual recombination processes. However, some of these features have been secondarily lost in some groups. Eumycetozoa, characterized by spore-bearing fruiting bodies, include Dictyostelia, Protostelia, and Myxogastria (Schilde, 2019).

Myxomycetes have also been classified as animals, plants, fungi and protists in the past. The vegetative stage, plasmodium, is a multinucleated, synchronized mitotic division, continuously multiplying and consuming food in an amoeba-like manner. Myxoamoeba and myxoflagellates, which occur before the formation of plasmodium, resemble protozoan-like organisms in the animal kingdom. Plasmodium develops into spore-producing fruit bodies as a result of growth and development. These spore-bearing mushroom-like fruit bodies and their ecological requirements also cause these organisms to resemble fungi (Lado and Eliasson, 2021).

The idea that myxomycetes are closely related to fungi has been accepted for many years. Like fungi, they were thought to be primarily found in temperate forest ecosystems. However, they were found to be part of a supergroup called Amoebozoa. Myxomycetes have been detected in all types of plant material and some animal material in terrestrial ecosystems and in or near aquatic ecosystems (de Basanta and Estrada-Torres, 2022).

According to the classification made by Kirk et al. (2001), there are three classes in the Myxomycota section: Protosteliomycetes, Dictyosteliomycetes and Myxomycetes. There are 5 orders in Myxomycetes; Echinostelida, Liceida, Physarida, Trichiida and Stemonitida, 14 families, 79 genera and 1152 species have been described (Lado, 2005-2025). The number of species in Türkiye is 311 and these species belong to 5 orders, 12 families and 50 genera (Baba and Sevindik, 2024).

The generative structure of myxomycetes is generally small in size. Depending on the genetic structure of the species and the plasmodium type, one or many sporophores can be produced from each plasmodium. Sporophores of Myxomycetes are usually including 6 parts: Hypothallus, Stalk, Columella or Pseudocolumella, Peridium, Capillitium or Pseudocapillitium and Spores (Lado and Pando, 1997). Columella is a dome-shaped, spherical, or elongated central sterile structure within the sporotheca that represents an extension of the stalk into the spore chamber, but may not resemble the stalk. It may be of various sizes and may serve as a supporting structure for the capillitium that may be attached in part or throughout its length. It may be a short protrusion or extend to the top of the sporangium. When a species has a columella, species identification is easier and is used as an important marker in keys to distinguish different species. It is a continuation of the stalk and may enter the spore pouch. Also, it may exhibit a spherical, conical or elongated structure that spreads from the base of the sporangium. It is on the hypothallus or capillitium in those without a stalk. It could reflect a similar structure to the stem and peridium, or it could have a very different structure. The columella is absent in order Liceida and Trichiida (Baba and Akgül, 2023).

The pseudocolumella is a free-hanging calcareous mass located at the centre of the sporophore, resembling a true columella but detached from the tip of the stalk or from the inner surface of the peridium. The pseudocolumella is a free columella that is not attached to the stalk. Pseudocolumella are found only in the order Physarida. The capillitium may be attached to the columella or pseudocolumella in some species. Pseudocolumella is mostly a calcareous rod or spherical structure and includes a fusion of lime knots collected in the center of spore masses. It does not depend on peridium or stem (Keller et al., 2017).

Development of Reproductive Structure in Myxomycetes:

The life cycle of myxomycetes consists of two stages; vegetative and generative stages. The vegetative stage is the free-living plasmodium and the generative stage is the fruiting body with unique structures and colors. The life cycle of myxomycetes undergoes a transformation from diploid multinucleate plasmodium to haploid fruiting bodies (Li et al., 2020). Myxomycetes produce spores that germinate as Myxoamoebo or Myxoflagellates. The Myxoamoebo or Myxoflagellates are trophic state of myxomycetes. Eventually, Myxoamoebo or Myxoflagellates develops into a plasmodium (Spiegel et al., 2004). The plasmodium is the most distinctive stage of the myxomycete, an assimilating structure that is essentially a naked, free-living, multinucleated, motile mass of protoplasm, varying in size and certain morphological details, although to a greater or lesser extent between species. The second stage is the fruiting body, which varies according to age, the particular plasmodium species involved, the species involved, and to some extent the nature of the substrate on which it grows and environmental factors (Gray and Alexopoulos, 1968). Four different species of plasmodium are known: protoplasmodia, aphanoplasmodia, phaneroplasmodia and trichiaceusplasmodia (Wang et al., 2017). As the plasmodium begins to develop into a generative structure, it condenses into a mass similar to the primordium in macrofungi. Each primordium secretes a hypothallus onto the substrate, which then grows and develops on the hypothallus, forming a stalk of fibrous material. As the stalk elongates, the sporotheca, the structures within it, and the stalk develop and grow, secreting intraprotoplasmic material from the primordium to the growing tip. Fructification is complete when the protoplasm breaks into uninucleate structures, which develop into spores. The young spores undergo meiosis and become haploid, with 3 of the 4 meiotic nuclei disintegrating and 1 remaining. When fruition reaches full maturity, the spores are released and the life cycle begins again (Alexopoulos et al., 1996; Stephenson et al., 2000).

The stalk is a structure secreted by the plasmodium during the life cycle from the plasmodium to the generative structure and separates from the sporotheca at the basal part of the peridium. The hypothallus is a part of the stalk that is present in some species and attaches to the substrate. It is a membranous structure that is continuous with the tubular region of the stalk in some species and filled with waste material or lime in others. In most species the stalk consists of a fibrous membranous tube, usually folded longitudinally to form striations. Lime crystals may also be deposited on both the inner and outer sides of the columella, capillitium, stalk and peridium. In stalked Myxomycetes, the columella begins at the basal part of the sporotheca and forms on the expanded stalk apex (Weldon, 1955). The columella consists of a thickened basal plate at the stalk apex, usually an expanded continuation of the stalk tube, formed by waste material or lime deposits, and a peridial membrane covering the basal plate and merging with the stalk. The structure, color, and shape of the columella vary from species to species. However, depending on the shape and size of the stem apex, it can be discoid, globose, or clavate. Most sessile, sporangial, and plasmodiocarpous species lack a columella. However, sometimes a thick peridial basal plate is seen, forming a flat, curved, wall-like pseudocolumella of variable structure and shape (Clark and Haskins, 2018).

There are four types of fruiting bodies in Myxomycetes: sporangium, plasmodiocarp, aethalium, and pseudoaethalium. Species with fruiting bodies in which the spores develop inside a membranous peridium-like structure, a sac, are called endosporous Myxomycetes. There are five recognized orders of endosporous myxomycetes: Echinosteliida, Liceida, Physarida, Stemonitida, and Trichiida (Baba H, 2015a).

The generative structure of Myxomycetes usually consists of two parts: sporotheca and stalk (Lado and Pando, 1997). Sporotheca is the spore-bearing part of the sporophore and consists of peridium, capillitium or pseudocapillitium, columella or pseudocolumella and spores. The other parts of the sporophore usually consist of two parts: stalk and hypothallus. However, not all of these structures are present in every sporophore. In some fruit bodies, hypothallus, stalk, columella/pseudocolumella or capillitium/pseudocapillitium may or may not be present. All these structures may not be present in all species and all fruiting bodies. All these structures and their characteristics are very important taxonomic criteria based on which classification is based (Baba H, 2012b).

The columella is a sterile structure that continues into the sporocyst of the peduncle or a thickening of the base of the sporocyst. They are elongated or knob-shaped structures that develop inside the sporotheca, which can be of different shapes and colors. In many stalked sporocarps, it is an extension of the peduncle and is usually of the same structure as the latter.

In sessile sporocarps, it is located at the base and may be formed by a simple thickening of the peridium or by a concentration of calcium carbonate. In these cases, it is usually subglobular or hemispherical in appearance. The columella can be cylindrical, usually pointed, as in many species of the Stemonitales, or spherical, as is often the case in the genus Diderma Pers., or sometimes comb-shaped, with a developed basal thickening in the plasmodiocarps. In some species of the Physaraceae, a columella-like collection of calcareous capillitium is seen in the central parts of the sporocyst. (Gøtzsche, 2019).

Pseudocolumella are columella-like structures resulting from the condensation of calcareous nodules of the capillitium within the sporotheca. It is never attached to the base of the sporotheca. A columella-like formation has nothing to do with a columella, but is often called a pseudocolumella. Pseudocolumella In calcareous genera, a mass of coalesced calcareous nodes resembling a columella but not attached to or part of the stem or base. In some stipitate species of the genus Didymium Schrad., a basal invagination of the peridium has been interpreted as a pseudocolumella (Lado and Rojas, 2020).

Different types of Columella and Pseudocalumella in Myxomycetes:

In a complete Myxomycete the fruiting body usually consists of 6 parts: hypothallus, stalk, columella, peridium, capillitium and spores. In some fruiting bodies a pseudocolumella or a pseudocapillitium may be present instead of the columella and capillitium. The capillitium elements may be attached to the columella or pseudocolumella. Not all of these 6 different components are present in all types of fruiting bodies in Myxomycetes. The columella or pseudocolumella structure is completely absent in the Liceida and Trichiida species. It is found in the orders Echinostelida, Physarida and Stemonitida. The pseudocolumella is found only in the order Physarida and is present as a calcareous mass within the spore mass Baba and Akgül, 2024).

The columella is an extension of the stem into the sporotheca, a continuation of the stem but may not resemble a stem. In a fruiting body, the columella may often appear as a dome-shaped structure or a continuation of the stem in the inner region of the peridium. It may also be a globular, conical, or elongated structure radiating from the base of the sporangium. In species without a stem, it is on the hypothallus or capillitium. It may have a similar structure to the stem and peridium, or it may have a very different structure (Baba H, 2015b).

The pseudocolumella is a mass of lime found only in the order Physarida, a Columella not attached to the stalk. The capillite elements may be attached to the columella or to the pseudocolumella. The pseudocolumella is mostly a mass of lime or a globular structure, consisting of a fusion of lime nodes collected in the center of the spore mass. It is not attached to the peridium or stem. The pseudocolumella is a free, globular to irregular, sometimes cylindrical lime mass found in the center of the fruiting bodies of various species, usually not attached to the stalk, but sometimes connected to the peridium by calcareous branches. In many species, the pseudocolumella is formed only by a denser aggregation of capillite lime nodes toward the center of the sporangium, or is either very pronounced or absent in different species of the same genus (Farr, 1976).

