Master's Thesis

Identification and prevalence mapping of plant-associated aerobic anoxygenic phototrophic (AAP) bacteria in Japan

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Murai Miyu: Identification and prevalence mapping of plant-associated aerobic anoxygenic phototrophic (AAP) bacteria in Japan

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Aerobic Anoxygenic Phototrophic (AAP) bacteria are prokaryotes, which live in aerobic conditions and are able to capture energy from solar radiation without producing oxygen. AAP bacteria are known as an aquatic bacterial group and numerous studies suggest that they play an important role in carbon cycling in the ocean. Recently, AAP bacteria have also been discovered in terrestrial environments, such as soils, plant phyllosphere and endosphere. However, there is little information about terrestrial AAP bacteria, including their prevalence, and interactions with plant hosts. Recently, Shared Light project (University of Jyväskylä) has collected plant-associated AAP bacteria from four different countries to examine the prevalence of the bacteria from boreal to arctic climate. Their results showed that AAP bacteria were present in nearly every plant species collected, with *Methylobacterium sp.* and *Sphingomonas sp.* being the most common bacterial genera.

In this study, four plant species were collected from two cities in Japan to investigate whether AAP bacteria also occur in warm East-Asian environments and to identify which AAP bacterial species are found in Japan. The isolated AAP bacteria were identified to the genus level and the results were compared with the previous data. Out of the total 24 leaf and 12 branch samples collected, 13% of AAP bacteria were found in the phyllosphere, 4% in the endosphere and 42% in branches. All collected plant species contained AAP bacteria, with a higher prevalence observed in the branches of *Pinus sp.* compared to other plant species. 9 different species of AAP bacteria were found in the collected samples, all belonging to the genus *Methylobacterium* sp. There was a clear difference between the AAP bacteria species isolated in Japan and from boreal–arctic climates, as *Sphingomonas* sp. was not found in samples from Japan. The sample size and the number of species collected in Japan were small, which might have affected the result. Further research is needed to determine the factors, such as host plant species, climate, temperature or humidity, that influence the differences in AAP bacterial species and their prevalence.

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Ekologian ja evoluutiobiologian maisteriohjelma	
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Aerobiset anoksygeeniset fototrofiset (AAP) bakteerit ovat prokaryootteja, jotka elävät aerobisissa olosuhteissa ja pystyvät hyödyntämään auringon valoenergiaa tuottamatta happea. Nämä bakteerit tunnetaan akvaattisena bakteeriryhmänä ja useat tutkimukset ovat osoittaneet niiden olevan merkittävässä roolissa meriekosysteemin hiilenkierrossa. AAP-bakteereita on lähiaikoina löydetty myös terrestriaalisista ympäristöistä kuten maaperästä sekä kasvien fyllosfääristä ja endosfääristä. Kuitenkin terrestriaalisista AAP-bakteereista on vain vähän tietoa, kuten niiden esiintyvyydestä sekä vuorovaikutuksesta kasvi-isäntien kanssa. Lähiaikoina Jaettu Valo -projekti (Jyväskylän yliopisto) on kerännyt neljästä eri maasta kasviassosioituneita AAP-bakteereita ja kartoittanut niiden esiintyvyyttä boreaalis–arktisessa ilmastossa. Heidän tuloksensa osoittivat, että lähes kaikissa kerätyissä kasvilajinäytteissä esiintyi AAP-bakteereita, joista suurin osa kuului *Methylobacterium sp.* ja *Sphingomonas sp.* sukuihin.

Tässä tutkimuksessa neljää kasvilajia kerättiin kahdesta eri Japanin kaupungista, tavoitteena selvittää AAP-bakteereiden esiintyvyyttä myös lämpimissä Itä-Aasian ympäristöissä ja millaisia lajeja löytyy Japanista. Eristetyt AAP-bakteerit on tunnistettu sukutasolle ja tuloksia vertailtiin aiempien tuloksien kanssa. Kerätyistä 24 lehtinäytteistä ja 12 oksanäytteestä AAP-bakteereja löytyi 13% fyllosfääristä, 4% endosfääristä ja 42% oksasta. Kaikki kerätyt kasvilajit sisälsivät AAP-bakteereita, mutta niitä löytyi *Pinus* sp. -lajin oksista enemmän kuin muista kasvilajeista. Japanissa kerätyistä näytteistä löytyi arviolta 9 AAP-bakteerilajia, josta kaikki lajit kuuluivat *Methylobacterium* sp. sukuun. Japanin ja boreaalis-arktisen AAP-bakteerilajiston välillä oli eroavaisuutta, sillä Japanin näytteistä *Sphingomonas* sp. sukua ei esiintynyt. Japanissa kerätyt laji- ja näytemäärät olivat pieniä, mikä voi vaikuttaa saatuun tulokseen. Lisätutkimuksia tarvitaan selvittääkseen mitkä tekijät, kuten isäntäkasvilajit, ilmasto, lämpötila tai kosteus, vaikuttavat AAP-bakteerilajiston eroavaisuuksiin ja niiden esiintyvyyteen.

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GLOSSARY AND ABBREVIATIONS

Glossary

Endosphere	Internal region in a plant
Phyllosphere	Leaf surface of the plant
Microbiome	Community of microorganisms in certain habitat

Abbreviations

AAP	Aerobic anoxygenic phototrophic
BChl *a*	Bacteriochlorophyll *a*
JaVa	Jaettu Valo, Shared Light (Name of research group)
NIRis	Near-infrared imaging system

1 INTRODUCTION

Aerobic anoxygenic phototrophic (AAP) bacteria are phototrophic prokaryotes which live in aerobic conditions and are capable of photosynthesis without producing oxygen as a by-product. AAP bacteria are widely represented in the ocean and nearly 1–7 % of the total bacterial population in the euphotic zone are AAP bacteria (Kobližek 2015). The high abundance and their higher growth rates compared to other oceanic bacteria may indicate their importance in carbon cycling (Ferrera et al. 2011). Moreover, they are widely distributed in a diverse range of aquatic ecosystems, such as freshwater, hot spring, brackish waters and marine environments. Some of the AAP bacterial species are also found in rhizosphere (Andreote et al. 2009), aquatic and terrestrial soil (Ritchie and Johnson 2012), even recently in plant phyllosphere and endosphere (Nissinen et al. 2023).

Plant phyllosphere is the environment on leaf surface, which is colonized by numerous metabolically diverse bacteria (Atamna-Ismaeel et al. 2012). It is poorly understood how plant microbiomes', especially phyllosphere microbiomes', diversity affects plant health and function, such as growth, immunity to disease (Stone et al. 2018) and the resistance to biotic or abiotic stressors (Trivedi et al. 2020). Some mutualistic microbes in the phyllosphere help plants in their growth, for example in nutrient uptake, resistance towards pathogens (Pieterse et al. 2014, Trivedi et al. 2020), or affecting the wettability of the leaf (Knoll & Schreiber 2000). The wettability and leaf cuticular permeability are the most essential features of the phyllosphere, because they regulate nutrient access through the leaf tissues and affect phyllosphere microorganisms communities (Bulgarelli 2013). According to Pieterse et al. (2014), plants have even been found to support mutualistic bacteria because of their beneficial features. For example, Pattnaik et al. (2017) stated in their research that most bacteria in the phyllosphere, which belong to the genus *Methylobacterium, had* an increasing effect on plant growth and seed germination.

Endosphere is the plant interior part where the complex microbial communities, endophytes, are colonizing. Endophytes can be anything between mutualists and pathogens but they mostly do not negatively affect the host plant performance (Compant et al. 2020). Mutualistic endophytic bacteria can positively affect plant growth and health (Fan et al. 2023) but they were also assumed to be good use for bioremediation due to their induction of pollutant tolerance (Pham et al. 2022). Bioremediation is the technique where a polluted environment is cleaned by removing toxic waste using microorganisms, mostly aerobes and anaerobes (Bala et al. 2022). According to Nissinen et al. (2023), AAP bacteria were consistently present in the endosphere of several perennial plants. Some of the AAP bacteria are capable of degrading pollutants for acquiring energy for themselves (Hiraishi et al. 2002) but yet there is too little information about AAP bacteria presence in plant phyllosphere or endosphere communities.

Furthermore, it is important to understand plant health, function, and the effect of photosynthesis, because this understanding is beneficial for e.g. agriculture. (Rastogi et al. 2012.) However, the function of AAP bacteria and their effect on host plants have not been studied at all. At this point, there are only limited studies about the presence of AAP bacteria in plant phyllosphere and endosphere, and their diversity, biogeography and interactions between plants (Nissinen et al. 2023).

