Investigation of phyllosphere Microorganism-Azolla Interaction: Insights into Incidence Rates and Metagenomic Analysis

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Research Article

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Abstract

Background: *Azolla* is a versatile aquatic fern that is rich in nutrients and possesses valuable antibacterial components, making it a useful green manure and medicinal raw plant material. However, the growth of *Azolla* is affected by microorganisms under different environmental conditions, the investigation on the interaction between microorganisms and *Azolla* is one of the crucial projects for *Azolla*exploration and application.

Results: In this study, we cultivated two different *Azolla* species, *Azolla imbricata* (Aim) and *Azolla pinnata* (Api), under identical condition to investigate their respective incidence rates. Metagenome analysis of phyllosphere microorganisms was performed to uncover the interaction between *Azolla*and microorganisms. Our results revealed significantly higher incidence rates in Aim compared to Api. The microbiological community taxonomy showed a predominance of Proteobacteria phylum and Burkholderiales order, with higher proportions in the Aim group. Conversely, the Api group had higher proportions of Cyanobacteria phylum, Nostocales order, Nostocaceae family, and *Trichormus*genus. Moreover, pathogenic bacteria exhibited a higher relative abundance in the Aim group. We further analyzed significant differences in KEGG pathways between the two groups and identified the top 10 enriched pathways. Additionally, according to the resistance gene analysis results, five resistant genes showed different patterns between Api and Aim groups. Expect AAC6-IIa, the other five resistant genes had a higher abundance in Aim than in Api.

Conclusions: The greater disease susceptibility of Aim compared to Api may be linked to the microbial community structure of the two species. The variations in microbial community structure could be influenced by the antibacterial components present in Api, whereas Aim may lack or have lower levels of these antibacterial components.These findings provide insights into the microorganisms-*Azolla* interaction, aiding the development of strategies to enhance *Azolla* growth and utilization.

Background

Azolla is an aquatic fern belonging to the genus *Azolla* (Family Salviniaceae). There are seven to nine species of *Azolla*, which can be classified into two subgenera. The first subgenus is *Rhizosperama*, which includes species such as *A. pinnata*, *A. nilotica* and *A. imbricata*. The second subgenus is the *Euazolla*, which consists of species like *A. filiculoides*, *A. mexicana*, *A.caroliniana*, *A. microphylla* and *A.rubra* [1]. It is well-known for its high nutritional value, making it an excellent source of plant nutrition in agriculture and horticulture. With its elevated levels of crude protein content, essential amino acids, vitamins, minerals, *Azolla* is highly valued as animal feed [2]. Additionally, *Azolla* exhibits rapid growth, with its biomass doubling within 2–5 days [3], thanks to its unique ability to fix atmospheric nitrogen through a symbiotic relationship with the cyanobacteria *Nostoc azollae* living special leaf cavities[4].

Azolla provides various ecological benefits, such as its use as green manure in rice fields, a carbon sink plant, and a phytoremediation agent [5–7]. Certain varieties of *Azolla* also possess antibacterial and antioxidant properties, making them valuable as medicinal raw materials [8]. Previous experiments with

ethanol extract of *Azolla filiculoides* have shown negative effect on *E. coli* and *S. aureus*, possibly due to undefined drug sensitivity [9]. Extracts of specific *Azolla* varieties have demonstrated protective effects against rat hepatotoxicity, with the main components displaying antioxidant, anti-inflammatory, and anti-apoptotic activities [10].

Azolla thrives on the surface of freshwater bodies such as ponds, lakes, and rivers. Due to the dynamic nature of the aquatic environment and varying water conditions, the growth and development of different *Azolla* species can differ significantly. Recent studies have highlighted the crucial role of the plant microbiome in both plant and ecosystem health. The beneficial interplay between plants and their phyllosphere endophytic microbiome is essential for maintaining the health of the plant-microbiome holobiont, while diseases are often correlated with microbial dysbiosis [11]. However, despite the potential significance of plant-microbe interactions, particularly in aquatic plants, our understanding of the plant microbiome and its contribution to host fitness remains limited. Investigating the microbiome of aquatic plants, such as *Azolla*, is still in its nascent stages, and there is much to learn about the complex relationships between plants and their microbial communities.

The objective of this study was to investigate the incidence rates and interaction between two different species of *Azolla* and their associated phyllosphere microorganisms. The research was conducted under controlled conditions to ensure consistent environment factors for both *Azolla* species. Metagenome analysis, a technique that allows for the study of the genetic material of an entire community, was employed to study the phyllosphere microorganisms. Specifically, DNA sequences of all microorganisms present in the phyllosphere of both *Azolla* species were analyzed to identify the microbial diversity and potential interactions with each *Azolla* species. The findings from this study could provide valuable insights into the ecology and potential applications of *Azolla*, with promising prospects in agriculture and environmental remediation.

Results

Statistics of pathogenic *Azolla* plants

Based on the observations, it is evident that the *Azolla* plants cultivated for two weeks have grown rapidly and fully covered the entire pond. The majority of the plants in both the Api and Aim groups appeared healthy. However, there seems to be an issue with *Azolla* plants infected with an unknown pathogenic fungus in both groups, which were cultivated for one month. These infected plants showed symptoms of disease, such as yellowing of the leaves, partial browning, or complete withering of certain plant parts, and loss of structural integrity (as shown in Fig. 1A, B). To further investigate, we conducted a count of pathogenic plants and the findings indicated that there were significantly more pathogenic plants in the Aim group compared to the Api group (as shown in Fig. 1C). Additionally, the disease incidence in the Aim group was significantly higher than in the Api group (as shown in Fig. 1D).

Sequence data summary

High-quality sequencing data were obtained from all six samples after confirming a clean data rate of > 97% through rigorous quality control analysis. The average Q20 and Q30 values were 97.8% and 93.59%, respectively, indicating excellent data quality. The average GC content was 52.46%, which felled within the expected range for the samples (as shown in **Supplementary table 1)**. To assess the consistency between biological replicates, correlation coefficient analysis was performed, and the results revealed that all samples were grouped individually within their respective replicates, indicating a high level of correlation (Fig. 2A). This was further supported by the principal component analysis (PCA) results (Fig. 2B). These findings indicate that the quality of the sequencing data met the necessary requirements for subsequent analysis, ensuring reliable results.