In order Echinostellida the columella is usually conical, cylindrical or fusiform. In the family Clastodermataceae the columella is short, reaching halfway down the sporotheca. The genus Barbeyella has minute, petiolate, globose sporangia with columella; the dark stalk is filled with dark granules, The columella is like an extension of the stem. Clastoderma A. Blytt has short or obsolete columella. A capillitium arises from the top of the columella in several branches, these branches fork several times at an acute angle, but do not merge into a network, the terminal branches are long and free or only connected at their ends by permanent fragments of the sporangial wall, branching and sparsely anastomosing threads arise from the columella or base of the sporangium, threads carrying the peridial platelets at their free ends, repeatedly forked as solid, pale brown threads. In *Clastoderma debaryanum* A. Blytt Columella is almost absent, causing early emergence of the few fine threads forming the capillitium by repeated bifurcations. Peridium disappears early, except for small fragments remaining at the ends of the capillitium and a narrow collar at the base of the columella. Capillitium ends persistent, smooth, with red-brown peripheral platelets, branched dichotomously two or more times between the columella and the periphery, tapering outwards (Figure 1).

In the family Echinosteliaceae the lower part of the stem is filled with granular material. Columella present or absent, sometimes a spore-like body attached to the top of the stem, usually a conical, cylindrical or fusiform columella. In Echinostelium de Bary and *Echinostelium minutum* de Bary Columella very short, conical, cylindrical or fusiform, light brown, not exceeding 3-10 µm in length. The spores are transparent, spherical, with very finely toothed, typically two spores attached to the base of the columella (Figure 2).

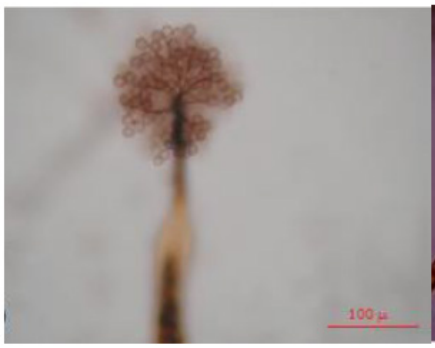

Figure 1. Clastoderma debaryanum

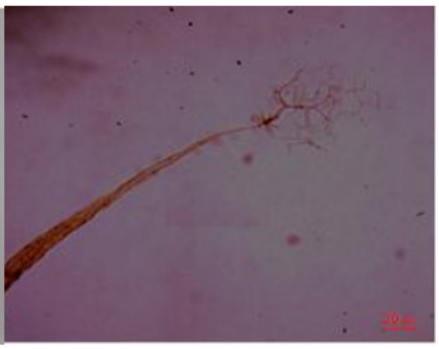

Figure 2. Echinostelium minutum

Order Liceida Fruiting bodies may be petiolate or sessile, with or without calcareous deposits on some parts. Members of this order have four types of fruiting bodies. True capillitium and columella are absent, sometimes pseudocapillitium consisting of irregular elements is present. In the family Reticulariaceae, the most important feature of *Siphoptychium* Rostaf. is the branching of the columella and the presence of perforations where the columella joins the peridium. Both of these features bring *Siphoptychium* close to *Alwisia* Berk. & Broome (the latter lacks a columella, but the capillitid threads have a structure and ornamentation similar to the branches of the columella in Siphoptychium).

Order Trichiida Fruiting bodies sporangia, rarely plasmodiocarps, petiolate or sessile, gregarious or compact, no calcareous deposits in the structure but sometimes present in the peridium. The stalk is filled with spore-like cells or granular materials. Columella is absent, never present. Members of this order are easily distinguished from other mycetozoa by the absence of a columella, spores usually brightly colored, capillitium threadlike, ornamented or smooth (Baba et al., 2024).

In the order Physarida, some parts of the fruiting bodies are calcareous; stalk, columella, capillitium or peridium. In the family Didymiaceae, columella may or may not be present. Occasionally, a pseudocolumella is usually prominent and well developed. Capillitium extends from sporangium base or columella to peridium wall, unbranched or several times branched outwards. *Diachea* Fr. stem and columella thick, erect, rigid, tapering upwards, white or yellowish, filled with minute, rounded lime granules. True columella always present. Columella continuous with stem, calcareous, also with lime granules or crystals. Capillitium non-calcareous, arising from numerous points of columella. This genus is scarcely distinguishable from *Lamproderma* Rostaf. except for the white lime mass filling the stem and columella. In *Diachea leucopodia* (Bull.) Rostaf. Columella

cylindrical, thick, calcareous, reaching almost to the apex of sporotheca. Capillitium threads arising from entire length of columella, pale at ends (Figure 3). *D. dictyospora* (Rostaf.) J.M. García-Martín, J.C. Zamora & Lado Columella pale brown, extending to the centre of the sporotheca, up to 0.5 mm high and 0.2 mm wide, lower part opaque dull brown, tip paler, filled with white lime. Tubular badhamoid capillitium, usually composed of non-lime threads or bands, arising from the columella and peridium, is present (Figure 4).

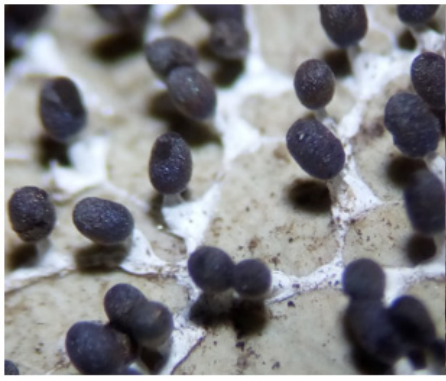

Figure 3. Diachea leucopodia

Figure 4. Diachea dictyospora

In the genus *Diderma* lime is present and more or less confined to the peridium and columella (when present). The capillitium is essentially non-calcareous and never has dilated calcareous nodes. Columella calcareous, usually well developed, distinct, sometimes reduced to a thickened or intrusive dome-like base, white to reddish brown. *Diderma montanum* (Meyl.) Meyl. Columella small, 0.4 mm max diameter, brownish-red, usually globose or sometimes hemispherical, sometimes petiolate, hemispherical to hemispherical at a limited base, rather smooth. Capillitium composed of fine purplish or transparent filaments, branched and anastomosed towards the periphery, radiating outwards from the columella, dark, loosely attached to the peridium, helically wound or wavy (Figure 5). *Diderma radiatum* (L.) Morgan Columella large, hemispherical to subglobose, convex, rough orange-brown to reddish brown (Figure 6).

Figure 5. *Diderma montanum*

Figure 6. *Diderma radiatum*

In the petiolate species of the genus Didymium the columella is formed during development on the enlarged petiole apex of the basal part of the sporotheca (Weldon, 1955). Thus the columella usually consists of an elongated continuation of the petiole tube, a thickened basal plate formed by continuous debris or lime deposits, and a peridial membrane covering the basal plate and fusing with the petiole. The shape of the columella may vary from discoid to globular or clavate, depending on the shape and size of the petiole apex. When the petiole apex is broad, a thick basal plate of the clavus type and a shallow umbilicus are produced. In most species of sporangia and plasmodiocarps the columella is absent but a thick peridial basal plate is usually present. This plate sometimes forms a columella such as the coiled wall seen in Didymium flexuosu (Clark and Haskins, 2018).

In petiolate *Didymium* species, the columella represents an extension of the stalk towards the sporangium. In some species, the columella appears continuous with the stalk in structure and morphology, while in others it may differ in structure, shape and colour. For example, some *Didymium* species have non-calcareous stalks but globular, calcareous columellae. In some species (especially *Diderma*), columellae can be found at the base of the fruiting body, ranging from a simple thickening within the peridium to a rather distinct dome-shaped or pulvinate mass. The characteristics of the columella are important criteria from a taxonomic point of view. *Didymium bahiense* Gottsb: Stem non-calcareous, conical, 50-75% of total height, thinner and orange-brown, yellowish above, translucent orange-red-brown below, almost black. Rounded calcareous pseudocolumella present at base of sporangia. Pseudocolumella, flat, discoid, white, small-laden. *Didymium columellacavum* Hochg., Gottsb. & Nann.-Bremek Sporotheca globose or hemispherical, a true columella, usually umbilicated below. Columella pale yellowish, hollow, filled with air and lime crystals, extending almost to centre of sporotheca, hemispherical, conical or cylindrical, sometimes with dilated tip or rarely funnel-shaped (Figure 7).

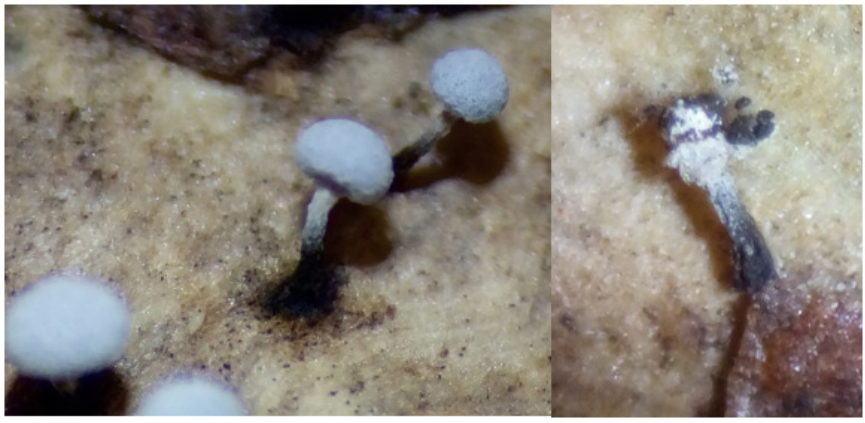

Figure 7. Didymium columellacavum

Didymium balearicum Ing Columella subglobose, calcareous, lemon yellow (Figure 8). *D. megalosporum* Berk and M.A. Curtis columella subglobose, discoid, yellow to orange-brown, rough or spiny above. *Didymium melanospermum* (Pers.) T. Macbr Columella large, hemispherical brown, calcareous, dark-colored, rough above, concave below (Figure 9).