1.1 AAP bacteria

AAP bacteria are taxonomically and phylogenetically heterogeneous but they have a distinctive features, such as presence of bacteriochlorophyll a (BChl *a)* in both reaction center and light harvesting complexes, high abundance of carotenoids but low abundance of photosynthetic unit in cells (Yurkov & Beatty 1998). They are not able to grow under anaerobic conditions, because they are facultative heterotrophs that require oxygen for their metabolism and growth (Yurkov & Csotonyi 2009).

AAP bacteria are a genetically extremely diverse group with over 100 genera (Villena-Alemany et al. 2024), which belong to *Proteobacteria* phylum and most members belong to the class of alpha-, beta- and gammaproteobacteria (Ritchie and Johnson 2012). Every photosynthetic prokaryote has a bacteriochlorophyll-based reaction center, where they utilize light for anoxygenic photosynthesis (Atamna-Ismaeel et al. 2012). Anoxygenic photosynthesis is a photosynthesis without producing oxygen (Yang et al. 2021). In bacterial photosynthesis, oxygen is not produced because they use diverse organic compounds as an electronic donor (Kobližek 2015), while in ordinally photosynthesis, light energy, carbon and water are used to produce oxygen because they use water as an electronic donor. Photosynthetic prokaryotes are capable of using both light and organic compounds for energy and carbon requirements (Lami et al. 2009) and because of it, bacteria can adapt to different environmental conditions by using different chemical reactions for carbon fixation and energy reproduction, such as maintaining growth and metabolism (Moo-Young 2011).

AAP bacteria have a photosynthetic pigment BChl *a*, which is found mostly in oxygenic, photosynthetic microbes (Ritchie and Johnson 2012) such as in purple phototrophic bacteria (Yang et al. 2021). Phototrophic prokaryotes use photons, light energy, for their energy supply. Thus, light is an energy advantage for AAP bacteria (Waidner & Kirchmann 2007). In oxygenic photosynthesis, carried out by plants and algae, chlorophylls absorb mostly visible light photosynthesis, with absorption peaks around 450–475 nm and 650–675 nm. The BChl a in AAP bacteria absorb the wavelength of 390 nm and 850–920 nm, in which the highest excitation is around 880 nm (Nissinen et al. 2023).

1.2 Background

The Shared Light (Jaettu valo, JaVa) is a project organized by researchers of University of Jyväskylä and the Steiner school of Oulu in Finland. The Shared Light project studies the biology of AAP bacteria in the northern ecosystem. The

aim of the Shared Light project (peda.net n.d.) is to clarify the interaction between plant-associated microbes and their host plants, and also how seasonal dynamics affect the well-being of plants.According to Yurkov & Csotonyi (2003), AAP bacteria are often found in extreme conditions, such as inside soils where microbes meet low or high heat, low water capacity, high ultraviolet radiation exposure and in high salinity and high altitude lakes (Yurkov & Hughes 2017).

The Shared Light project has collected AAP bacteria from Finland, from a boreal-subarctic climate and plant samples were collected in different years between 2021–2023 in all seasons. From Finland, plant species were collected from 10 different places with 5 different vegetation types, which were alpine/oroarctic zone (Utsjoki, Kaldoaivi, Kevo and Kilpisjärvi,), northern boreal zone (Rovaniemi and Kuusamo), middle boreal zone (Oulu), southern boreal zone (Jyväskylä) and hemiboreal zone (Turku and Espoo). (Nissinen et al. 2023).

This thesis is an extension of the Shared Light project. Because AAP bacteria were often collected from extreme climates and their presence were studied, in contrast the bacteria were collected from a non-extreme climate, from Japan. The first discovery of aerobic bacteria that contain BChl *a*, was reported by Shiba et al. (1979) when they studied the marine bacteria of aerobic marine environments from the bottom of Tokyo Bay. They collected beach sand, the surface water of seawater and seaweeds as a sample (Shiba et al 1979). Moreover, they have been found even from a terrestrial hot spring (Muramatsu et al. 2022). The vegetation of Japan is mostly temperate broadleaf forest or subtropical evergreen and the climate is mostly temperate and in summer times temperatures are often over 30 °C or more, and high humidity with over 75% (WorldData 2015). The plant samples were collected only in the southern-east part of mainland Honshu, from the capital of Japan, Tokyo, and its neighboring prefecture, Chiba.

1.3 Research questions

AAP bacteria were discovered in plant phyllosphere and endosphere recently. Therefore there is little information available about their abundance and prevalence in plant species, for example which kind of plants they foster in and what kind of climate they occur in (Nissinen et al. 2023).

The main research question of this study is: "Can AAP bacteria be found in Japanese vegetation?" According to the null hypothesis, there should not be any AAP positive bacteria in the vegetation of Japan. However, as mentioned in the introduction of this thesis, AAP bacteria have been found in aquatic ecosystems in Japan, for example from the Tama river (Sato-Takabe et al. 2020), hot spring and even in seaweeds. Therefore, my first hypothesis is that AAP bacteria can be found in the vegetation of Japan. Another reason is that according to Nissinen et al. (2023), AAP bacteria are found in most of the plant species collected in Finland. In their collected samples, AAP bacteria were present in 20-100 % of the plant phyllosphere and 0-91 % of the endosphere.

The second research question of this study is: "Assuming AAP bacteria are found in Japanese vegetation, in which plant species are they found?" According to Nissinen et al. (2023), AAP bacteria were constantly present in the plant endosphere, especially in perennial leaves or photosynthetic tissues. However, some plant samples, such as *Betula sp.*, did not have any AAP bacteria in the endosphere (Nissinen et al. 2023). *Betula sp.* is a deciduous plant, which drops leaves in winter and thus their leaves are not perennial. Therefore, the hypothesis is that leaf perenniality affects the prevalence of AAP bacteria.

The third research question is: "Assuming AAP bacteria are found in Japanese vegetation, do Japanese microbial species differ from Finland?" According to Nissinen (2023), AAP bacteria are commonly found in cold climates. The Shared Light project has collected plant samples from several countries but none of them were collected from warm environments, such as subtropical climates. AAP bacterial species which the Shared Light project extracted from plant samples from Finland were *Sphingomonas* sp. from *Sphingomonadales* family and *Methylobacterium* sp., from *Methylobacteriaceae*

family, *Lichenihabitans* sp. from *Rhizobiaceae* family and *Aurantimonas* sp. from *Aurantimonadaceae* family (Nissinen et al. 2023) All these bacteria belongs to *Alphaproteobacteria* class.

Methylobacterium taxa are commonly found around boreal (Atamna-Ismaeel et al. 2012, Zervas et al. 2019) to arctic environments (Nissinen et al. 2023), in addition, *Methylobacterium* sp. taxa were also found in the desert (Li et al 2020). As reported by Trotsenko et al. (2001), the members of *Methylobacterium* sp. in the phyllosphere are highly resistant to dehydration, elevated temperatures and UV ionizing radiation. There was also an article by Atamna-Ismaeel et al. (2012) about phyllosphere AAP bacteria collected from clover (*Trifolium repens),* rice, soybean and thale cress (*Arabidopsis thaliana)*, all of which were collected from the Czech Republic in April 2011. Moreover, tamarisk (*Tamarix nilotica*) leaves were collected from Israel. As result, a total of two families of AAP bacteria was found, which were *Methylobacterium* sp. and *Rhizobiales* sp. These same AAP bacterial families were also found in Zervas et al. (2019) from wheat collected in June 2018 in Denmark. In addition, they also found *Alsobacter* sp., *Sphingomonas* sp. and *Roseomonas* sp., which are from *Alphaproteobacteria* class as well.

Because the AAP bacterial species that were found in Denmark, Israel, Czech Republic and Finland were mostly similar, my hypothesis is that there are no differences in bacterial species between Finland (the boreal – subarctic climate) and Japan (temperate – subtropical) either.

2 METHODS

Materials were collected from Japan, which were compared with the data collected by the Shared Light project (2021-2023). All of the isolated AAP positive strains were identified to genus level.

The plant samples of Japan were collected from Tokyo, Toneri park (35,47°N, 139,46°E) and University of Chiba, Nishichiba Campus area (35,44°N, 139,54°E) in July. Four plant species (Table 1.) were collected from both of the

cities. Phyllosphere and endosphere samples were isolated from each plant species. Branch samples were collected only from *Pinus sp.* and *Ginkgo biloba* (Table 1). The plant species collected for this study were preferred according to their perennially, the similarity of genus with the plant species collected by the Shared Light project, and the frequency of species occurrence in Japan.