Taxonomic composition of the microbial communities

The taxonomic composition of the two groups was determined by blasting against the NCBI-NT database. In the annotation results, 195 OTUs were identified as archaea, 10857 as bacteria, 986 as eukaryotes, and 147 as viruses. Bacteria accounted for the highest proportion, followed by eukaryotes. A total of 142 bacterial species were found to be present in both *Azolla* samples when analyzed at the phylum level. Cyanobacteria and Proteobacteria were the most abundant phyla in both Api and Aim, together comprising more than 70% of the overall population (as shown in Fig. 3A). Proteobacteria was the most abundant phylum in both samples, comprising 42.39% of all phyla in Api and 51.35% in Aim. At the order level, the most abundant orders within Proteobacteria were Burkholderiales (21.54% and 27.98% in Api and Aim, respectively), Hyphomicrobiales (2.72% and 5.01% in Api and Aim, respectively), and Cellvibrionales (0.62% and 1.28% in Api and Aim, respectively) (as shown in Fig. 3B). Cyanobacteria was the second most abundant phylum in both samples, comprising 42.19% of all phyla in Api and 22.40% in Aim. The most abundant orders within Cyanobacteria were Nostocales (43.92% and 22.73% in Api and Aim, respectively) and Pseudanabaenales (0.75% and 0.86% in Api and Aim, respectively) (as shown in Fig. 3B). At the genus level, a total of 2,271 genera were identified from all samples. Trichormus, Calothrix, Methyloversatilis, and Haliscomenobacter were the dominant bacteria. The relative abundance of Trichormus was as high as 27.49% in Api and 12.06% in Aim. The relative abundance of other genera is less than 10% (as shown in Fig. 3C). The relative abundance of Haliscomenobacter showed the largest difference between Api (1.25%) and Aim (4.06%). LEfSe analysis was performed based on the bacterial data at the phylum level. The results showed different biomarkers for the two groups: the Proteobacteria phylum, Burkholderiales order, Betaproteobacteria class, and others had a higher proportion in the Aim group (as shown in as shown in Fig. 3D **and E**), while Cyanobacteria phylum, Nostocales order, Nostocaceae family, Trichormus genus, and others had a higher proportion in the Api group (as shown in Fig. 3D **and E**).

We have also identified numerous OTUs annotated as eukaryotes. The Streptophyta phylum accounted for over 90% of the relative abundance of all the identified fungi (as shown in Fig. 4A). Within the Streptophyta phylum, we observed the presence of Vitis, Glycine, Selaginella, Cajanus, Marchantia, and several another fungi with low abundance (as shown in Fig. 4B). Further analysis using LEfSe revealed that Magnoliopsida class, Bacillariophyta phylum, Bacillariophyceae class, and others had a higher proportion in the Aim group (as shown in Fig. 4C **and D**), while the Streptophyta phylum, *M. polymorphic* species, *Marchantia* genus, and others had a higher proportion in the Api group (as shown in Fig. 4C **and D**).

Pathogenic microorganism

Based on the information from the Pathogenic Bacteria Database (https://globalrph.com/bacteria/), we have identified 70 pathogenic bacteria in our results (Fig. 5A). Most of these pathogenic bacteria showed a higher relative abundance in the Aim group. Furthermore, we have performed LEfSe analysis, which revealed that 13 bacteria were identified as significantly different between the two groups (Fig. 5B). Among these, Actinomyces, Porphyromonas, Salmonella, Achromobacter, Stenotrophomonas maltophilia, Clostridium, Nocardia, and Enterobacter cloacae complex were found to have higher abundance in the Aim group compared to the Api group. On the other hand, Enterobacter cloacae, Enterobacter, Pseudomonas aeruginosa, and Nocardia showed higher abundance in the Api group compared to the Aim group.

KEGG functional analysis

Functional capabilities of the microbial community were assessed through a metagenomic study for KEGG functional analysis. Base on our criteria, pathways accounting for more than 1% of all paths were considered dominant. Subsequently, 20 predominant pathways were identified in the two groups (Fig. 6A). Among them, ribosome, oxidative phosphorylation, photosynthesis, quorum sensing, and two-component system were the top five enriched KEGG pathways. Further analysis was conducted to evaluate KEGG pathways that showed significant differences between the two groups. The results revealed that pathways such as Staphylococcus aureus infection, melanogenesis, chloroalkane and chloroalkene degradation, sphingolipid metabolism, biosynthesis of various secondary metabolites, Vibrio cholera infection, apoptosis, RAS signaling pathway, stilbenoid, diarylheptanoid and gingerol biosynthesis, and butanoate metabolism were among the top 10 significantly enriched KEGG pathways (Fig. 6B).

Resistance gene analysis

122 contigs were identified, out of which 19 were found to be associated with Antibiotic Resistance Ontology (ARO). The analysis of resistance gene abundance revealed that the most prevalent resistance gene was adeF (as shown in Fig. 7A). Furthermore, when comparing the different resistance genes

between Api and Aim, we conducted Metastats analysis using the CARD database. The results revealed the identification of five distinct genes: AAC6-IIa, cmlA9, qacEdelta1, tetD, and ANT3-Ii-AAC6-IId_fusion_protein (as shown in Fig. 7B). Among these genes, AAC6-IIa and qacEdelta1 were both found to be present in the Proteobacteria phylum.

Discussion

The intricate interactions between plants and microbes play a crucial role in shaping the health and resilience of plant ecosystems. Within the phyllosphere, the aerial parts of plants, microbial communities form through complex associations influenced by various factors, including plants, waters, and soil conditions [12]. The composition and diversity of these phyllosphere microbial communities are believed to be influenced by the plant's genetic makeup and architectural characteristics, thus leading to variations in microbial assemblages [13]. Understanding the dynamics of plant-microbe interactions in the phyllosphere is essential for unraveling the mechanisms that contribute to plant health and ecosystems stability.