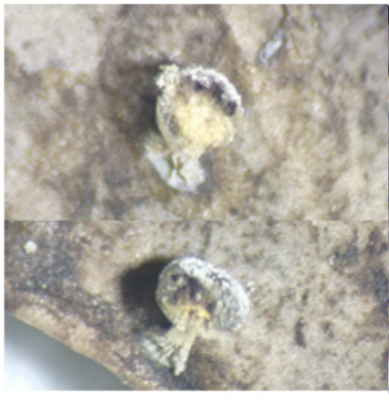

Figure 8. Didymium balearicum

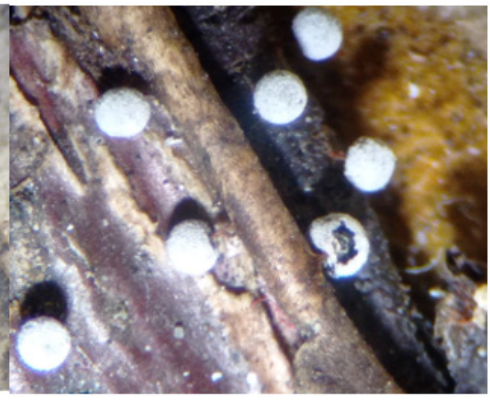

Figure 9. Didymium megalosporum

D. squamulosum (Alb. and Schwein.) Fr columella light colored, white or pale, discoid or hemispherical, thickened, umbilicated. It is an enlarged, spherical structure formed from the base of the sporotheca (Figure 10).

Neodiderma spumarioides (Fr. & Palmquist) X.F.Li, B.Zhang & Yu Li Columella convex or hemispherical, rough, globose, white or yellowish, calcareous. Capillitium pale-tipped, brown filaments, finely colored fila-

ments, occasionally ornamented with nodules, sparsely branched and anastomosed, rising from the columella (Figure 11).

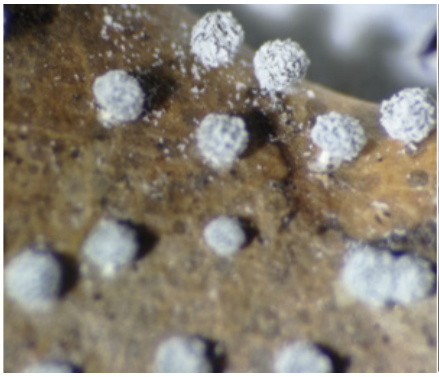

Figure 10. Didymium squamulosum

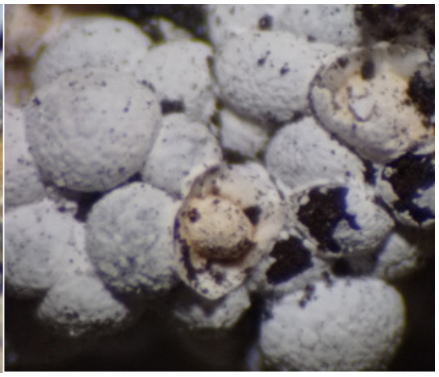

Figure 11. Neodiderma spumarioides

In the family Elaeomyxaceae and the genus *Elaeomyxa* Hagelst., wax is found in the form of granules, globules or inclusions in the stem, columella, often in the capillitium and wall.

In the family Physaraceae, fruiting bodies are in the form of sporangium or plasmodiocarp. The stem is present or absent, rarely extends as a columella within the sporangium. Columella is absent, rarely present, sometimes a calcareous pseudocolumella is present.

In the genus *Badhamia* Berk. Columella are present or absent. *Badhamia affinis* Rostaf thick tubules of capillitium, weakly branched and unconnected or sparsely connected, forming a rough basal lime plate, forming a sparse irregular network with broad expansions at the corners, radiating from a basal columella filled with white lime. *Badhamia panicea* (Fr.) Rostaf Pseudocolumella sometimes occurs with a denser development of the capillitium near the centre of the sporangium. Capillitium usually with a few non-calcareous tubules and often with many tubules, forming a pseudocolumella with broad expansions at the corners (Figure 12). *Badhamia utricularis* (Bull.) Berk. stalks weak, peridium thin, capillitium white (Figure 13).

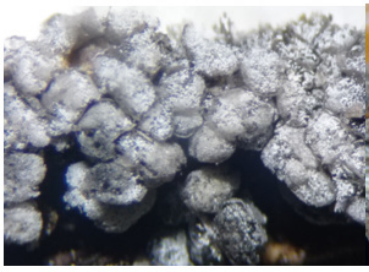

Figure 12. Badhamia panicea

Figure 13. Badhamia utricularis

In *Claustria* Fr. genera Columella absent, sometimes with a pseudocolumella. In *Craterium* Trentep genera Capillitium hyaline, thread-like tubes, often aggregated in the center to form a pseudocolumella. *Craterium aureonucleatum* Nann.-Bremek capillitium a net which mass together in the centre to form an orange-yellow pseudocolumella, the nodes sometimes contain yellow crystalline discs. *C. brunneolum* (W.Phillips) J.M. García-Martín, J.C. Zamora & Lado Columella absent, sometimes presents a pseudocolumela. *C. leucocephalum* (Pers. ex J.F. Gmel.) Ditmar Dehiscence circumscissile, occasionally somewhat irregular but in globose sporocarps always leaving a deep, goblet-shaped cup. Capillitium slender, hyaline tubules, often massed at the centre to form a prominent pseudocolumella (Figure 14).

In *Fuligo* Haller and *Fuligo cinerea* (Schwein.) Morgan Columella none. Capillitium tubules, lime nodes sometimes merged into a pseudocolumella in the middle of the tubes.

In *Leocarpus* Link genera columella none, but a pseudocolumella often present. Limeless threads consisting of a network of pale, slender, branching, mixed with broad, yellow tubules with expansions, yellowish or brownish lime-knots, filled with calcareous granules, occasionally massed in the centre to form a pseudocolumella. *Leocarpus fragilis* (Dicks.) Rostaf Columella pulvinate, large, filled with lime, creme to pinkish. Capillitium abundant, firmly attached to columella and peridium, limeless threads mixed with broad, yellow tubules with expansions, filled with calcareous granules, with hyaline blunt ends, occasionally massed in the centre to form a columella (Figure 15).

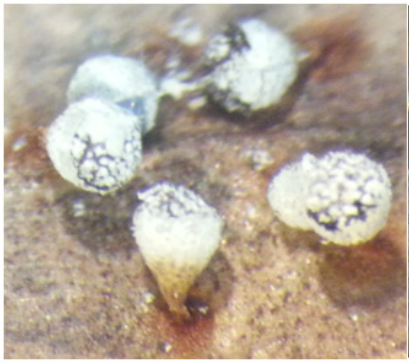

Figure 14. Craterium leucocephalum

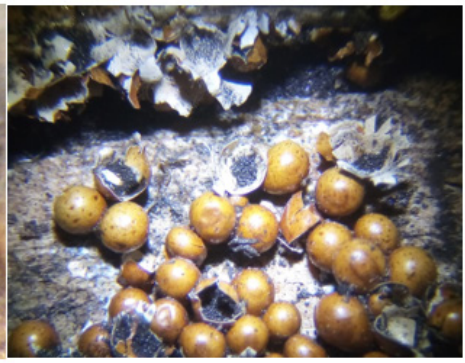

Figure 15. Leocarpus fragilis

In the genus *Nannengaella* J.M. García-Martín, J.C. Zamora & Lado the stem is calcareous, completely filled with lime. The columella is calcareous, usually dome-shaped or a conical extension of the stem. In some

cases, a calcareous pseudocolumella is present. *Nannengaella leucopus* (Link) J.M.García-Martín, J.C.Zamora & Lado The columella is usually absent, sometimes present in the form of a short conical protrusion. The capillitium is rather loose, sometimes clustered to form a central pseudocolumella *Nannengaella mellea* (Berk. & Broome) Massee The columella is small, conical, white or yellowish, clear, snow-white, with rather large angular stellate nodes.

There is no columella in the genus *Physarum* Pers. Capillitium netted, non-calcareous tubules connecting the calcareous nodes, sometimes the nodes agglutinate and form a pseudocolumella. *Physarum murinum* Lister Columella short, hemispherical or blunt-conical. *Physarum robustum* (Lister) Nann.-Bremek Pseudocolumella present, formed by aggregation of calcareous nodes of the capillitium, as a flat basal thickening, whitish. Capillitium netted, dense with sparse filiform nodes, rigid and radiating from a pseudocolumella, oblong to fusiform calcareous nodes, white, interconnected by colorless tubules up to 2 μm in diameter. *Physarum schroeteri* Rostaf Columella large, subglobular or clavate, yellow. Capillitium composed of stiff yellow filaments radiating from the columella, branched and anastomosed, sparse narrow yellow nodes, few, small, linear or fusiform nodules (Figure 16). *P. clavisporum* G. Moreno, A. Sánchez, A. Castillo & Illana Sporotheca spherical or oval, plasmodicarps straight or tortuous. Peridium paired in plates of different sizes, irregularly opening, capillitium very abundant, composed of short and transparent filaments, connecting the nodules to the peridium, completely covering the inside of the sporotheca (Figure 17).

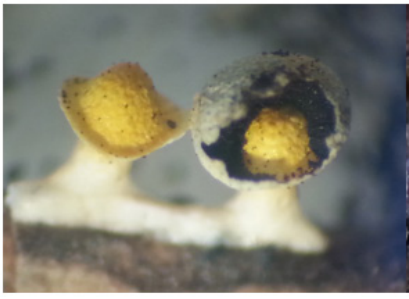

Figure 16. Physarum schroeteri

Figure 17. Physarum clavisporum

In order Stemonitida has got columella, the capillitium, consisting of simple, branched arising from the columella or from the base of the sporophore, is present. The columella is usually present and extends from the centre or apex of the sporangium. Family Stemonitidaceae stalk tapers upwards and continues as a more or less elongated columella within the sporangium. The columella is well-developed as a true columella in some

species. The capillitium, arising from numerous points on the columella, consists of a network of dark fine brown threads, mostly well developed and branched, persistent and smooth.

In *Amaurochaete* Rostaf. Columella black, rising from a dark, membranous hypothallus, irregularly branched and anastomosing, merging into the strands and threads of the capillitium. *Collaria* Nann.-Bremek. Columella cylindrical, thin, reaching 33-50% of the sporotheca and anastomosing there densely, dividing into 2 or more main branches. *Collaria arcyrionema* (Rostaf.) Nann.-Bremek. ex Lado Columella cylindric, slender, reaching 33-50% the height of the sporotheca, with some expansions in the axils, giand rise to the densely anastomosed outer capillitium. Capillitium threads arise either from the apex of the stem or from the tip of a very short columella. *Collaria lurida* (Lister) Nann.-Bremek. Stalk black, 50-75% of the total height. Columella cylindric, short, rarely reaching to 50% the height of the sporotheca and divided at the tip into several stout branches which giand rise to most of the capillitium.