Table 1. Collected plant species in Japan.

Common name of the species	Species	Branch samples
Pine	*Pinus sp.*	Yes
Japanese maple	*Acer palmatum*	No
White clover	*Trifolium repens*	No
Maidenhair tree	*Ginkgo biloba*	Yes

Three leaf samples were collected from each plant species and three branch samples were collected from *Pinus sp.* and *Ginkgo biloba* from each city. Each leaf and branch samples were collected from different individuals. The exact locations of the sampling from Tokyo, Toneri park, can be seen at figure 1 and in Chiba, University of Chiba campus area in figure 2. Thus from one city, there were 12 leaf samples (3 leaf samples from each species from different individuals x 4 species) and 6 branch samples (3 branch samples from each species from different individuals x 2 species) collected. Leaf and branch samples were collected with sterile scissors into sterile boxes and stored at 4 °C at the maximum of three days before processing.

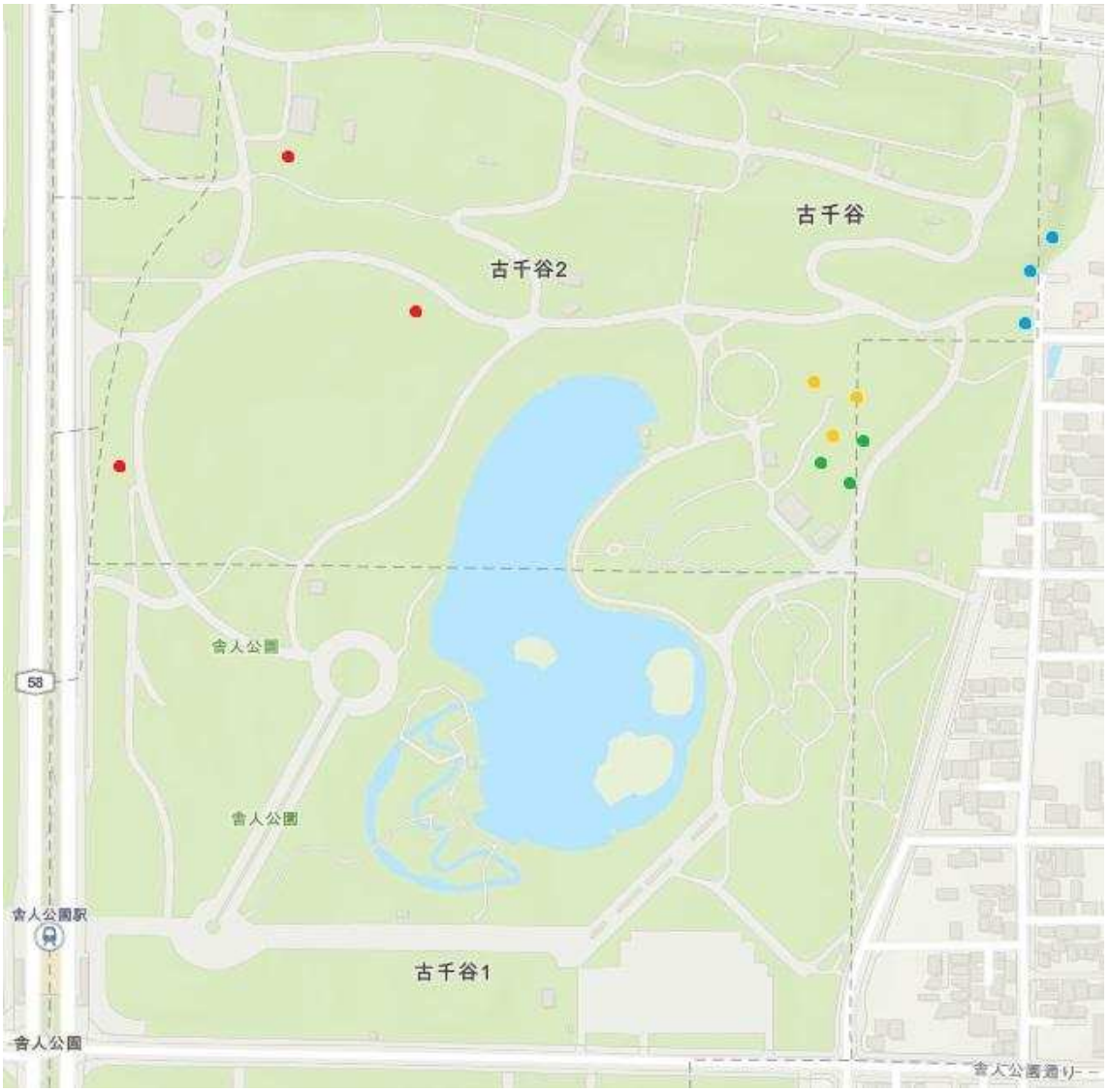

Figure 1. The sampling area from Tokyo Toneri park. Each dot represents the collected leaf and branch samples from different individual plants, and the colors of the dots represent different plant species: *Trifolium repens* (red), *Pinus sp.* (green), *Acer palmatum* (yellow) and *Ginkgo biloba* (blue).

Figure 2. The sampling area from University of Chiba campus area. Each dot represents the collected leaf and branch samples from different individual plants, and the colors of the dots represent different plant species: *Trifolium repens* (red), *Pinus sp.* (green), *Acer palmatum* (yellow) and *Ginkgo biloba* (blue).

2.1 Methods for phyllosphere samples

From the collected leaves, phyllosphere bacteria were isolated into 50 ml conical tube and 10 ml of isolation buffer (20 mM potassium phosphate buffer, KPi, pH 6,5, Silwet-L77 0,025%) using an ultrasonic bath (Ultrasonic cleaner US-104) for

4 minutes. 2 x 1 ml of the suspension buffer was moved into two 1,5 ml Eppendorf tubes, which were centrifuged for 3 minutes at 13 000 g. 900 μl of supernatant were removed and the remaining pellet was resuspended into one tube (total of 200 μl). Resuspended bacteria were diluted to 10^{-1}, 10^{-2}, 10^{-3} using 20 *mM* KPi (pH 6,5) buffer. The isolates were divided into three different dilutions (10^{-1}, 10^{-2}, 10^{-3}) for avoiding overgrowth of bacterial colonies in Petri dishes and to make bacterial colony calculation easy.

2.2 Methods for leaf endosphere and branch samples

The same leaf which was used for phyllosphere bacteria isolation were used for the endosphere bacterial isolation as well. The phyllosphere of leaf and branch samples were sterilized by submerging in 3% sodium hypochlorite for 3 minutes. After hypochlorite, sterilized samples were submerged into sterile water for another three minutes. This sterile water step was repeated three times to ensure that hypochlorite is no longer in the samples. The tissues of the branch and leaves were moved into sterile plastic bags and 5 ml of isolation buffer (20 mM (KPi), pH 6,5) were added to each plastic bag. They were crushed carefully so the endosphere bacteria could be released to the isolation buffer. 1 ml of homogenized buffers with endosphere bacteria were transferred into 1,5 ml Eppendorf tubes. Resuspended bacteria were diluted to 10^{-1}, 10^{-2}, 10^{-3} using 20 *mM* KPi (pH 6,5) buffer.

2.3 Plating and imaging

100 µl of each dilution series were plated into ½ x R2A (pH 6,5) agar plates. Plates with phyllosphere samples contained an additional amount of fungicide nystatin (50 mg/ml), because phyllosphere samples are expected to be more prone to fungi growth compared to the endosphere samples. Plates were incubated at room temperature for 3–5 days, after which they were transferred into 4 ºC for 3–5 weeks.

After 3 weeks when plates had bacterial colonies, they were imaged by a near-infrared imaging system (NIRis) to check their AAP positivity. NIRis is an

imaging system used for discovering BChl *a* containing bacterial colonies by detecting UV-induced NIR-fluorescence (Franz et al. 2023). There are two different lamps used in NIRis (Figure 3). LED-based white lamps were used to capture reference images for detecting the location of bacterial colonies from NIR fluorescence images. LED-based lamps (Glossday, 128 LED UV Flashlight) were used in UV-induced NIR fluorescence imaging. The used camera was Raspberry Pi, PiNoir Camera V2, 913-2673 and the detailed setting of the NIR imaging system is written in Table 2.