In this study, we found that the Aim group had a higher percentage of Proteobacteria phylum, Burkholderiales, and both Alpha- and Beta-proteobacteria class, while the Api group had a higher percentage of Cyanobacteria phylum, Nostocales order, Nostocaceae family, and Trichormus. Our findings are consistent with previous research by Laura [14], which also reported the dominant role of Proteobacteria and Alpha- and Beta-proteobacteria classes in *Azolla* and the ditch water. Interestingly, we also found a significant difference in the relative abundance of pathogenic bacteria between Aim and Api groups, with Aim showing a higher proportion of infection accompanied by more abundant pathogenic bacteria. Previous studies have demonstrated that *Azolla pinnata* extract is rich in antioxidants and antimicrobial compounds, with over 35 chemical compounds identified as having insecticidal, pesticidal, and antimicrobial properties [15–17]. Therefore, we speculate that Api possesses phyto-constructs with antimicrobial activity and can be utilized as a natural plant-based antimicrobial, while Aim may contain fewer antimicrobial active compounds.

The KEGG enrichment results revealed several significantly enriched pathways, including staphylococcus aureus infection, melanogenesis, chloroalkane and chloroalkene degradation, sphingolipid metabolism, biosynthesis of various secondary metabolites, vibrio cholera infection, apoptosis, RAS signaling pathway, stilbenoid, diarylheptanoid and gingerol biosynthesis, and butanoate metabolism. Among these pathways, the RAS signaling pathway is an intracellular signaling system that regulates cellular proliferation, differentiation, survival, and gene expression [18, 19]. Inhibition of the immune system may be particularly essential in proliferating cells with elevated Ras/MAPK signaling, as reported by Anan Ragab et al. [20]. Therefore, the difference in the RAS pathway between the two *Azolla* groups may be one of the reasons for their difference in disease resistance. Additionally, the enrichment of pathways related to aureus infection and vibrio cholera infection based on the different genes between Api and Aimgroups suggests the potential pharmaceutical value of Api.

According to the resistance gene analysis results, five resistant genes showed different patterns between Api and Aim groups. Expect AAC6-IIa, the other five resistant genes had a higher abundance in Aim than in Api. Interestingly, different varieties of *Azolla* in the same water area have different concentrations of resistance genes. This discrepancy may be due to the resistance of these genes to the antibacterial components produced by Api, but not to the conventional antibacterial components in water. Microorganisms carrying these genes may be one of the main causes of disease in Aim.

Conclusions

Base on the analysis of composition, it was observed that Api had a higher abundance of Cyanobacteria phylum, Nostocales order, Nostocaceae family, Trichormus genus, but a lower abundance of Proteobacteria phylum, Burkholderiales order, and Betaproteobacteria class compared to Aim. This difference in taxonomic composition may explain why Api were more resistant to disease compared to Aim.

The higher relative abundance of pathogenic bacteria in Aim plants compared to Api plants suggests that Aim plants may be more susceptible to disease. This could be due to the presence of antimicrobial components in Api that provide resistance against pathogenic bacteria. These antimicrobial components may inhibit the growth of pathogenic bacteria, leading to lower abundance of pathogenic bacteria in Api plants.

Furthermore, the identification of drug-resistant genes in several genera suggests that drug-resistant bacteria may be present in both Aim and Api plants. However, it is possible that these drug-resistant bacteria in Aim plants may be less resistant to the antibacterial components produced by Api plants, which could explain the lower abundance of pathogenic bacteria in Api plants.

In conclusion, the taxonomic composition and presence of antimicrobial components in Api plants may contribute to their higher resistance to disease compared to Aim plants. The presence of drug-resistant bacteria in both Aim and Api plants highlights the need for further research to understand the complex interactions between plants, bacteria, and antimicrobial components in the context of disease resistance.

Materials and methods

Plant materials

Two species of *Azolla*, namely *Azolla pinnata* R.Brown (accession number, IRRI 7001, referred to as “Api” for short) and *Azolla imbricata* (Roxb.) Nakais (accession number, NARC 500, referred to as “Aim” for short), were selected as plant materials. Api was originally collected from Australia in 1982 and introduced from International Rice Research Institute (IRRI) in Philippines in 1984. Aim was collected from China in 1980 and preserved at the National Azolla Germplasm Resource Center (NARC), in China. Both Azolla species were identified by National Azolla Germplasm Resource Center, in China.

Experimental settings

The experimental site was located in Fuzhou city, China (26°7’58”N, 119°19’58”E). The air-dried paddy soil was crushed and sieved to remove impurities, and then used as the cultivation substrate in each small pond, which was filled with tap water and maintained with a 5 cm soil layer and 7 cm water depth. The composition of the cultivation substrate was shown in Supplementary Table 2.

All plants were cultivated under natural illumination, at ambient temperature, with 80% relative humidity. The cultivation was carried in November. The ambient temperature data can be found in Supplementary Fig. 1.

Sample collection

We first collected 10 gram of *Azolla* plants for each species that had been cultured for two weeks as our samples for sequencing. To ensure the samples were free from contaminants, we conducted surface disinfection using a modified method based on our previous study [21]. The whole plants were carefully rinsed under running water for 10 minutes and the roots were removed to obtain frond rich tissue. The fronds were then washed with ddH_2O to remove epiphytes and contaminants. To further disinfect the fronds, we soaked them in 70% ethanol for 40 seconds, followed by a step in 2.5% sodium chloride for 10 minutes. Afterward, the fronds were immersed in sterile Milli-Q water for 5 minutes, repeated three times. The fronds were then carefully dried with sterile filter paper and briefly freezen with liquid nitrogen for 2 minutes. To ensure accuracy, each sample was repeated three times to obtain reliable results. Finally, the sediment-free fronds were stored at -80℃ for further analysis.

Disease investigation and Statistical analysis

In addition, we also conducted a statistical analysis of disease incidence in *Azolla* plants cultured for one month. Total plants and diseased plants were counted separately for both Api and Aim in each pond, with the diameter of the pond was 20 cm, each sample was repeated three times. Incidence rate = (Number of diseased plants/ Total number of plants) * 100.

The statistical analysis of the number of pathogenic *Azolla* and disease incidence of *Azolla* were performed using one-way analysis of variance (ANOVA) using the prism 9.0 software (GraphPad Software, CA, USA).