Comatricha Preuss Columella usually reaching nearly to the apex of the sporangium. *Comatricha elegans* (Racib.) G. Lister Columella absent or short, rarely reaching half way up the sporotheca, at first divided into a few main branches, splitting abruptly into several thick branches sometimes repeatedly dichotomously branched. Capillitium thin, main branches radiating upwards from the stalk apex or columella tip, dichotomously branched and often anastomosed into a net in the basal part, tapered gradually towards the periphery where they form many free ends (Figure 18). *Comatricha ellae* Härk Columella 50-66% of sporotheca height, reaching the centre, 2-4 branches usually directed at right angles to the columella. Capillitium dark, flexuose, arising from all parts of the columella, branched and anastomosed to form a surface reticulum with no or few free ends. *Comatricha laxa* Rostaf Columella black and opaque, merging into the capillitium nearly reaching the apex. *Comatricha longipila* Nann.-Bremek It reaches the top of the sporotheca and has a columella structure that merges with the capillitium. The primary branches of the capillitium are perpendicular to the columella, with free ends.. *Comatricha nigra* (Pers. ex J.F. Gmel.) J. Schröt Columella is half to three-quarters the height of the sporangium, sometimes reaching almost to the top. Capillitium forms a network attached to the columella. *Comatricha pulchella* (C. Bab.) Rostaf Columella tapered, reaching the apex with the capillitium. Capillitium flexuous reddish-brown, primary branches at right angles to the columella, branched, flexuous, forming an internal net, looped at the surface but not forming a surface net, consisting of thin threads through- out, without free ends (Figure 19).

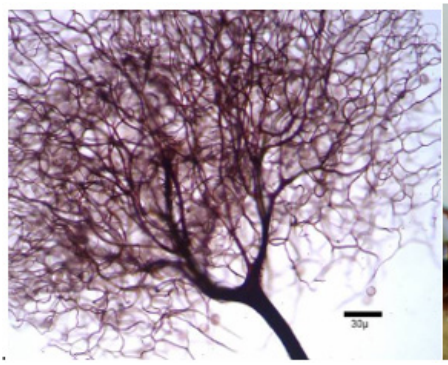

Figure 18. Comatricha elegans

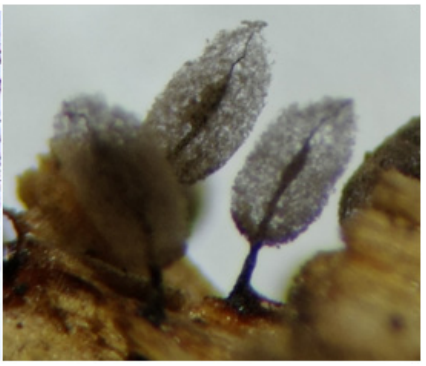

Figure 19. Comatricha pulchella

Enerthenema Bowman genera and *Enerthenema papillatum* (Pers.) Rostaf Stem thick, stout, tapering upwards, entering the sporangium and extending to its apex, continuing to the apex of the sporangium as a columella, where it widens into a cup-like disk to which the capillitium is attached. The columella reaches the apex of the sporotheca and forms a small, shiny disk up to 0.2 mm in diameter, to which the capillitium is attached to the margin (Figure 20). *Lamproderma* Rostaf. Stem more or less long, tapering upwards and entering the sporangium as a short columella. Columella cylindrical or clavate, half to two-thirds the height of the sporangial cavity, rarely shorter or absent, almost always blackish. Capillitium arising mainly from the apex of the columella, they approach the periphery. *Lamproderma laxum* H. Neubert Stem black, Columella 33-50% of the height of the sporotheca, cylindrical, rounded at the apex (Figure 21). *Lamproderma scintillans* (Berk. and Broome) Morgan Columella cylindrical, blunt, reaching the centre of the sporotheca. Capillitium radiates from the apex of the columella, hyaline at the base, later smooth and dark brown filaments.

Figure 20. Enerthenema papillatum

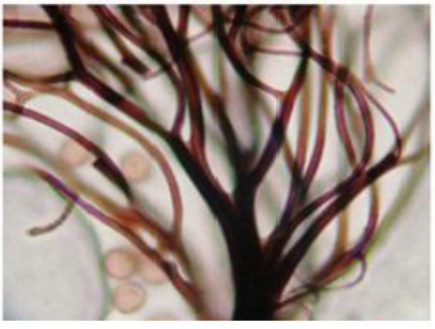

Figure 21. Lamproderma laxum

Macbrideola H.C. Gilbert Columella large, globose. Capillitium absent or represented by a slight branching at the tip of the columella, more or less flexible and tapering towards the periphery. *M. cornea* (G. Lister and Cran) Alexop columella reaching the centre of the sporotheca and penetrating into the sporotheca up to 100-110 μm. *Macbrideola decapillata* H.C. Gilbert Stalk black, columella give rise to short projections, rounded or branching reaching about two-thirds of the length of the sporotheca. *Macbrideola martinii* (Alexop. & Beneke) Alexop. Columella reaching to the centre of the sporotheca (Figure 22). *Macbrideola synsporos* (Alexop.) Alexop. Columella reaching the centre of the sporotheca. Capillitium lax, arising from the tip and sometimes the sides of the columella, branched and anastomosed (Figure 23).

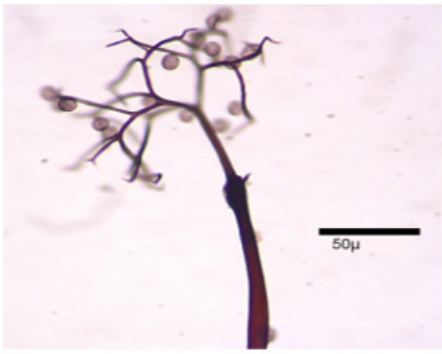

Figure 22. Macbrideola martinii

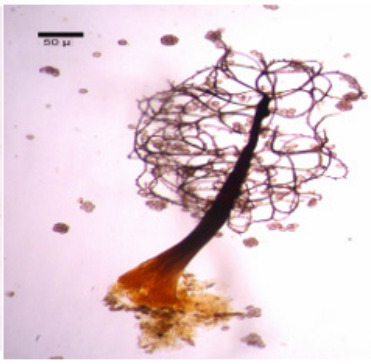

Figure 23. Macbrideola synsporos

Meriderma Mar. Mey. & Poulain Columella cylindrical, reaching the center. Capillitium arising from the length of the columella, rigid, straight, frequently branched and sometimes anastomosed, dense, uniformly dark brown. *Paradiacheopsis* Hertel The stalk is very long, continuing to the centre of the sporotheca as a columella, contains opaque, black and compactly converged fibrils. Capillitium lax originating from the tip of the columella and containing 2 (3) thin, black branches and drooping with very thin and extended ends. The primary branches of the capillitium are distinctly perpendicular to the columella. *P. fimbriata* (G. Lister & Cran) Hertel ex Nann.-Bremek. Columella tapered and ending abruptly. Capillitial threads usually branched dichotomously once or twice, thin at the connection to the columella, swollen and club-shaped at their tips (Figure 24). *P. longipes* Hooff and Nann.-Bremek Columella reaches to the centre of the sporotheca. Capillitium usually 2-4 branches arising from the apex of the columella. *Stemonaria* Nann.-Bremek., R. Sharma & Y. Yamam. differ from *Comatricha* Preuss because of the longitudinally rigid, fibrous, or homogeneous construction of the stipe and columella. *Stemonitis* Gled.

Stalk extending into the sporangium as a columella. Capillitium arising from the entire length of the columella. *Stemonitopsis* (Nann.-Bremek.) Nann.-Bremek. columella usually extending apex, capillitium arising from the columella along its entire length consisting of thread like tubules. *S. reticulata* (H.C. Gilbert) Nann.-Bremek.& Y. Yamam. Columella reaching 75% of the sporotheca (Figure 25).

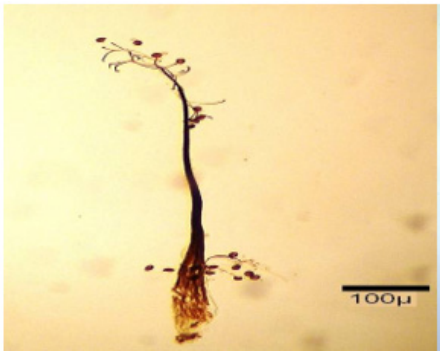

Figure 24. Paradiacheopsis fimbriata

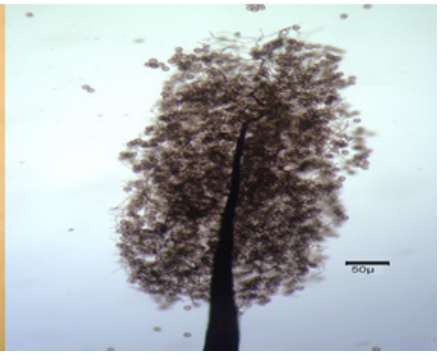

Figure 25. Stemonitopsis reticulata

Symphytocarpus Ing & Nann.-Bremek. Columella absent or when present, rather degenerate, irregular in shape. Capillitium connected to the columella. *Tasmaniomyxa* S.J. Lloyd, Leontyev, G. Moreno, López-Vill. & Schnittler Columella cylindrical, opaque. No lime deposits observed. *Valtocarpus* Gmoshinskiy, Prikhodko, Bortnikov, Shchepin & Novozh. Columella is irregular, branched.

Conclusion:

Columella A central sterile structure, dome-shaped, globose or elongated, within the sporotheca. It represents an extension of the stalk between the spores and the capillitium. It may be of various sizes, shapes and colours. The capillitium may be attached partly or completely along the length of the columella. It may be short or may extend to the apex of the sporotheca. Pseudocolumella A calcareous mass suspended freely in the centre of the sporotheca, resembling a true columella but separated from the tip and base of the stalk or from the inner surface of the peridium. When present, the columella or pseudocolumella is an important character in the species description of Myxomycetes and is used in keys to identify different species. This structure is absent in Liceida and Trichiida.