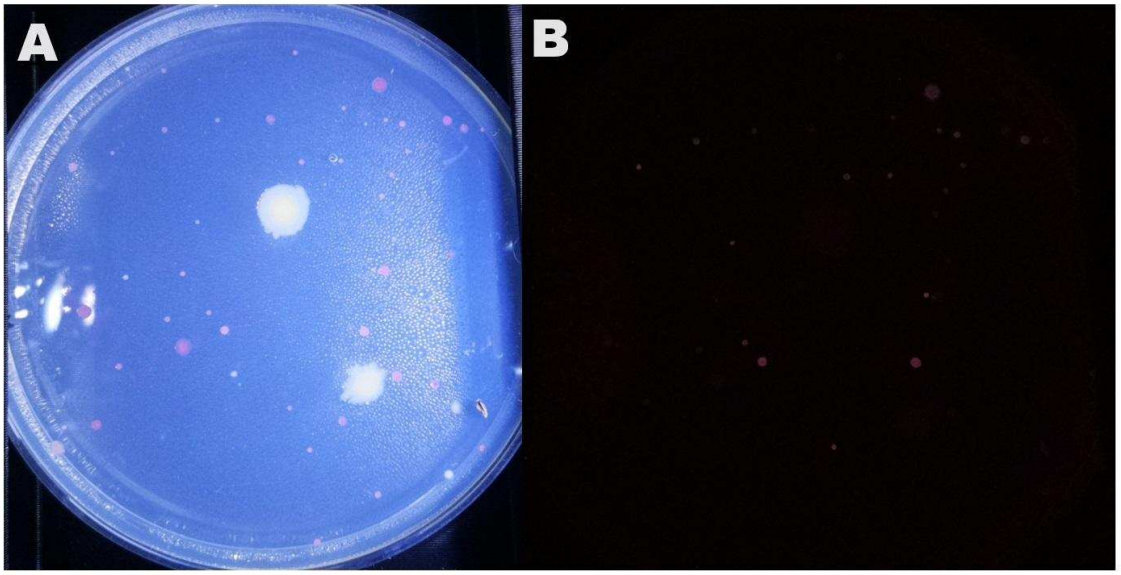

Figure 3. Pictures of bacterial colonies taken by NIRis. In picture A, Petri dishes were pictured by white light to see the location of all bacterial colonies. In picture B, Petri dishes were pictured through 1" Bandpass filter (Thorlabs, FB880-70 3) by UV-induced NIR fluorescence. The pink dots on the right side picture are bacterial colonies which are AAP positive.

Table 2. The detailed settings from Franz et al. (2023) of NIRis imaging device, Raspberry Pi computer program.

Program	Setting, Unit
Camera ISO (Light sensitivity)	800
Shutter speed	0.35 sec
Resolution	1280 x 1024 pixel

After checking the AAP positivity from Petri dishes by NIRis, the fluorescing AAP bacterial colonies were replated to other plates for ensuring the purity of AAP positive bacteria for sequencing.

2.4 Ensuring purity of AAP positivity by re-plating

When re-plating strains, a single AAP positive colony was picked using an inoculation loop, re-plating was done at least two times to ensure the purity of bacterial colonies. Replated Petri dishes were stored at room temperature for 2–3 days and moved to 4 ºC for 5–7 days. Bacteria were re-plated every time when bacterial colonies appeared to be impure. This is for example different colored or sized bacterial colonies in one re-plated Petri dish.

2.5 Harvesting DNA templates for sequencing

From the plates containing pure strains, one single bacterial colony was picked up by using an inoculation loop and it was added into 8-striped PCR tubes, which has 16 μl of sterilized water (MilliQ), 20 μl of Dreamtaq Green, 1 μl of 27F primer and 1492R primer (both 10 µM) (Lane 1991) and 2 μl of templates each. The rest of the bacteria colonies in the plates were picked up by an inoculation loop and transferred into cryotubes with R2B-glycerol medium and stored at –80 ºC for later use or storing bacterial DNAs. PCR tubes were put into PCR (C1000 Touch Thermal Cycler) and were run in a program written in table 3. cryotubes were stored at –80 ºC and PCR tubes at –20 ºC.

Table 3. PCR running program in C1000 Touch Thermal Cycler.

Steps	Duration	Temperature
1.	5 min	94 °C
2.	30 sec	94 °C
3.	1 min	55 °C
4.	1,5 min	72 °C
	Repeat steps 2. – 4. total of 35 times.	
5.	10 min	72 °C
6.	10 min	12 °C

DNA templates were Sanger sequenced and partially 16S rRNA sequenced and the aligned rRNA genes were classificated on Silva 16S rRNA database (Pruesse et al. 2012). The evolutionary history of isolated rRNA genes were inferred by Maximum Likelihood (ML) method and Tamura-Nei model (Tamura & Nei 1993) using MEGA11. The Neighbor-Joining and BioNJ algorithms were applied to a pairwise distance matrix, which were estimated by using the Tamura-Nei model and selecting the topology with superior log-likelihood value for obtaining the initial tree for the heuristic search.

3 RESULTS

The AAP bacteria were found in every plant species (*Pinus sp., Trifolium repens, Acer palmatum, Ginkgo biloba)* collected from Japan. Of the 24 leaf (24 phyllosphere and 24 endosphere samples) and 12 branch samples collected, four out of 24 (17%) of leaf samples and five out of 12 (42%) of branch samples were positive. There were no endosphere AAP bacteria found in any leaf sample in both cities. From all of the AAP positive leaf and branch samples (total of 9 samples), 2 samples of *Ginkgo biloba,* 5 samples of *Pinus sp.,* 1 sample of *Trifolium repens* and 1 sample of *Acer palmatum* were AAP positive. There were no AAP bacteria found from the leaf of *Ginkgo biloba* but from *Pinus sp.*,

two samples out of five (40%) were AAP positive from the phyllosphere and the rest were from the branch samples (60%). Out of the total 36 collected leaf and branch samples, nearly 25% turned out to be AAP positive (Figure 4). The branch sample AAP positivity rate was 42% and the leaf sample was 17%. Leaf samples are the combination of all leaf phyllosphere and endosphere samples. From the endosphere, AAP bacteria were found only in *Pinus* sp. From all AAP positive samples (n=9), five out of nine (56%) was from branch and four out of nine (44%) was from leaf samples, which of all was from the phyllosphere.

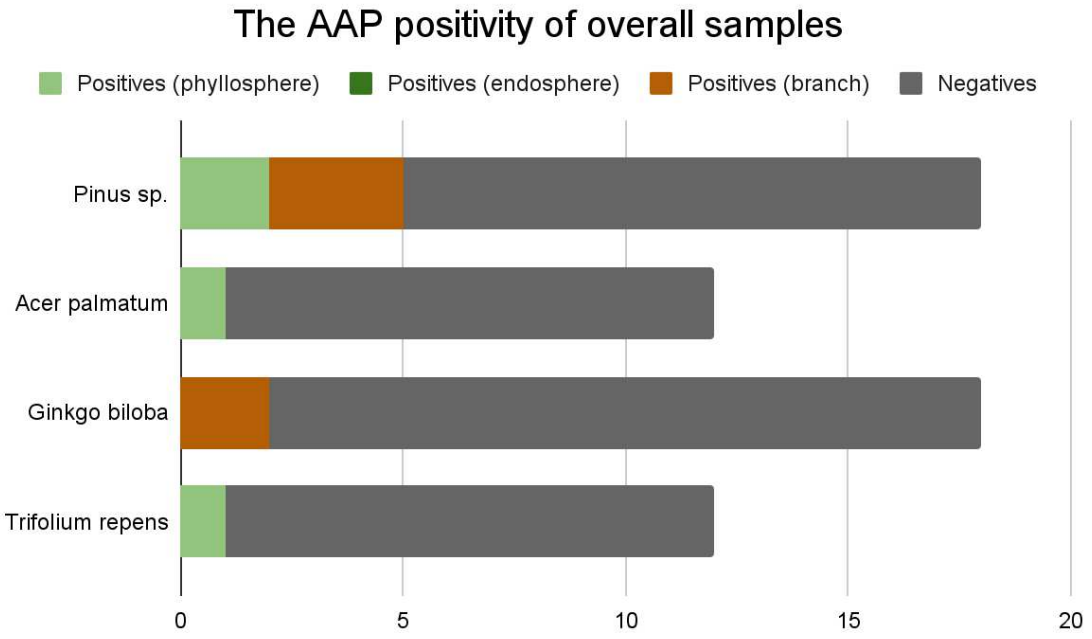

Figure 4. AAP positivity rate from all of the collected plant samples. X axis tells the amount of samples (n). All plant species had a total of 3 leaf samples each (3 leaves from one species x 4 plant species x 2 cities, which were processed into phyllosphere and endosphere isolation). In addition to, *Pinus sp,* and *Ginkgo biloba* had 6 branch samples (3 from Tokyo, 3 from Chiba for each species)

3.1 Chiba samples

The total of 12 leaf and 6 branch samples collected from Chiba, three out of 12 (25%) of leaf and three out of six (50%) branch samples were AAP positive. From the AAP positive leaf samples, two third (67%) were from *Pinus sp*. and one third (33%) were from *Acer palmatum*. From the AAP positive branch samples, two third (67%) was from *Ginkgo biloba.* and one third (33%) was from

Pinus sp. There were no AAP positive bacteria found in *Trifolium repens.* The total of 18 collected leaf and branch samples, 6 out of 18 (33%) turned out to be AAP positive (Figure 5). The branch sample AAP positivity rate was 50% and the leaf sample 25%.