Metagenomic library construction and sequencing

To isolate metagenomic DNA from frond-rich tissues, we utilized the PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc.). DNA samples were collected and stored at -80°C until DNA extraction. To minimize potential bias during the DNA extraction process, we established three biological replicates for each group, with each replicate involving the extraction of DNA from three samples. Subsequently, the extracted DNA was sheared into fragments of approximately 300 bp using an M220 Focused-ultrasonicator (Covaris Inc., Woburn, MA, USA) to construct a paired-end library. The DNA templates were then processed with the TruSeqTM Kit following the manufacturer's instructions (Illumina, CA, USA).

Finally, pair-end sequencing (2 * 150 bp) was conducted at Allwegene Technology Co., Ltd. (Beijing, China) following standard protocols.

Sequence data analysis

To ensure data integrity, the raw readings were trimmed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) with the parameters of minimum quality score of 20 and minimum length of 50 bp. The SOAPdenovo software (http://soap.genomics.org.cn/) was then used to assemble the clean readings. Prodigal [22] was used for open reading frames (ORFs) prediction and annotation in each sample. CD-HIT was used to predict non-redundant ORFs and clusters of non-redundant ORFs [23]. Samples were blasted against the NCBI-NT database for taxonomic annotation. The BLAST program was used to match protein-coding reads against protein sequences in the NCBI-NR database, and the results with the best hit compared to the NCBI microbial taxonomy database. Clusters of Orthologous Groups of Proteins (COGs) were generated by aligning the ORFs to the eggNOG database (Version 4.0) using BLASTP (BLAST Version 2.2.28+) with an optimal E-value threshold of 1^{e-5}. STAMT was employed for pairwise statistical comparisons of COG categorization [24], and the Mann-Whitney rank sum test in SigmaPlot (Version 12.5, Systat Software, USA) was used to determine significance between samples. To identify KEGG pathways, a BLAST search (BLAST Version 2.2.28+) was performed with an optimized E-value threshold of 1e-5 against the Kyoto Encyclopedia of Genes and Genomes database (KEGG GENES, Kyoto Encyclopedia of Genes and Genomes). KOBAS 2.0 (KEGG Orthology-Based Annotation System) was used for functional annotation of genes [25]. Statistical analysis of sequencing data was carried out according to the parameters of the software.

Declarations

Ethics approval and consent to participate

The plant materials used in this research complies with international, national and/or institutional guidelines. All experiments on plants were conducted in accordance with the cultivars protection regulations of the Agricultural Ecology Institute, Fujian Academy of Agricultural Sciences.The *Azollapinnata* was originally preserved in the International Rice Research Institute (Philippines), and it was legally introduced by the National Azolla Germplasm Resource Center (China) in the late 1980s. The *Azolla imbricata* was collected from Fujian and kept in the National Azolla Germplasm Resource Center (China) in the late 1980s. All of the *Azolla* varieties have been identified by researcher Longfei Tang, and plant samples were kept in the Agricultural Ecology Institute, Fujian Academy of Agricultural Sciences. The National Azolla Germplasm Resource Center (China) belongs to the administrative management of the Agricultural Ecology Institute, Fujian Academy of Agricultural Sciences. The National Azolla Germplasm Resource Center is a legal institution in China to legally collect and preserve *Azolla* plants.

Consent for publication

Not applicable.

Availability of data and materials

The sequence data generated and analyzed in this study are available at NCBI (https://www.ncbi.nlm.nih.gov) under accession numbers (PRJNA983057).

Competing interests

The authors declare that they have no competing interests.

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Author’s Contributions

Conceived and designed the study: Yan-Qiu Yang. Performed research: Yan-Qiu Yang, Su-Fang Deng, You-Quan Yang. Analyzed the data: Yan-Qiu Yang. Contributed materials/reagents: Zhao-Yang Ying. Wrote the paper: Yan-Qiu Yang.

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Figures

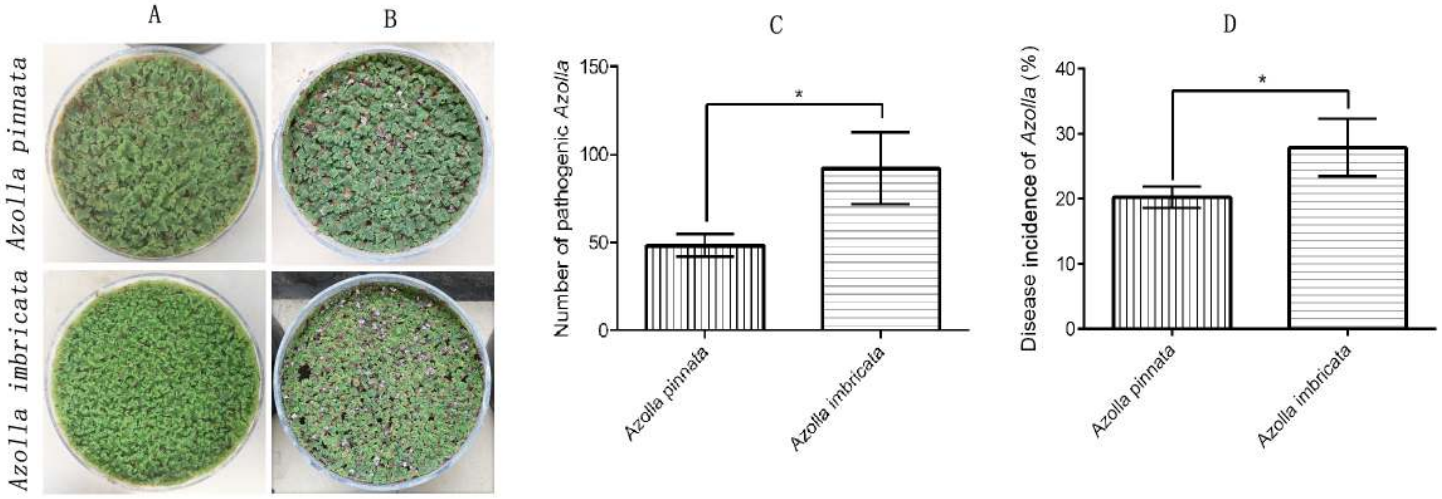

Figure 1

Phenotype and pathogenic statistics analysis of different species of *Azolla*

(A) Phenotype of different species of *Azolla* after two weeks of growth in the pond; (B) Dysbiosis features of unknown fungus in different species of *Azolla* cultivated in the pond for one month; (C) Number of pathogenic plants in different species of *Azolla* . *, $p < 0.05$. (D) Disease incidence of different species of *Azolla*. *, $p < 0.05$.