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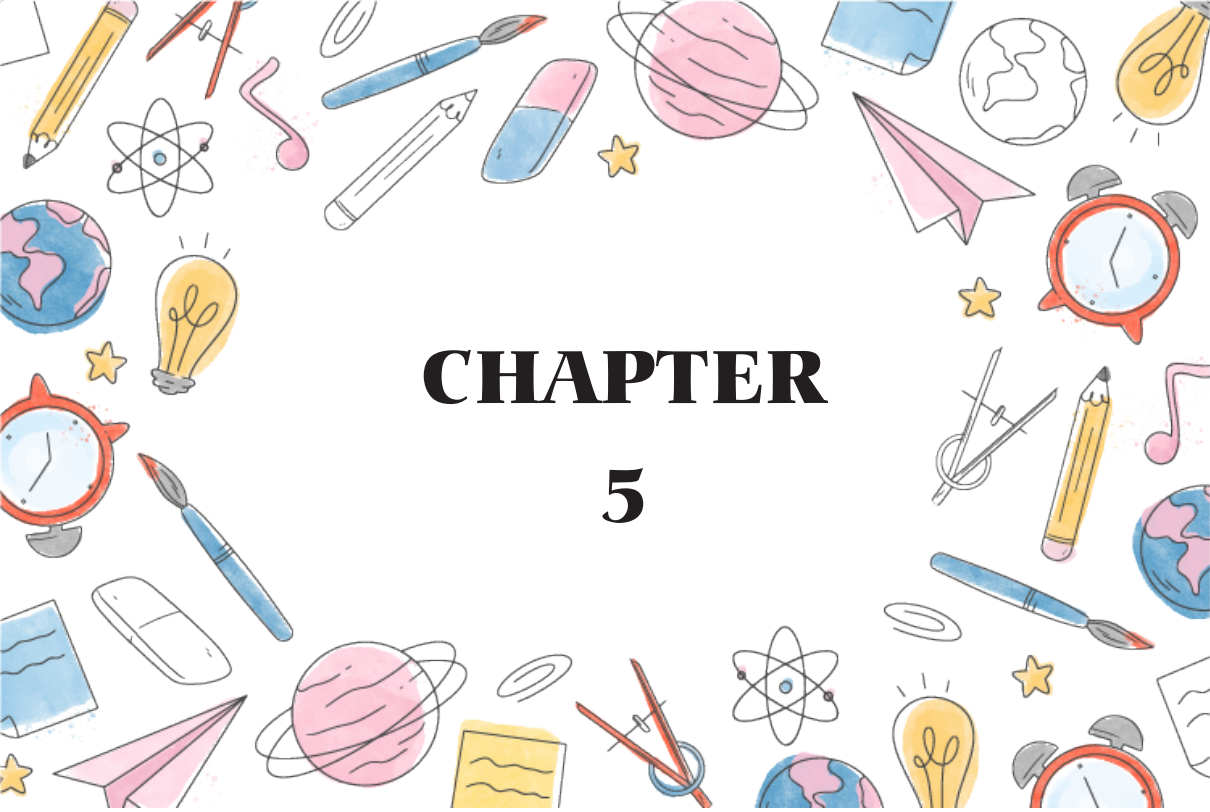

CHAPTER 5

THE SILENT THREAT OF PESTICIDES IN AQUATIC ECOSYSTEMS: HISTOPATHOLOGICAL ALTERATIONS IN FISH

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1. INTRODUCTION

The extensive application of pesticides in agriculture has led to growing concerns about their unintended consequences on aquatic ecosystems. Once introduced into water bodies through runoff, leaching, or irrigation discharge, these chemicals may accumulate and exert harmful effects on non-target aquatic organisms, particularly fish. Among the various manifestations of pesticide toxicity, histopathological alterations in fish tissues have garnered significant attention due to their reliability as early indicators of environmental stress.

Exposure to pesticides can result in distinct morphological and structural damage across multiple organ systems, including the gills, liver, kidneys, digestive tract, muscles, and nervous tissues. Such cellular and tissue-level disruptions often impair key physiological functions, affecting respiration, detoxification, osmoregulation, and neural signalling. This chapter delves into the spectrum of histopathological responses observed in fish following pesticide exposure, offering insight into organ-specific vulnerabilities and the potential use of these alterations as biomarkers for aquatic health assessment.

2. PESTICIDES

Pesticides are chemical or biological substances employed to suppress or eliminate harmful organisms that threaten crops and other living systems. This term broadly encompasses various categories such as insecticides (targeting insects), herbicides (aimed at weeds), and fungicides (used against fungal pathogens). These agents are commonly applied in agricultural fields, private gardens, and public areas to manage unwanted species. However, when pesticides enter aquatic environments, they pose significant ecological risks and may adversely affect both aquatic ecosystems and human health (Hassaan and El Nemr 2020).

Pesticides are crucial components in contemporary agricultural production, serving a vital function in safeguarding crops against weeds and detrimental insects. Their extensive utilization by agriculturists has been associated with substantial enhancements in crop yields. The swift increase of the global population in the 20th century would not have been sustainable without parallel advancements in food production, to which pesticides have significantly contributed. Approximately one-third of global agricultural production depends on pesticide usage. In the absence of these inputs, fruit yields may decline by as much as 78%, vegetable production by 54%, and grain yields by roughly 32%. Pesticides are deemed essential for enhancing productivity and alleviating the impact of plant-related diseases globally (Tudi et al., 2021).

In this context, it is crucial to examine the historical evolution of pesticide usage, their diverse classifications, specialized applications, environmental behavior, and potential ecological impacts. Research demonstrates that agricultural development has a historical presence in various regions globally, and the progression of pesticide application reflects this pattern. Pesticides can be classified according to their chemical makeup, functional groups, mechanisms of action, and toxicity profiles. Although these compounds are designed to eradicate pests and inhibit weed proliferation, they may also demonstrate detrimental effects on non-target creatures, including birds, fish, beneficial insects, and plants. Furthermore, they can accumulate in the atmosphere, soil, water, and vegetation, presenting dangers to larger ecosystems (Tudi et al., 2021).

Pesticides can be classified in a variety of ways based on their appearance, physical properties, formulation types, the target pest or disease group and its biological stage, the chemical group of the active ingredient, their level of toxicity, and method of application (Figure 1).

According to the type of organisms they are designed to control, pesticides are categorized as follows:

Insecticides: Used to eliminate harmful insects such as ants and other pest species.

Herbicides: Target unwanted plants including weeds and aquatic vegetation.

Fungicides: Applied to manage fungal infections in crops.

Rodenticides: Designed to control rodents such as mice and rats.

Nematicides: Used against plant-parasitic nematodes.

Molluscicides: Target mollusks like slugs and snails.

Algicides: Used to suppress the growth of algae in aquatic environments.

Acaricides: Effective against mites, ticks, and other small arthropods.

Based on their chemical composition, pesticides are typically grouped into three main categories:

Inorganic pesticides: Include compounds containing heavy metals such as arsenic, mercury, fluoride, and copper.

Synthetic organic pesticides: Comprise industrial chemicals such as organochlorines, organophosphates, organosulfurs, and carbamates.

Natural organic pesticides: Derived from natural sources, including plant-based compounds like rotenoids, pyrethrins, and nicotine derivatives (Korkmaz 2018).

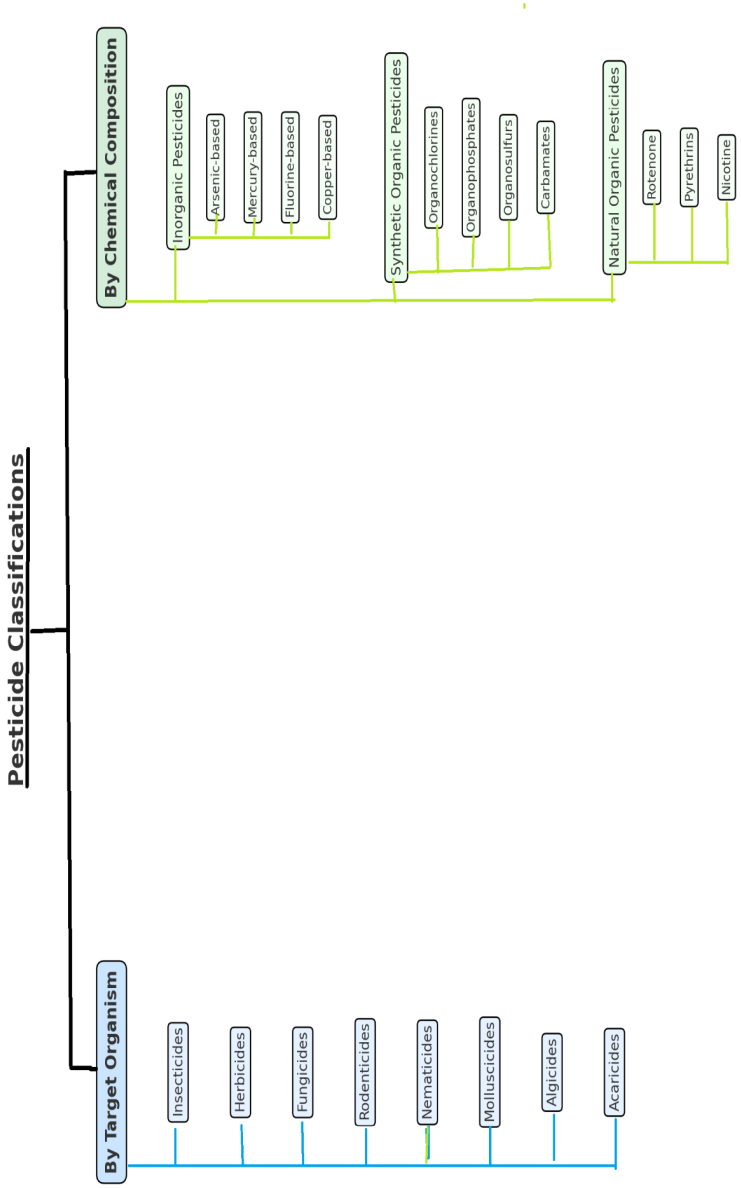

Figure 1: Classification of pesticides

The majority of pesticides possess organic molecular structures and exhibit hydrophobic characteristics. Due to their low solubility in water, they tend to bind to suspended particles and eventually accumulate in the sediment layers of aquatic ecosystems. This accumulation poses long-term ecological risks by introducing persistent toxic residues into benthic habitats, potentially impacting aquatic organisms and disrupting ecological balance (Korkmaz 2018).

3. PESTICIDE POLLUTION IN AQUATIC ECOSYSTEMS

Pesticides, which are widely used to combat harmful organisms in agricultural production, are toxic compounds that can affect not only the target organisms but also the entire ecosystem. It is known that pesticides are transported from agricultural areas to aquatic ecosystems through direct application, surface runoff, erosion, atmospheric transport and groundwater infiltration (Tang et al., 2021) (Figure 2). Especially in developing countries, intensive pesticide use, combined with inadequate environmental controls, leads to dangerous levels of pesticide accumulation in streams, lakes and wetlands (Li et al., 2015). In the study published by Tang et al. (2021) in Nature Geoscience, 64% of agricultural areas worldwide were determined to be at risk of pesticide pollution with global modeling developed based on 92 active pesticide compounds. Approximately 31% of these areas were included in the "high risk" class. The risk analysis covers the effects of pesticides on four different environmental components - soil, surface water, groundwater and atmosphere. This comprehensive model is important in terms of quantitatively revealing the environmental burden of agricultural areas caused by pesticides.