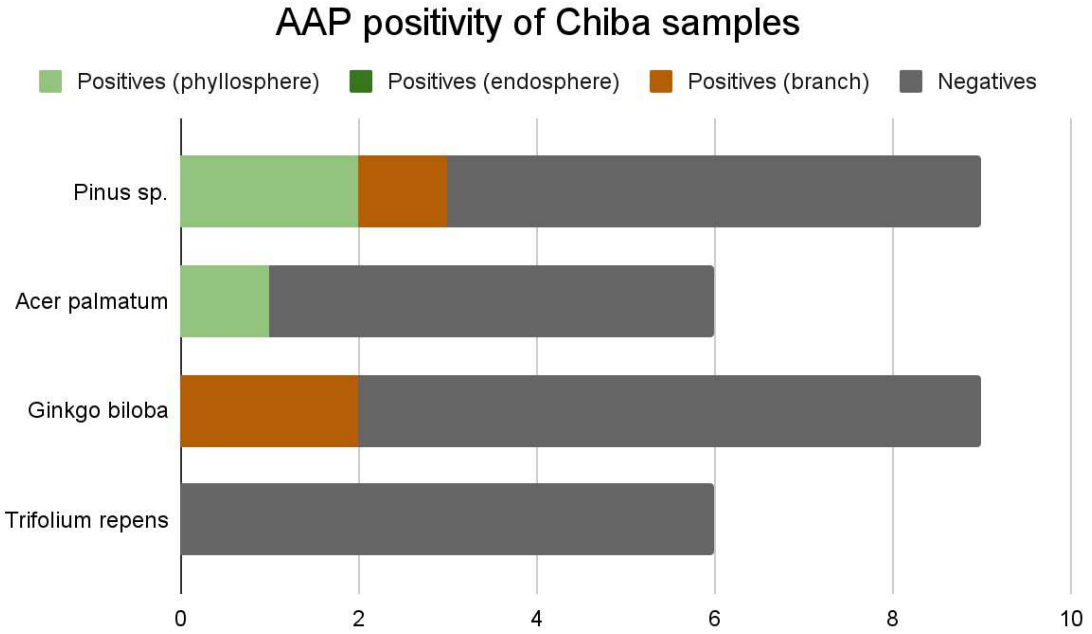

Figure 5. AAP positivity rate from all of the Chiba plant samples. X axis tells the amount of samples (n). All plant species had a total of 3 leaf samples each and in addition, *Pinus sp,* and *Ginkgo biloba* had 3 branch samples each.

3.2 Tokyo samples

The total of 12 leaf and 6 branch samples collected from Tokyo, one out of 12 (8%) of leaf and two out of six (33%) branch samples were AAP positive (Figure 6). From the AAP positive leaf samples, *Trifolium repens* had an AAP positive bacteria in its phyllosphere. From the AAP positive branch samples, only *Pinus sp.* had an AAP positive bacteria. There were no AAP positive bacteria in *Acer palmatum* and *Ginkgo biloba*. The total of 18 collected plant samples, 3 out of 18 (17%) turned out to be AAP positive. The branch sample AAP positivity rate was 33% and the leaf sample 8%.

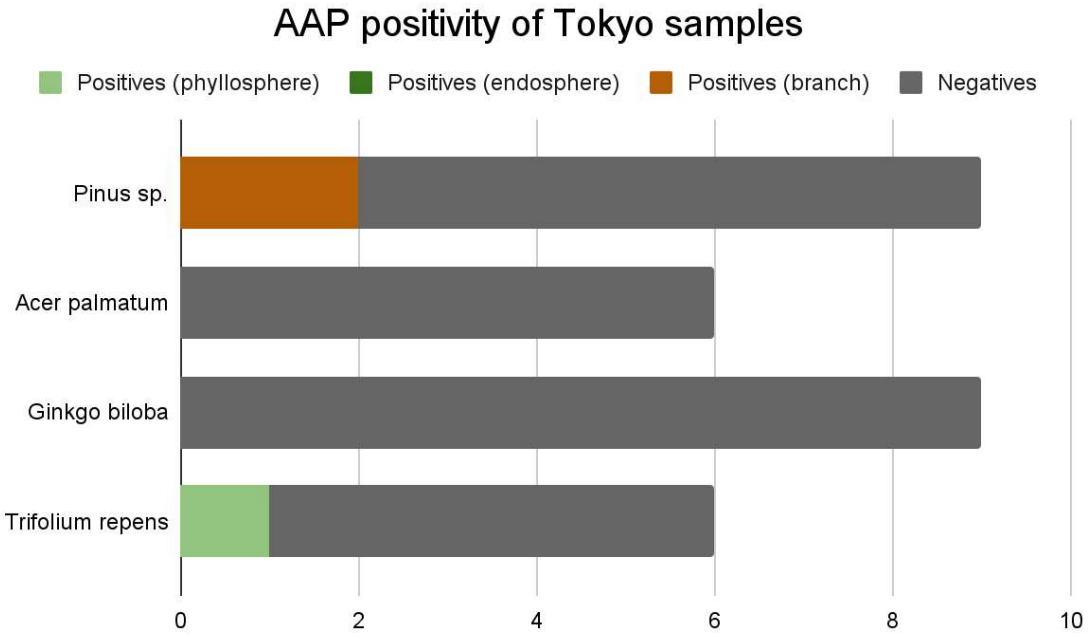

Figure 6. AAP positivity rate from all of the Tokyo plant samples. X axis tells the amount of samples (n). All plant species had a total of 3 leaf samples each and in addition, *Pinus sp,* and *Ginkgo biloba* had 3 branch samples each.

3.3 Phylogenetics of rRNA sequences

The total of 33 bacterial colonies isolated for sequencing, 3 of them were AAP negative based on NIRis imaging at the re-plating stage. These negative isolates were two from Chiba and one from Tokyo. Species were *Ginkgo biloba* from Chiba and *Trifolium repens* from Tokyo. The total number of bacterial colonies were calculated from each plate of isolates and it was compared with the total number of AAP positive bacterial colonies (Table 4). From all bacterial colonies detected, nearly 28,9 % were AAP positive bacteria. In Chiba, the AAP positive colony rate from all bacterial colonies was 33,5 % and in Tokyo 22,3 %.

Table 4. The total number and the percentage of AAP positive colonies from all bacterial colonies. The amount of the same replicates means that bacterial colonies were isolated from the same plates.

City	The amount of same replicates	Species	Location of the plant	AAP positive colonies (n)	Total colonies (n)	Positive colony rate from all colonies (%)
Chiba	4	*Ginkgo biloba*	branch	14	55	25,5
Chiba	1	*Ginkgo biloba*	branch	2	2	100
Chiba	5	*Pinus sp.*	phyllosphere	9	121	7,4
Chiba	1	*Pinus sp.*	branch	4	38	10,5
Chiba	3	*Pinus sp.*	phyllosphere	23	57	40,4
Chiba	3	*Pinus sp.*	phyllosphere	66	251	26,3
Chiba	1	*Acer palmatum*	phyllosphere	1	4	25
Tokyo	3	*Pinus sp.*	branch	5	15	33,3
Tokyo	1	*Pinus sp.*	branch	1	73	1,4
Tokyo	3	*Pinus sp.*	branch	49	270	18,2
Tokyo	4	*Pinus sp.*	branch	62	112	55,4
Tokyo	1	*Trifolium repens*	phyllosphere	1	29	3,5
			AVERAGE:	19,8	85,6	28,9

The total of 30 isolates from Japan were sequenced by Sanger sequencing using a partial 16S rRNA sequence. After sequencing, one isolate was discarded due to its insufficient sequence length.