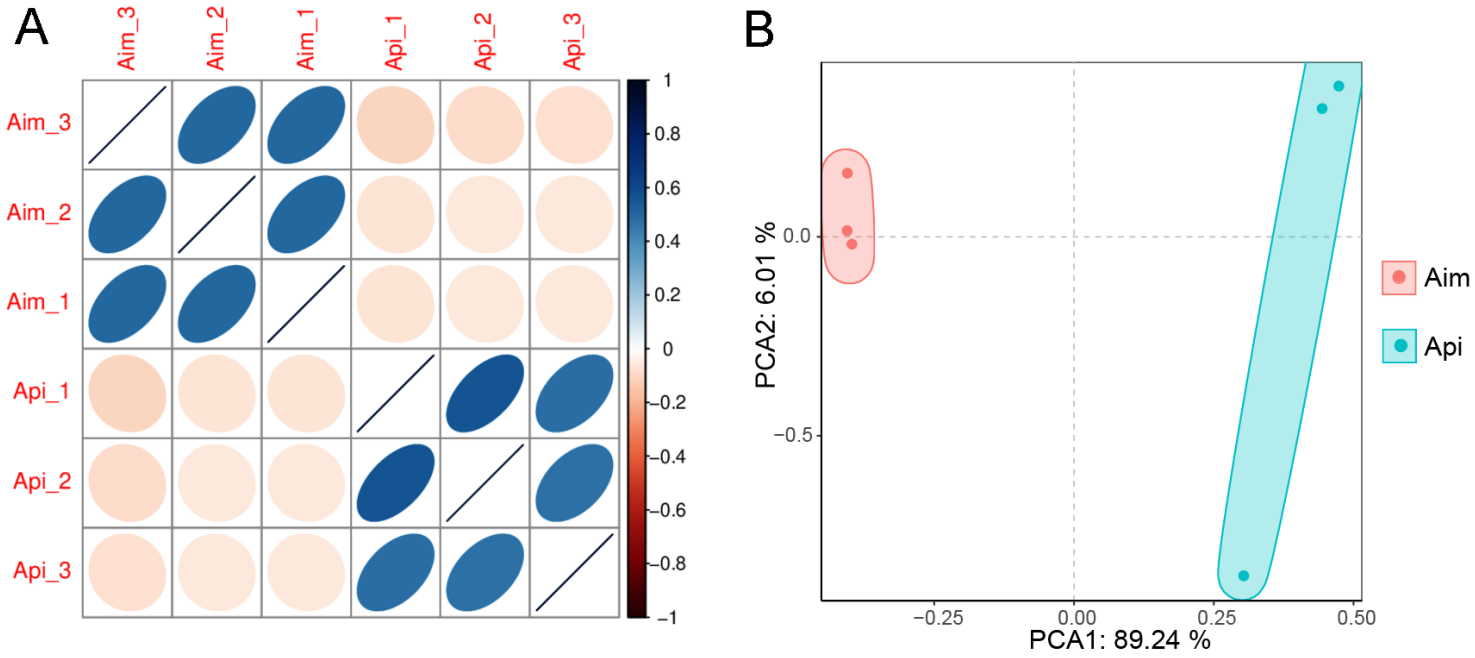

Figure 2

The analysis of *Sequence data for two species of Azolla*

(A) Biological correlations of the samples. The presence of significant correlations between two species is indicated by red ellipses for negative correlation and blue ellipses for positive correlations. (B) PCA of the samples. “Aim” represents *Azolla imbricata,* and “Api” represents *Azolla pinnata*.