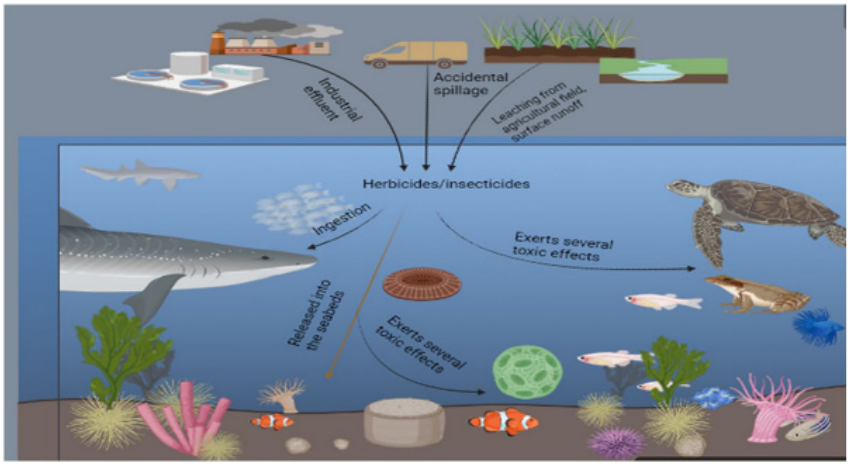

Figure 2: *Pesticide pollution in aquatic ecosystems (Okagu et al., 2023)*

Pesticide pollution can cause acute and chronic toxicity in aquatic ecosystems. Water-soluble pesticides can be directly taken in by fish and other aquatic organisms, which can cause cellular, physiological and behavioral disorders. In particular, pesticide mixtures increase their effects through synergistic toxicity and can cause serious harm even at low doses. Studies on aquatic invertebrates such as *Chironomus dilutus* have shown that pesticide mixtures can have more toxic effects than individual compounds (Maloney et al., 2018).

The spread of pesticides poses significant risks not only to ecosystem health but also to human health. People become exposed to various exposure routes through pesticides reaching drinking water sources, consumption of fish containing pesticide residues, or evaporation of these substances into the atmosphere (Nicolopoulou-Stamati et al., 2016). In fact, it has been widely reported in the literature that pesticides have direct effects on human health, such as cancer, hormonal disorders, and neurotoxicity. Advanced spatial modeling shows that areas at high pesticide risk often coincide with areas where biodiversity is high, water scarcity is common, and income levels are low (Tang et al., 2021). In this context, watersheds in countries such as China, India, South Africa, and Argentina have been defined as critical thresholds. The effects of pesticides on biodiversity are not limited to direct toxicity, but also manifest themselves through indirect effects such as habitat loss, population decline, and reduced genetic diversity (Maggi et al., 2020). These findings reveal that pesticides should not only be considered as a means of increasing agricultural productivity, but should also be addressed in a holistic manner in terms of the sustainability of aquatic and terrestrial ecosystems. Within the framework of sustainable agricultural practices, reducing pesticide use, disseminating biological control methods and monitoring toxicological effects are among the urgent priorities in terms of environmental and public health.

4. PESTICIDE EXPOSURE IN FISH

Pesticides are widely used chemicals in modern agriculture to combat pests and increase crop yield. However, their extensive use has led to their accumulation in various ecosystems, particularly aquatic environments. Fish, being integral components of these ecosystems, are highly vulnerable to pesticide contamination. These pollutants can reach water bodies through agricultural runoff, leaching, or atmospheric deposition, ultimately affecting fish physiology and health (Ekubo & Abowei, 2011; Islam et al., 2022).

Pesticide contamination poses multifaceted risks to fish populations. Toxicity can manifest in various forms, including oxidative stress, immune

suppression, reproductive dysfunction, and behavioral abnormalities. Histopathological changes such as cellular degeneration, necrosis, and tissue deformities in vital organs like gills, liver, and kidneys are frequently documented in exposed fish species (Mela et al., 2007; Ahmed et al., 2013). These effects compromise the survival, growth, and reproductive success of aquatic organisms.

Sources of pesticide pollution are diverse. In addition to direct applications in agriculture, improper disposal of pesticide containers, industrial activities, and domestic uses contribute to environmental contamination. Once introduced into aquatic systems, pesticides may undergo bioaccumulation and biomagnification, posing long-term threats not only to aquatic fauna but also to human health through the consumption of contaminated fish (Hampel et al., 2015; Bhat et al., 2017).

Histological biomarkers have emerged as crucial tools for assessing environmental pollution. The gills, liver, and kidney of fish are particularly sensitive to chemical stressors. Gills, due to their large surface area and direct contact with the external environment, exhibit immediate responses such as lamellar fusion, epithelial lifting, and hypertrophy upon exposure to toxicants (Au, 2004; Cengiz & Unlu, 2006).

Liver tissue, being a primary site for detoxification, often displays structural damage under chemical stress, including hepatocellular vacuolation, nuclear abnormalities, and sinusoidal dilations. Similarly, kidneys exhibit tubular degeneration and glomerular shrinkage as typical signs of pesticide-induced nephrotoxicity (Figueiredo-Fernandes et al., 2006; Barhoumi et al., 2012). Such pathological observations provide essential insights into sub-lethal effects and potential ecological risks.

Classification of pesticides is essential for understanding their mechanisms and environmental behavior. They are commonly categorized based on their target organisms into insecticides, herbicides, fungicides, and rodenticides. Among these, insecticides are the most prevalent in aquatic contamination due to their persistent and bioactive nature. Despite regulatory restrictions, many insecticides remain in use and continue to enter aquatic ecosystems through non-point source pollution (Akdoğan et al., 2012).

Toxicity evaluation through bioassays is fundamental in ecotoxicology. Acute toxicity tests, such as LC50 determination, help identify the concentration of a substance that causes mortality in 50% of the test organisms within a specified period. Fish species are ideal models for such tests due to their ecological relevance, ease of maintenance, and sensitivity to contaminants (Nazifi et al., 2000; Stara et al., 2019).

Preventive strategies and regulatory frameworks are vital to mitigate pesticide pollution. Sustainable agricultural practices, integrated pest management (IPM), and the use of biopesticides are promising alternatives. Environmental monitoring programs and stricter legislation on pesticide application and disposal can significantly reduce the ecological footprint of these chemicals (Carvalho, 2017; de Souza et al., 2020).

In conclusion, pesticide exposure in fish is a pressing environmental issue that demands urgent attention. The cumulative impacts of pesticide residues on aquatic life and ecosystem health underscore the need for interdisciplinary approaches involving toxicology, ecology, and environmental policy. Future research should focus on long-term and low-dose exposure studies, biomarker development, and eco-friendly alternatives to chemical pesticides.

4.1.1. Hematological Changes

Pesticide exposure can cause significant decreases or increases in erythrocyte (RBC), hemoglobin (Hb), hematocrit (HCT), total leukocyte (WBC) and platelet counts in fish. In particular, compounds such as fipronil, cypermethrin, diazinon and chlorpyrifos cause erythrocyte deformations, anemia and immune deficiency (Tahir et al., 2021; Rohani, 2023). For example, fipronil exposure in *Labeo rohita* caused decreases in RBC, Hb and HCT levels, and increased WBC and neutrophil counts (Ghaffar et al., 2021).

4.1.2. Biochemical Changes

As a result of the toxic effects of pesticides on the liver, significant increases in liver enzymes such as AST, ALT, ALP, and decreases in parameters such as serum total protein and albumin have been reported. These changes indicate that liver and kidney functions are impaired (Dogan and Can, 2011; Ghelichpour et al., 2017; Rohani, 2023; Mustafa et al., 2024). In addition, increases in the levels of MDA and ROS, which are markers of oxidative stress, and a decrease in the antioxidant defense system (SOD, CAT, GPx) have been reported (Korkmaz and Orun, 2020; Mustafa et al., 2024).

4.1.3. Genotoxic Effects and DNA Damage

Some organophosphate pesticides have significant genotoxic effects. In molecular biological tests such as micronucleus tests and comet assays, DNA chain breaks and nuclear anomalies have been observed (Tahir et al., 2021). Compounds such as dichlorvos have been shown to interfere with

DNA replication and cause chromosomal abnormalities (Mustafa et al., 2024).

Genetic damage markers such as DNA chain breaks, micronucleus formation, and nuclear anomalies have been reported as a result of pesticide exposure. These effects have been documented in detail in *Carassius auratus*, *Oreochromis niloticus* and *Cyprinus carpio* species. (Çavaş and Könen, 2007; Zeid and Khalil, 2014; Ambreen and Javed,2018; Tasneem and Yasmeen, 2018).

4.1.4. Histopathological Effects

Pesticide exposure causes significant histopathological changes in many organs, especially in the gills, liver, kidney and intestinal tissues. The most frequently observed effects include lamellar fusion, epithelial hypertrophy, hyperemia and necrosis in the gills; hepatocyte necrosis, vacuolization and intravascular hemorrhage in the liver; and glomerular deformation, tubular degeneration and cellular infiltration in the kidneys (Rohani, 2023; Mustafa et al., 2024). These structural deteriorations cause adverse effects on vital functions such as respiration, metabolism and osmoregulation in fish.

4.1.5. Mechanism of Action of Pesticides via Oxidative Stress

Heavy metals and pesticides cause oxidative damage by increasing the production of free radicals (ROS) in cells. This can trigger apoptosis by affecting the cell membrane, protein structures and nucleic acids. Pesticides can also disrupt gene expression by activating redox-sensitive transcription factors (NF-κB, p53) (Mustafa et al., 2024).

4.1.6. Endocrine System Disorders and Reproductive Effects

Some pesticides act as endocrine disrupting compounds (EDCs) and negatively affect gamete formation, fertilization and embryo development, especially through estrogen-like effects. This is among the factors that threaten the sustainability of fish populations (Mustafa et al., 2024).

4.1.7. Ecological and Human Health Consequences

Exposure of fish to pesticides causes negative consequences not only at the individual level, but also at the population and ecosystem level. In addition, the bioaccumulation and biomagnification of these chemicals pose a threat to human health. Consumption of fish containing pesticide

residues can lead to serious health problems in humans, such as endocrine disorders, genetic damage, and liver-renal toxicity (Rohani, 2023).