Based on phylogenetic analyses by Neighbor-Joining with ML and Tamura-Nei model, estimates of 9 different species of AAP bacteria were found from the 29 isolates, all belonging to *Methylobacterium* sp. (Figure 7).

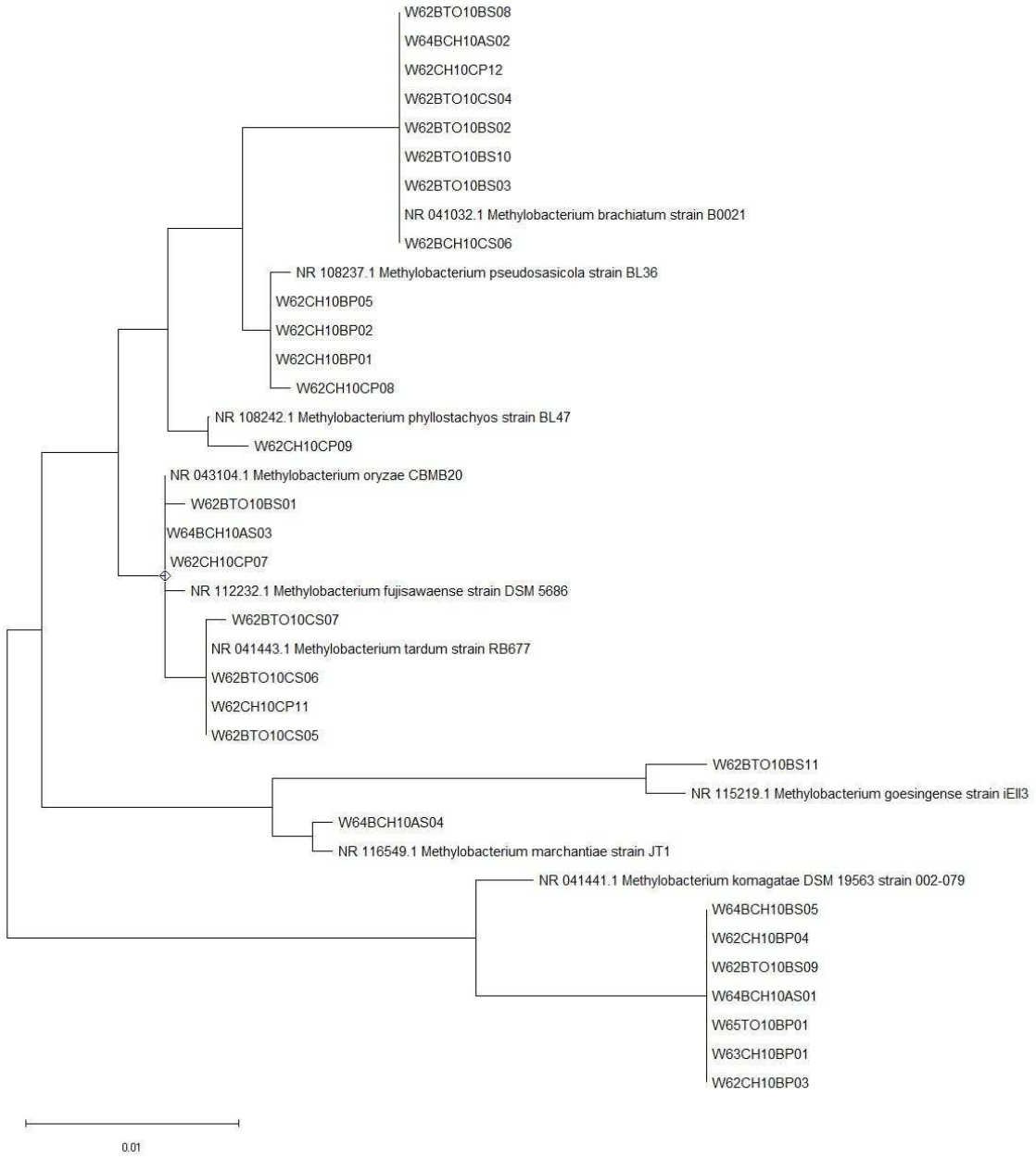

Figure 7. Phylogenetic analyses of all positive sequences from Japan by Neighbor-Joining with Tamura-Nei model. The marked 9 bacterial species represent the closest reference species from Silva 16sS rRNA gene database. The first part are the sample species name (W62 = *Pinus sp.*, W63 = *Acer palmatum*, W64 = *Ginkgo biloba*, W65 = *Trifolium repens*), the second part is the city (CH = Chiba, TO = Tokyo) and the third part is from what part of the plant is sample taken (S = endosphere, P = phyllosphere). Branch samples are written as (B) after the sample species name.

According to the Neighbor-Joining phylogenetic analyses, most isolates were identified as *Methylobacterium brachiatum* (n=8), and secondly was *Methylobacterium komogatae (n=7)*. There were some differences in the presence

of AAP bacterial species between Tokyo and Chiba (Figure 8). *Methylobacterium brachiatum* was more present in plant samples from Tokyo compared to plant samples from Chiba but *Methylobacterium komogatae* was more present in the samples from Chiba. *Methylobacterium phyllostachyos* and *Methylobacterium marchantiae* were present only in the samples collected from Chiba and *Methylobacterium goesingense* and *Methylobacterium fujiwaense* were only present in the samples collected from Tokyo.

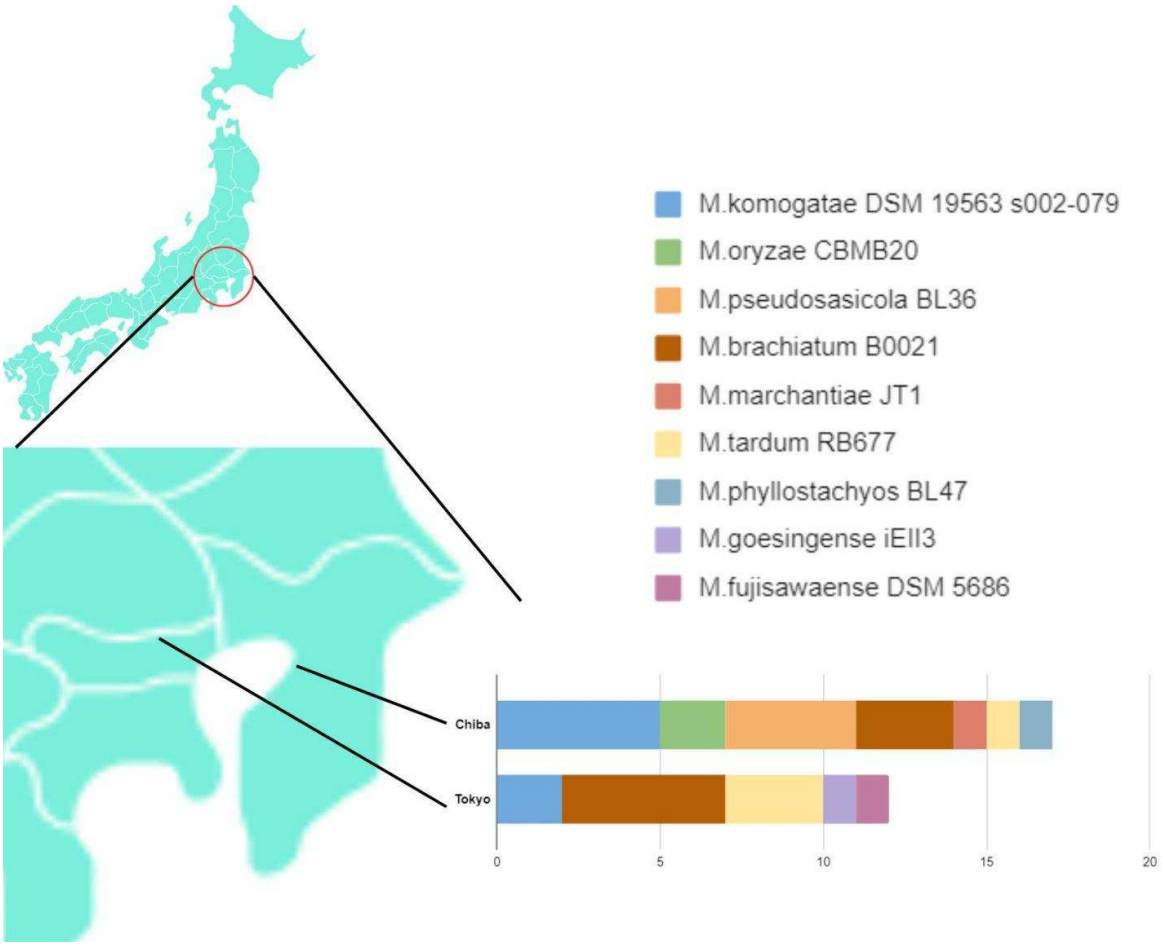

Figure 8. AAP positive bacterial species in their sampling sites. The bar represents the isolated AAP positive bacterial species and their amount (n) in each site. Different AAP bacterial species were indicated by different colors.