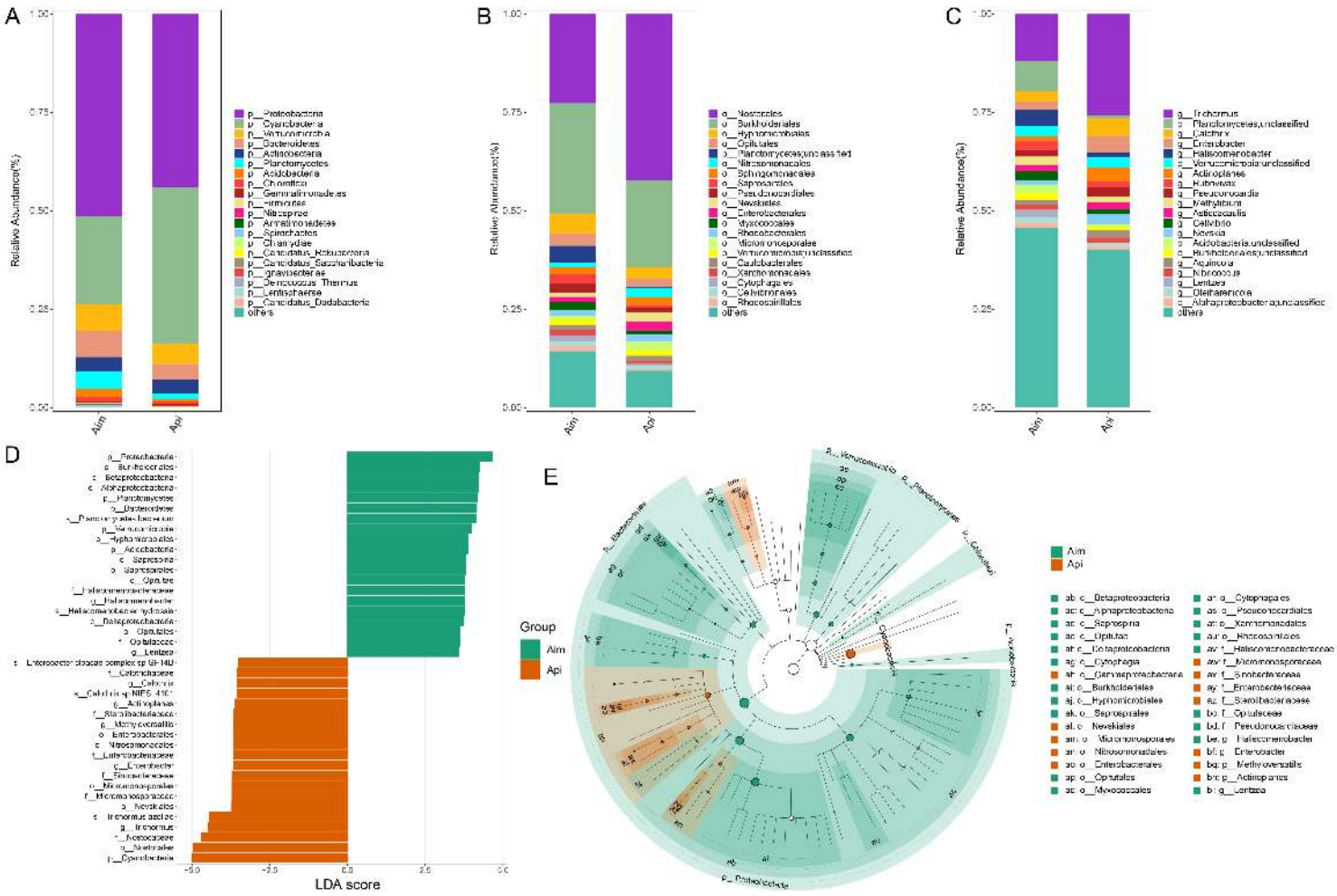

Figure 3

Taxonomic composition of bacteria communities

(A), (B) and (C) represent the relative abundance of bacteria at the phylum, order and genus level, respectively. (D) and (E) shows the graphical summaries of LEfSe (Linear discriminant analysis Effect Size) results. “Aim” represents *Azolla imbricata,* and “Api” represents *Azolla pinnata*.

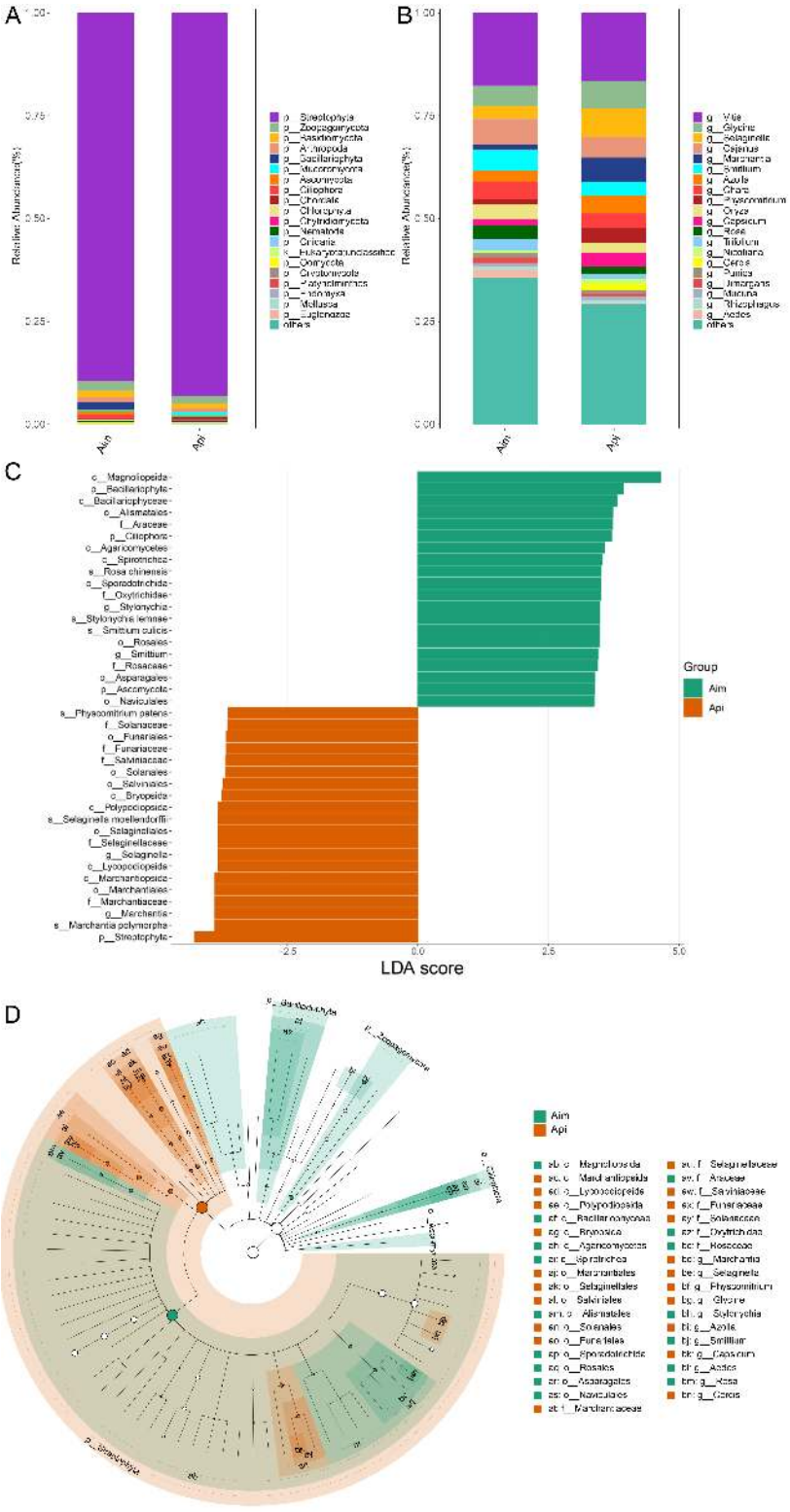

Figure 4

Taxonomic composition of fungi communities

(A) and (B) represent the relative abundance of fungi at the phylum and genus level, respectively. (C) and (D) display the graphical summary of LEfSe results. “Aim” represents *Azolla imbricata,* and “Api” represents *Azolla pinnata*.