The damages caused by pesticides in aquatic ecosystems threaten the physiological, histological, and genetic integrity of fish. Therefore, reducing pesticide use, disseminating biological control methods, and regular monitoring of pesticide residues are of great importance. In addition, the harmful effects of pesticides can be reduced by measures such as breeding more resistant species in fish farms and supplementary use of phytobiotics.

5. HISTOPATHOLOGICAL EFFECTS OF PESTICIDES ON FISH

Pesticides, which are widely used today to increase agricultural production, can harm not only target organisms but also many living creatures living in aquatic environments. Pesticides carried from agricultural areas to lakes, rivers and other freshwater resources through surface runoff, evaporation or mixing with groundwater cause serious physiological and textural effects on non-target organisms, especially fish.

Fish are frequently used bioindicator species in environmental toxicity assessments due to their sensitivity to chemical changes in their environment and their position in the trophic chain. One of the most important biomarkers used to understand pesticide exposure in these organisms is histopathological changes. Organs that are metabolically active and in direct contact with environmental pollutants, such as gills, liver, kidneys and intestines, stand out as the most sensitive tissues to pesticides.

Lamellar fusion, lifting and increase in mucus cells in gills; hepatocyte vacuolization, necrosis and melano-macrophage accumulation in the liver; In the kidneys, deteriorations such as tubular degeneration and glomerular damage are among the most common findings regarding the negative effects of pesticides on tissues. These structural changes can disrupt the life functions of fish and negatively affect both individual health and biological balance at the population level. In this section, histopathological deteriorations caused by different pesticide groups in fish tissues will be discussed in detail; their mechanisms of action and ecological consequences will be evaluated in the light of scientific data.

5.1. Effects of Pesticides on Gills: Structural Deterioration and Mucus Secretion Changes

The investigation of histopathological changes in fish tissues has emerged as a critical tool in aquatic toxicology, particularly for monitoring the ecological impact of environmental pollutants such as pesticides.

Among the various tissues, gills are the primary interface between fish and their surrounding environment, making them especially susceptible to waterborne toxicants. Damage to gill structures can disrupt vital physiological processes, including respiration, ion regulation, and excretion, thereby threatening the organism's homeostasis.

Gill tissues are particularly vulnerable due to their extensive surface area and thin epithelial lining, which facilitates efficient gas exchange but also increases exposure to dissolved toxicants. Histopathological examinations commonly reveal structural alterations such as lamellar fusion, epithelial hyperplasia, aneurysms, necrosis, and hemorrhaging, especially in fish exposed to organophosphates and synthetic pyrethroids (Cengiz & Unlu, 2006; Ezemonye & Ogbomida, 2010).

Sublethal concentrations of pesticides like diazinon, deltamethrin, and fenitrothion have been reported to induce various histopathological lesions in the gills, including epithelial detachment, swelling, and cellular degeneration (Cengiz, 2006; Banaee et al., 2011). Such disruptions compromise the structural integrity of lamellae, reducing the efficiency of oxygen uptake and osmoregulation.

In experimental settings, acute and chronic exposures to malathion and glyphosate have led to significant pathological changes in fish gills, such as epithelial protrusion, cellular atrophy, and intraepithelial edema. These effects are dose- and time-dependent, with higher concentrations causing more extensive tissue damage (Selvarani et al., 2019; Hassan et al., 2022).

Nile tilapia (*Oreochromis niloticus*) exposed to lambda-cyhalothrin and chlorpyrifos exhibited prominent gill abnormalities, including vascular congestion, hypersecretion of mucus, epithelial necrosis, and aneurysm formation (Zahran et al., 2018; Subburaj et al., 2020). These histological markers are crucial indicators of pesticide-induced stress and cellular damage.

Epithelial hyperplasia, often observed in pesticide-exposed fish, reflects a defensive response aimed at minimizing toxicant entry. However, excessive cell proliferation can thicken the gill epithelium, reduce gas exchange efficiency, and elevate metabolic stress. These compensatory mechanisms may help short-term survival but impair long-term physiological stability (Rahbar et al., 2021).

Other pesticides, such as butachlor, captan, and tebuconazole, have also been associated with histological disturbances like epithelial detachment, eosinophilic infiltration, and vascular dilation. These lesions suggest

that even low-level pesticide residues in water bodies can lead to severe gill damage in fish (Boran et al., 2012; Vajargah et al., 2019).

Gill alterations including lamellar lifting, telangiectasia, and hemorrhaging have also been documented in various fish species exposed to herbicides like Roundup. Notably, these pathological features often appear at the lowest tested concentrations, indicating the high sensitivity of gill tissues to chemical stress (Günal et al., 2019; El-Garawani et al., 2022).

Fish gills have critical functions in terms of both respiration and ion balance, and are among the most vulnerable tissues to the toxic effects of pesticides. Gills are the entry gates for pesticides due to their large surface area and direct contact with the external environment, as well as the regions where the first and most obvious histopathological effects of exposure are observed (Tahir et al., 2021; Deveci et al., 2021). The most frequently reported gill damages in fish exposed to pesticides can be listed as lamella fusion, shortening of secondary lamellae, hyperplasia and hypertrophy in epithelial cells, lifting of lamellae (epithelial separation), necrosis, edema and vascular congestion (Kadiru et al., 2022; Mansour et al., 2025). For example, it has been reported that diazinon, an organophosphate pesticide, causes changes such as lamellar lifting, epithelial necrosis, and mucus cell increase in fish (Deveci et al., 2021). Deltamethrin, a pyrethroid group, causes secondary lamellae shrinkage and intravascular hemorrhage (Ghafarifarsani et al., 2023). Mucus cells are secretory cells that serve a defensive function in the gill epithelium and retain harmful particles. It has been reported that there is a significant increase in the number of these cells after pesticide exposure, that the viscosity of the mucus secretion changes, and that this has negative effects on gill gas exchange (Oğuz et al., 2022; Soman & Arun, 2021). It has been observed that fungicides, especially tebuconazole, stimulate gill mucus cells and cause excessive mucus production, resulting in lamellae adhesion and decreased respiratory efficiency (Oğuz et al., 2022). Histopathological observations reveal that pesticides can cause irreversible damage to the gill epithelium and that these damages can seriously affect the general physiological condition, ion balance and life span of the fish. These findings are important in terms of showing that pesticides cause serious damage not only to the target pests but also to the basic species in aquatic ecosystems.

Overall, gill histopathology serves as a powerful diagnostic and biomonitoring approach for assessing pesticide pollution in aquatic environments. Future studies should incorporate molecular techniques to understand underlying mechanisms and to develop standardized histopathological indices for regulatory monitoring purposes.

5.2. Effects of Pesticides on the Liver: Hepatocyte Degeneration, Lipid Peroxidation and Oxidative Damage

The liver in fish serves as a crucial hub for metabolic activity, xenobiotic detoxification, and nutrient storage. As the primary organ involved in the biotransformation of toxic compounds, it is highly responsive to environmental pollutants, especially pesticides. Due to its anatomical position and high vascularization, it readily accumulates pesticides, particularly those with lipophilic properties, which facilitates their passage through cellular membranes and deposition in hepatic tissues (Deveci et al., 2021; Ghafarifarsani et al., 2023).

Histopathological damage in the liver is among the most reliable indicators of pesticide-induced toxicity. Structural anomalies like hepatocyte necrosis, vacuolization, sinusoidal congestion, hypertrophy, and nuclear pyknosis are frequently documented. These changes not only affect liver architecture but also impair essential physiological functions such as protein synthesis, bile production, and enzymatic regulation. The presence of melano-macrophage centers, which are clusters of pigmented phagocytic cells, often signals chronic exposure and ongoing immune responses to persistent stress (Pacheco & Santos, 2002; Akaishi et al., 2004).

Lipid peroxidation is a primary consequence of pesticide-induced oxidative stress in liver tissue. The excessive generation of ROS due to chemical exposure damages polyunsaturated fatty acids in cell membranes, leading to the formation of reactive aldehydes like MDA. These oxidative products not only serve as markers of stress but also propagate cellular injury by altering DNA, proteins, and lipids (Kadiru et al., 2022; Oğuz et al., 2022).

In response to ROS accumulation, antioxidant enzyme activities such as those of SOD, CAT, GPx, and GSH are often suppressed. This enzymatic imbalance compromises the cell's ability to neutralize oxidative damage, ultimately triggering cellular apoptosis and necrosis. Prolonged pesticide exposure may also activate apoptotic markers such as caspase-3, indicating programmed cell death due to chemical insult (Jiang et al., 2014; Mansour et al., 2025).

Numerous studies on species like *Oreochromis niloticus*, *Ctenopharyngodon idella*, and *Cyprinus carpio* confirm a spectrum of histological alterations associated with pesticide exposure. These include hydropic degeneration, sinusoidal dilation, bile duct proliferation, fibrosis, inflammatory infiltration, and hepatocellular atrophy. Pesticides such as diazinon, thiamethoxam, sumithion, chlorpyrifos, and deltamethrin have shown potent hepatotoxic potential even at sublethal concentrations (Kabir et al., 2019; El Euony et al., 2020; Subburaj et al., 2020).

Carbamate and pyrethroid pesticide groups have also demonstrated substantial hepatotoxicity. For example, exposure to carbofuran, cypermethrin, and cyfluthrin resulted in extensive vacuolar degeneration and fatty infiltration in liver tissues of fish. These effects were often dose-dependent, with more severe damage observed at higher concentrations (Sepici-Dincel et al., 2009; Mirvaghefi et al., 2012; Kenthao et al., 2020).

Biochemical assessments often align with histopathological data. Elevated serum ALT and AST levels are strong indicators of hepatocyte membrane leakage and cellular lysis. Such enzymatic markers provide early evidence of liver dysfunction, complementing microscopic findings (Bojarski & Witeska, 2020; Chang et al., 2020). In addition, reduced albumin synthesis and increased bilirubin levels suggest impaired hepatic performance and bile transport disturbance.

Pesticide-induced liver injury is not uniform across species, and interspecies variation may be attributed to differences in metabolic rate, detoxification capability, and tissue sensitivity. Fish exposed to the same pesticide under identical experimental conditions may exhibit different histopathological patterns, emphasizing the need for species-specific toxicological assessments (Figueiredo-Fernandes et al., 2007; Raeeszadeh et al., 2018).

In some instances, subchronic exposure even to low concentrations of herbicides like glyphosate (Roundup) or fungicides like tebuconazole and captan caused significant liver impairment. These included atrophic hepatocytes, fibrotic scarring, and proliferation of Kupffer cells. Observations in zebrafish, rainbow trout, and grass carp demonstrate that even environmentally relevant concentrations can induce severe hepatocellular stress and histological remodeling (Ferreira et al., 2010; Cao et al., 2016; Li et al., 2019).