In Chiba samples, the AAP bacterial species diversity was highest in endosphere samples (6 different AAP bacterial species from the total of 7) and the lowest in phyllosphere (2 different AAP bacterial species from the total of 7).

Methylobacterium komogatae was the only AAP bacterial species found in phyllosphere, endosphere and branches (Figure 9). In Tokyo samples, the AAP bacterial species diversity was highest in branch samples (5 different AAP bacterial species from the total of 5) and the lowest in the endosphere (no AAP bacterial species found). *Methylobacterium komogatae* was the only species found both in the phyllosphere and in branches (Figure 10).

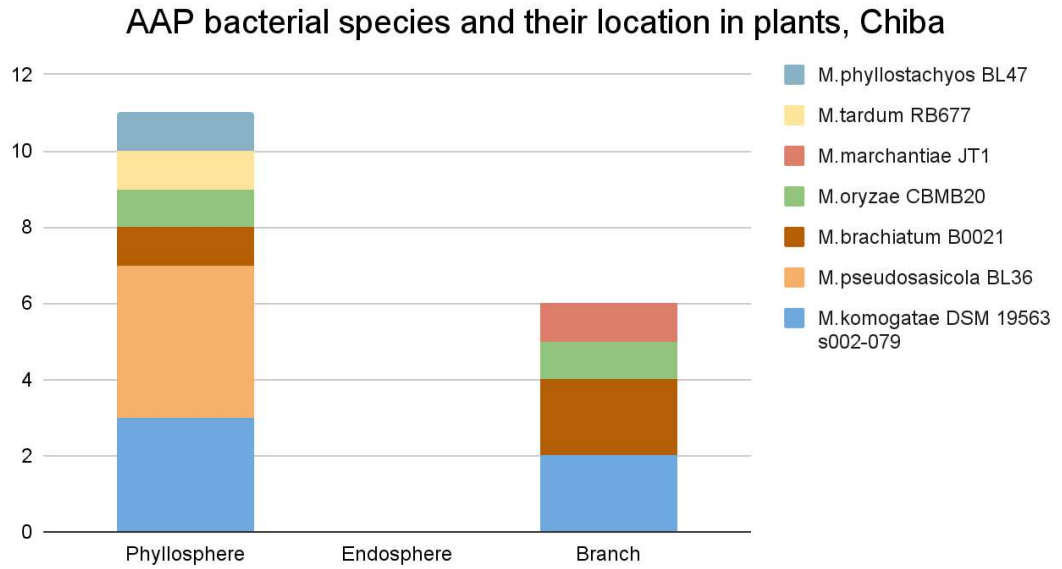

Figure 9. AAP bacterial species in different plant locations in Tokyo. Different colors represent AAP bacterial species and X-axis represents where the bacteria occurred in plants.

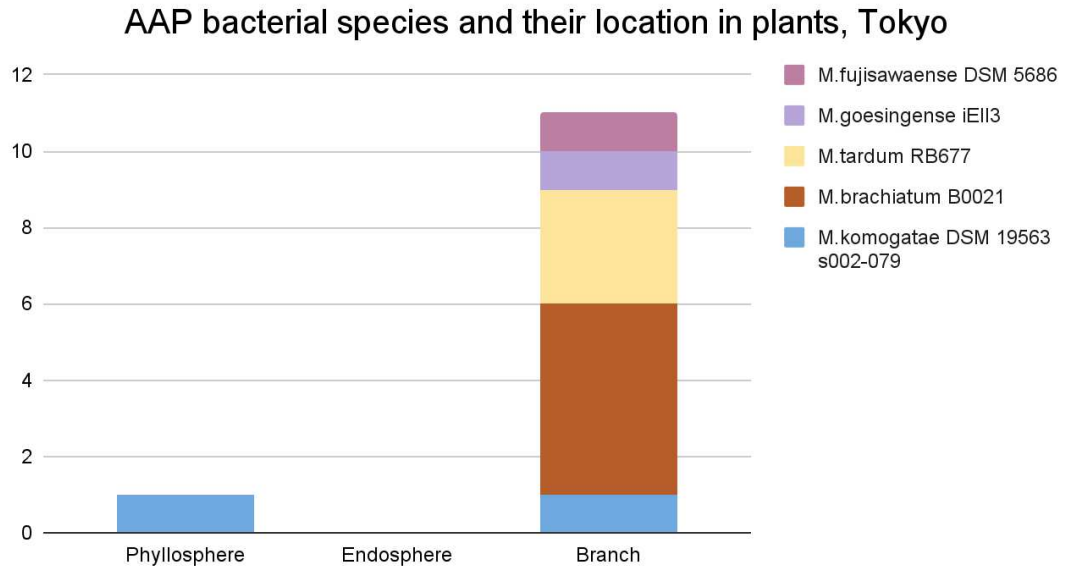

Figure 10. AAP bacterial species in different plant locations in Tokyo. Different colors represent AAP bacterial species and X-axis represents where the bacteria occurred in plants.

In both cities, AAP positive bacteria was found mostly in *Pinus* sp. compared to other plant species. In Chiba, only one AAP positive bacterial species, *Methylobacterium* sp., was found in *Trifolium repens* (Figure 11) and *Acer palmatum* in Tokyo (Figure 12). In Chiba, there were an estimated 4 different AAP bacterial species in *Ginkgo biloba*. In Tokyo, there were no AAP positive bacteria found in *Ginkgo biloba*.

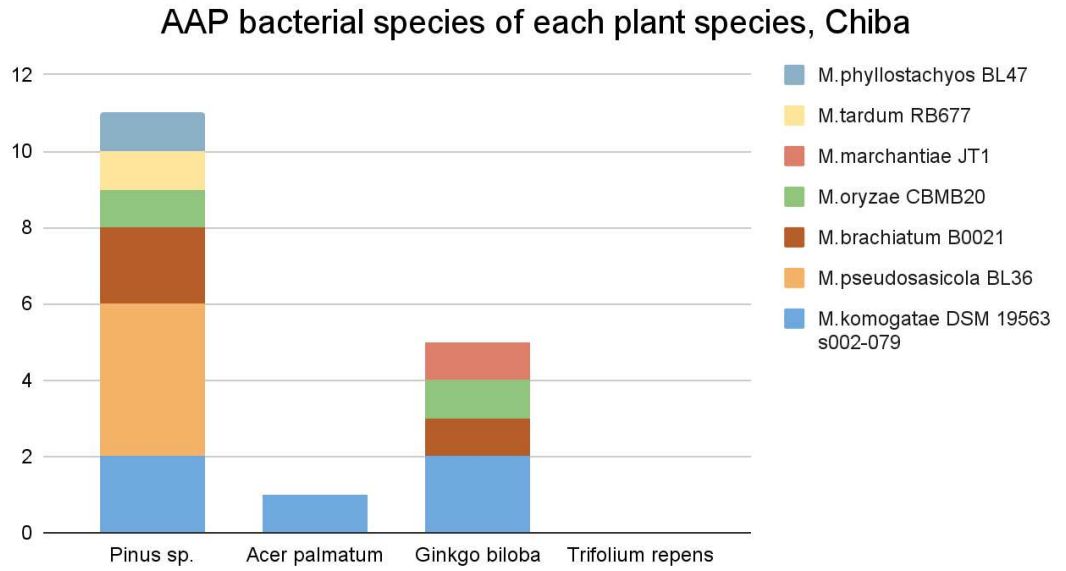

Figure 11. AAP bacterial species in different plant species in Chiba. Different colors represent AAP bacterial species and plant species are written in X-axis.