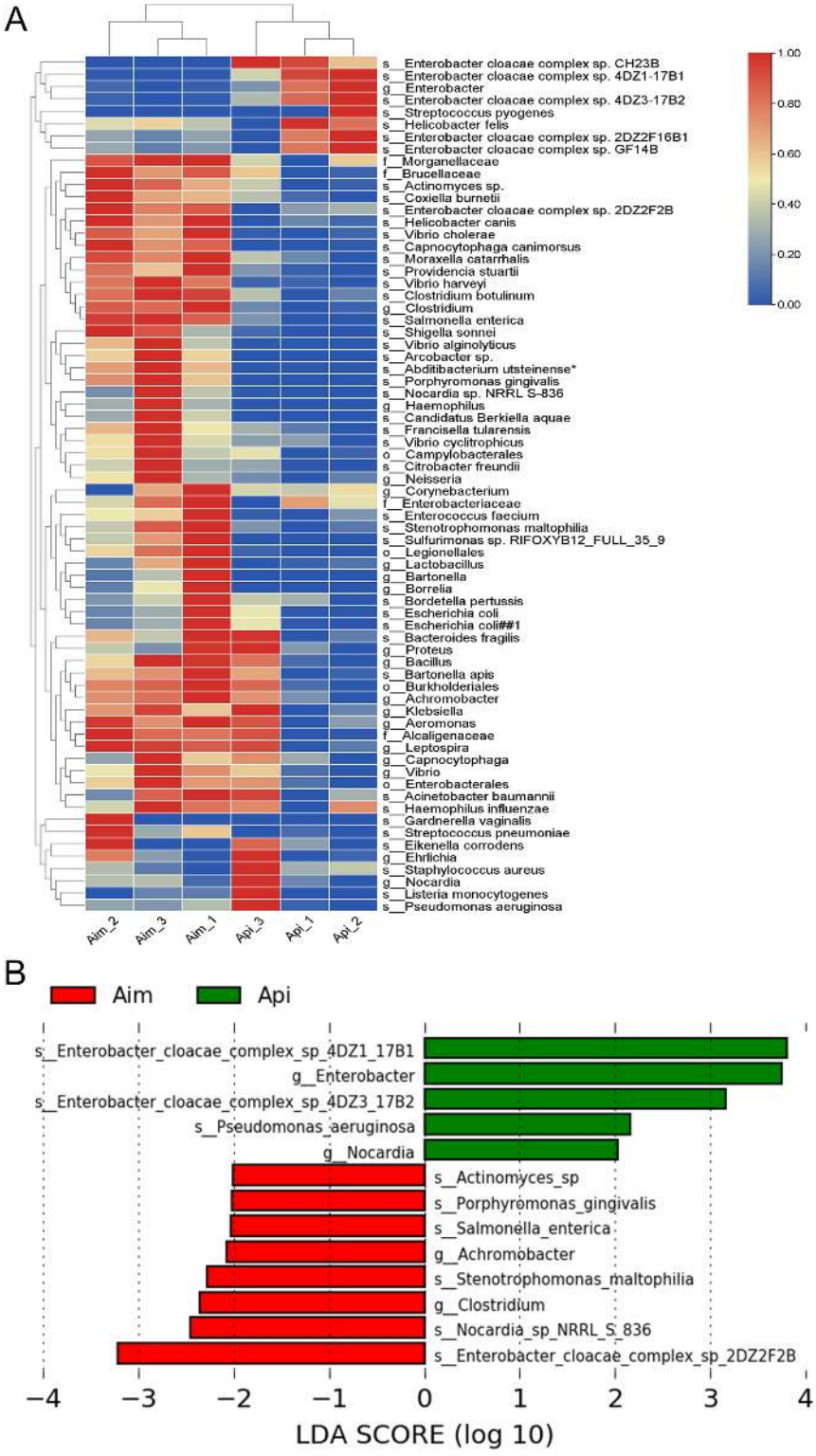

Figure 5

Pathogenic microorganism analysis

(A) Heatmap of Pathogenic microorganism relative abundance in all samples. High abundance is denoted by the color red, while low abundance is represented by the color blue. (B) Graphical summary of LEfSe results. “Aim” represents *Azolla imbricata,* and “Api” represents *Azolla pinnata*.