In conclusion, the fish liver is a sentinel organ that responds rapidly to environmental contaminants, particularly pesticides. Its structural and biochemical changes reflect the severity of toxicant exposure and provide a valuable index for environmental risk assessments. Future research should focus on elucidating the molecular pathways involved in pesticide-induced hepatotoxicity, while also developing standardized histopathological scoring systems for regulatory applications in ecotoxicology.

5.3. Renal Effects of Pesticides in Fish: Glomerular Damage and Tubular Degeneration

In teleost fish, kidneys are multifunctional organs responsible for excretion, osmoregulation, and hematopoiesis. Due to their continuous interaction with the aquatic environment and their role in metabolite filtration,

fish kidneys are particularly vulnerable to environmental pollutants such as pesticides. The nephrotoxic effects of pesticides manifest in both structural and functional disruptions, reflecting systemic toxicity (Deveci et al., 2021; Ghafarifarsani et al., 2023).

Glomerular lesions are a hallmark of pesticide-induced renal injury. These often include glomerular shrinkage, dilated Bowman's capsules, and congestion of glomerular capillaries. Degradation of podocytes impairs the filtration barrier, reducing the kidney's ability to eliminate metabolic waste. These changes are well-documented in fish exposed to diazinon, endosulfan, and other organophosphates and organochlorines (Al-Otaibi et al., 2019; Vajargah et al., 2021).

Tubular injuries are equally significant. Common findings include cytoplasmic vacuolization, epithelial necrosis, pyknosis, and detachment of tubular cells. Hyaline droplet degeneration and hemorrhage may accompany these effects, severely disrupting ion transport and water reabsorption, leading to impaired osmoregulatory functions (Soman & Arun, 2021; Oğuz et al., 2022).

In addition, renal histology frequently reveals signs of inflammation, such as leukocyte infiltration, and decreased hematopoietic tissue, indicating systemic immunosuppression. The severity of lesions depends on several factors, including the class of pesticide, dosage, exposure duration, and species-specific sensitivity (Kadiru et al., 2022).

Numerous histopathological investigations have reported nephrotoxic effects in species like *Oreochromis niloticus*, *Oncorhynchus mykiss*, and *Mystus tengara*. Exposure to pesticides like captan, clothianidin, deltamethrin, and cypermethrin induced a wide range of abnormalities, including tubular degeneration, glomerular atrophy, hemorrhages, and necrosis (Boran et al., 2012; Haque et al., 2017; Dogan et al., 2022).

Fungicides such as tebuconazole have also demonstrated renal toxicity, though the extent may be less severe than in liver or gill tissues. Histopathological signs include Bowman's capsule dilation, hyaline droplet formation, and mild necrosis. These effects have been observed 72–96 hours post-exposure in *Capoeta capoeta* and Van fish, indicating a time- and dose-dependent toxic response (Bacchetta et al., 2014; Barnhoorn & van Dyk, 2020).

Interestingly, some studies report that kidneys are less affected than the liver or gills, which could be attributed to shorter exposure periods or lower pesticide concentrations. However, chronic exposure or higher doses often result in severe renal impairment, making the kidney a critical

biomarker organ for environmental toxicity assessment (Tabassum et al., 2016; Othmène et al., 2020).

Research conducted in the Lake Van Basin emphasized the ecological threat posed by tebuconazole accumulation in aquatic habitats. Given its persistence and usage in agriculture, its presence in fish tissues, water, and sediments poses a serious risk to endemic fish species. These findings support the need for stricter regulation and continuous monitoring to protect aquatic biodiversity (Oguz et al., 2022).

In conclusion, fish kidneys demonstrate clear and quantifiable responses to pesticide exposure, particularly through glomerular and tubular degeneration. These histological alterations are indicative of underlying physiological disruption and reflect the broader ecological consequences of unchecked pesticide use in aquatic systems.

5.4. Effects of Pesticides on the Digestive System: Intestinal Epithelial Disruption, Enzymatic Activity, and Metabolic Alterations

The digestive system of fish is not only crucial for nutrient absorption but also acts as a primary interface with environmental contaminants, particularly pesticides. Upon exposure, pesticides can induce substantial morphological, biochemical, and physiological changes in the gastrointestinal tract, affecting the health and survival of aquatic organisms (Soman & Arun, 2021).

Histopathological examination of intestinal tissues in pesticide-exposed fish often reveals severe alterations such as villus atrophy, epithelial necrosis, mucosal lifting, cellular vacuolation, and infiltration of immune cells. These changes impair the absorptive capacity of the intestine and weaken local immune defenses. Fungicides like tebuconazole and insecticides such as thiamethoxam have been shown to disrupt epithelial integrity and reduce nutrient uptake (Mansour et al., 2025; Oğuz et al., 2022).

Studies on fish exposed to deltamethrin, thiodan, and other pesticides have demonstrated villus disintegration, irregular mucosal architecture, and reduced epithelial elasticity. These effects compromise intestinal function and contribute to growth retardation, especially in sensitive species such as *Gambusia affinis* and *Cirrhinus mrigala* (Cengiz & Unlu, 2006; Velmurugan et al., 2007).

Biochemical indicators further confirm intestinal damage. Altered levels of serum ALT, AST, and ALP enzymes signal cellular injury and leakage, while reductions in digestive enzyme activities—such as amylase, lipase, and protease—suggest impaired breakdown of macronutri-

ents. These effects, combined with disrupted intestinal structure, impair metabolic homeostasis (Kadiru et al., 2022; Ghafarifarsani et al., 2023).

Pesticide exposure also affects systemic metabolism. Changes in glucose, cholesterol, and triglyceride levels in the bloodstream indicate stress responses involving carbohydrate and lipid metabolism. These alterations may serve as early biomarkers of sublethal pesticide toxicity and contribute to reduced feed conversion efficiency (Soman & Arun, 2021).

Additional research shows that compounds like lindane, carbaryl, and chlorpyrifos can cause dose-dependent intestinal toxicity. In species such as *Channa punctatus* and *Ctenopharyngodon idella*, histological evaluations revealed necrosis, hemorrhage, cellular degeneration, and mucosal disruption, particularly at higher pesticide concentrations (Forouhar Vajargah et al., 2017; Stalin et al., 2019).

Comparative studies indicate that intestinal tissues exhibit a concentration- and time-dependent response to pesticide exposure. Long-term exposure, even at low doses, may cause chronic inflammation, villus fusion, and epithelial detachment. Furthermore, variations in sensitivity among species suggest the importance of considering biological variability in ecotoxicological assessments (Shamloofar et al., 2015; Forouhar Vajargah et al., 2017).

Although the protective mechanisms of intestinal epithelium against pesticide-induced injury remain underexplored, current evidence points to the intestine as a sensitive and early target organ for environmental toxicants. Histological and enzymatic changes in this tissue provide critical insights into the health impacts of pesticide pollution in aquatic ecosystems.

In conclusion, the intestinal tract in fish is a vulnerable yet underappreciated target of pesticide toxicity. Structural disintegration, enzymatic suppression, and metabolic imbalance resulting from pesticide exposure can significantly affect the growth, immunity, and survival of fish populations. Therefore, incorporating gastrointestinal endpoints into routine ecotoxicological evaluations is essential for comprehensive aquatic risk assessments.

5.5. Toxic Impacts of Pesticides on the gonads

Pesticides are known to impair the reproductive health of fish by inducing structural and functional abnormalities in the gonads. These effects include disruptions in spermatogenesis and oogenesis, ultimately reducing reproductive success and fertility. Both testicular and ovarian tissues are sensitive targets, and histopathological alterations in these organs have been widely reported across different fish species.

In males, exposure to pesticides such as diazinon has been shown to significantly decrease spermatozoa count, cause vacuolization within testicular tubules, and result in swollen seminiferous tubules. Necrotic spermatocytes are also frequently observed, indicating direct damage to spermatogenic lineage cells (Dutta & Meijer, 2003; Banaee et al., 2009). These lesions disrupt normal spermatogenesis and may contribute to reduced fertilization rates.

In females, ovarian histopathology typically includes disorganized vitellin vesicles, degenerative cytoplasm, and vacuolization of follicular epithelial cells. Such changes impair oocyte maturation and can hinder ovulation and fecundity (Banaee et al., 2008).

In Gangetic mystus (*Mystus cavasius*), cypermethrin exposure led to oocyte shrinkage, granulosa layer disruption, and deformation of oocyte walls, further highlighting the vulnerability of reproductive tissues to pesticide toxicity (Uddin et al., 2022).

These gonadal alterations not only affect the immediate reproductive capability of fish but also influence the viability and quality of the resulting embryos and larvae. Reproductive toxicity caused by pesticides may thus contribute to population-level declines in fish, especially in contaminated aquatic environments. Such findings underline the importance of including gonadal histopathology as a critical endpoint in ecotoxicological assessments.

CONCLUSION

This chapter has provided a comprehensive examination of the histopathological effects of pesticides on fish, focusing on structural alterations in critical organs such as the gills, liver, kidneys, digestive tract and gonad tissues. The evidence presented highlights that pesticides are not confined to affecting target pests; rather, they pose substantial biological risks to non-target aquatic vertebrates by impairing vital physiological functions.

Histopathological changes represent highly sensitive and observable biomarkers of pesticide toxicity. Even at sublethal concentrations, these tissue-level alterations serve as early warning indicators and should be incorporated into ecological risk assessments and environmental monitoring programs. In fish, these structural disruptions can reveal the intensity and nature of contaminant exposure within aquatic ecosystems.

To mitigate such risks, integrated and proactive strategies are required. These include reducing the reliance on synthetic pesticides, promoting alternative agricultural practices, encouraging the use of biopesticides, and establishing buffer zones around pesticide application areas to minimize ru-

noff. Additionally, regular biological monitoring of aquatic organisms-particularly fish-should be implemented, and histopathological assessments should be integrated into national environmental policy frameworks.

Public awareness and education are also essential. Training farmers in the responsible use of pesticides and involving local communities in conservation planning can enhance the effectiveness of protection efforts. Stakeholder engagement and community-based monitoring programs can help ensure that environmental strategies are grounded in both scientific evidence and societal participation.

In conclusion, the protection of aquatic ecosystems and fish populations depends not only on reactive responses to pollution but also on science-based, collaborative, and anticipatory management approaches. The data and insights presented in this chapter are intended to contribute both to scientific understanding and to the formulation of informed environmental policies.

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