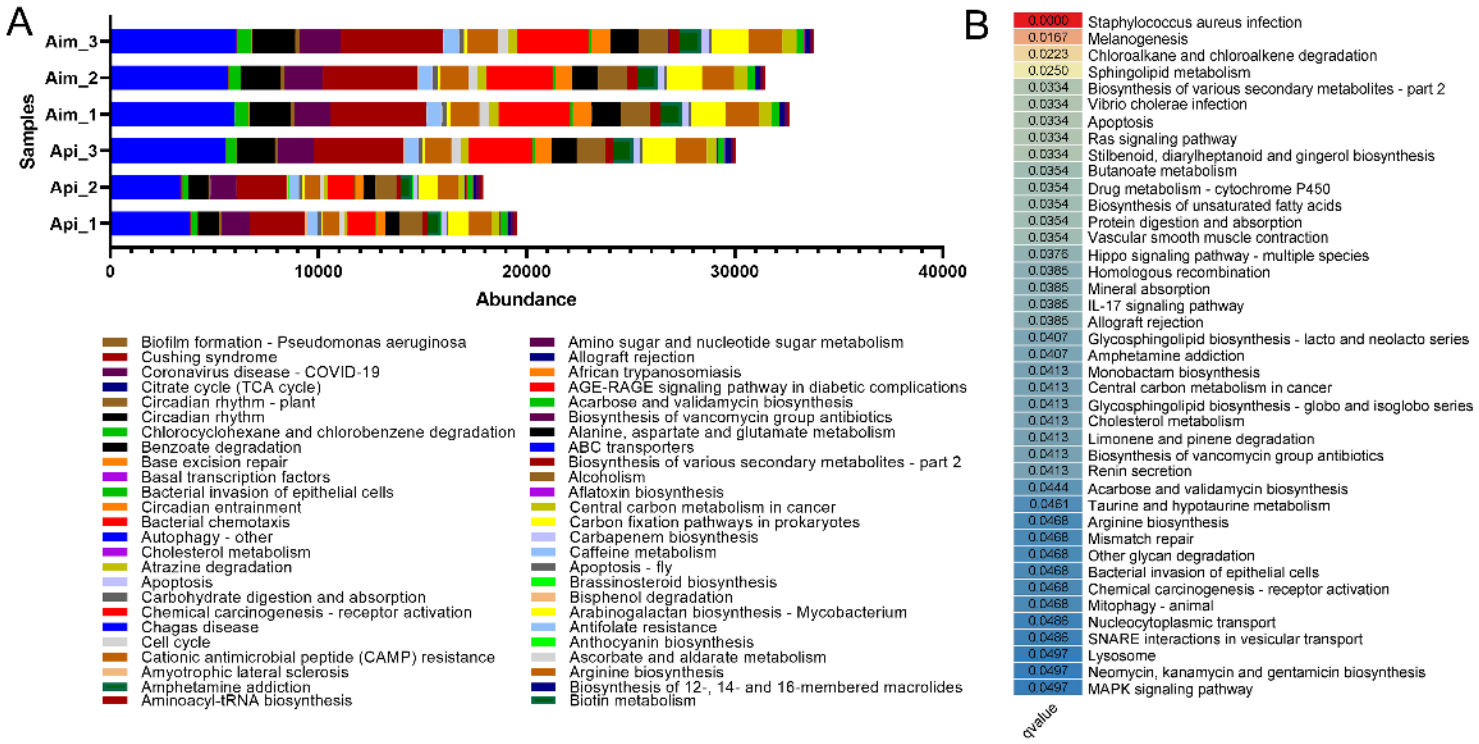

Figure 6

The KEGG functional analysis

(A) Dominant pathways are defined as those with a relative abundance greater than 1.00% of the total observed pathways. (B) The q-value of enriched pathways is represented by color, with a redder color indicating a smaller q-value. “Aim” represents *Azolla imbricata*, and “Api” represents *Azolla pinnata*.

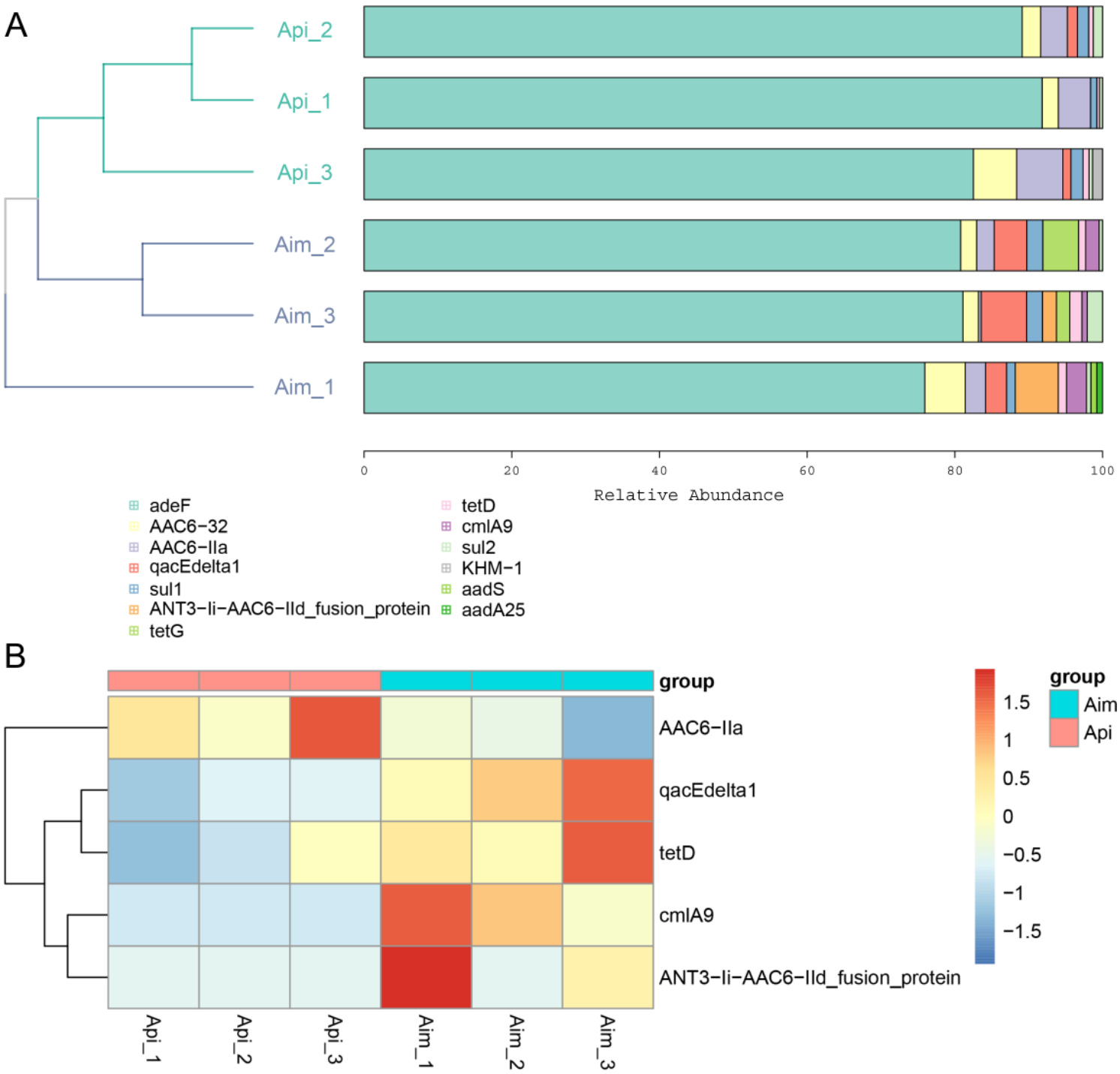

Figure 7

Resistance gene analysis

(A) Resistance gene in different samples. (B) Heatmap of the significantly different genes, Redder color indicates higher abundance. “Aim” represents *Azolla imbricata*, and “Api” represents *Azolla pinnata*.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfigure1DailytemperatureinformationforNovember2021.tif
- supplementarytable1Summaryofrawsequencingdataquality.docx
- supplementarytable2CompositionofCultivationSubstrate.docx