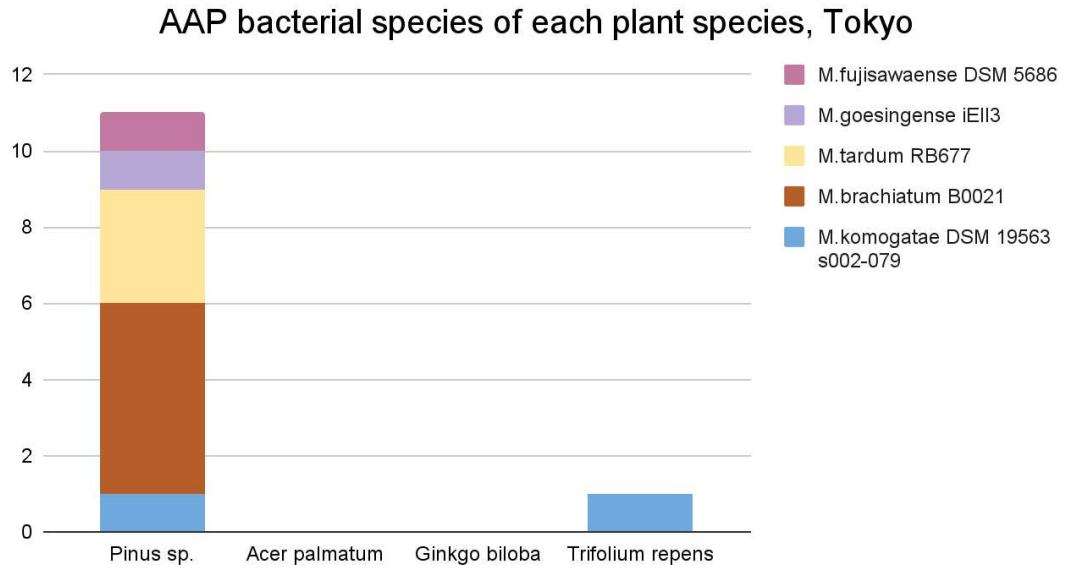

Figure 12. AAP bacterial species in different plant species in Tokyo. Different colors represent AAP bacterial species and plant species are written in X-axis.

4 DISCUSSION

The aim of this study was to examine the presence of AAP bacteria in Japan, in warm environments. This study showed that plant-associated AAP bacteria were found in Japan, both from phyllosphere and branch endosphere, but none

from the leaf endosphere), even though their abundance was much lower than expected. Hence, the zero hypotheses "There are no AAP bacteria found in Japanese vegetation" were not supported. Overall, results showed nearly 25% of leaf endosphere and phyllosphere, and branch samples contained at least one AAP positive bacterial colony (Figure 4). The prevalence of AAP bacteria in the Tokyo plant sample showed to be lower compared to Chiba plant samples (Figure 5 and 6). Figure 4 showed that there were no AAP positive bacteria found in the endosphere of any plant species and in the phyllosphere of *Ginkgo biloba.*

The second aim of this study was "Assuming AAP bacteria are found in Japanese vegetation, in which plant species are they found?". AAP bacteria were found in every plant species but they were considerably abundant in *Pinus sp.* (Figure 4). In total, *Acer palmatum* and *Trifolium repens* had only one AAP positive leaf sample out of six and there were no AAP bacteria found in the phyllosphere of *Ginkgo biloba*. These three plant species (*Trifolium repens, Acer palmatum* and *Ginkgo biloba*) have annual leaves which means that they drop leaves off before winter or in *Trifolium repens*, the plant dies in winter.

In contrast, *Pinus* sp. has perennial needles and overall, AAP bacteria was found in two needle samples out of six. As mentioned in results, AAP bacteria were found slightly more abundant (56%) in branches compared to leaves. This means that seven out of nine (78%) plant samples were found somehow from the perennial plant or area. Thus, AAP bacteria could have a tendency to prefer perennial plants or plant areas rather than annual plants or areas. The hypothesis for this study question "leaf perenniality affects the prevalence of AAP bacteria" was supported, even though this conclusion is not reliable due to a small sample size.

The third hypothesis, which was "AAP bacterial species would be similar to the boreal-arctic climate, e.g. Finland" was partially not supported. There were few same bacterial species found in the research of Nissinen et al. (2023), for example, *Methylobacterium brachiatum* was found in *Picea abies* (European spruce) and *Vaccinium vitis-idaea* (Cranberry), *Methylobacterium*

phyllostachyos was found in *Picea abies* and other plant species and *Methylobacterium oryzae* was found in *Vaccinium myrtillus* (blueberry). Overall, of the nine observed AAP bacterial species in Japan, three species were also found in Finland. This means that about one third of the AAP bacterial species observed in Japan were also prevalent in Finland. *Methylobacterium brachiatum* was mostly present in branches of *Pinus* sp. in Japan isolates (Figure 10, 11, 12 and 13), even though they were mostly present in the phyllosphere in the result of Nissinen et al. (2023).

The major difference between the two study sites, Chiba and Tokyo, was the complete absence of other genuses besides *Methylobacterium* sp. in the isolates from Japan. In Nissinen et al. (2023), a total of 23 plant species were collected in Finland, in which 12 plant species were screened in replicated manner (3–24 replicate per one plant species) and 11 plant species had single samplings (one replicate per species). AAP bacteria were found from all 12/12 (replicated samples) and 5/11 (single samples). Thus, out of 23 plant species collected, 17 contained at least one AAP positive bacteria. AAP bacteria were found mostly in *Vaccinium vitis-idaea* and following *Vaccinium myrtillus* (Table 5).

Table 5. A: Collected plant species and their amount of AAP positive bacterial strain in Finland (Nissinen et al 2023).

Species common name	Species	The total amount of observed AAP+ bacterial colonies (n)
Lingonberry	*Vaccinium vitis-idaea*	211
Blueberry	*Vaccinium myrtillus*	134
European spruce	*Picea abies*	87
Birch	*Betula* sp.	79
European red pine	*Pinus sylvestris*	38
Purple saxifrage	*Saxifraga oppositifolia*	28
Aspen	*Populus tremula*	26
Raspberry	*Rubus idaeus*	21
Others	(48)	274
Total:	57	898

The total of 88 isolates sequenced, *Sphingomonas* sp. (49 isolates out of 88) and *Methylobacterium* sp. (34 isolates out of 88) from *Alphaproteobacteria* class were the most commonly found in the Shared Light project in Finland. In addition, there was found *Lichenibacterium* sp. (4 isolates out of 88) from *Rhizobiaceae* family and *Aurantimonas* sp. (1 isolates out of 88) from *Aurantimonadaceae* family (Nissinen et al. 2023).

In the isolates from Japan, no other AAP bacterial species (such as *Aurantimonadaceae* sp. or *Liechenihabitans* sp.*)* reported by Nissinen et al. (2023) were present (Figure 7). According to an article written by Zervas et al. (2019), 21 unique isolates from the wheat phyllosphere of Denmark, the majority of which belonged to *Methylobacterium* genus. The few other isolates belonged to *Rhizobium* sp., which are also from the *Rhizobiaceae* family, *Alsobacter* sp. and *Sphingomonas* sp. (Zervas et al. 2019).

AAP bacterial species diversity comparison at figure 8 has shown that Chiba samples had more species diversity compared to Tokyo samples.

Surprisingly, AAP bacterial species of the phyllosphere samples had considerably greater diversity in Chiba samples but the phyllosphere diversity of Tokyo was poor (Figure 9 and 10). According to the result of Nissinen et al. (2023), the positive rate of AAP bacteria was greater in the phyllosphere compared to the endosphere but the Tokyo result in Figure 10 showed otherwise.

When plant samples were collected from Japan in July, there was a continuous heat wave. Several plant species, especially ground-level plants like *Trifolium repens*, suffered from over radiation of UV light and drought. The plant phyllosphere may have represented an extreme condition for phyllosphere microbes. This could be a possible reason why only *Methylobacterium* sp. was found in Japan.

The collected samples should have been kept cool, willingly at 4 ºC until samples are processed but transporting plant samples from Tokyo took about 1,5 h. On the way back, the cooler box might have warmed too much. Although not expected, unavoidable, minor variations in the handling of the samples, considering transportation temperature and storage condition, could have potentially affected the results.

AAP bacterial species had a slower growth rate than assumed. Some re-plated bacteria consistently appeared AAP negative for a long duration, thus often additional few days were added. Therefore some of the AAP positive bacterial colonies may have been undetected because they showed negative while imaging in NIR imaging, likely due to incomplete bacterial colony growth or lacking fluorescent components. The reason for the slow growth rate compared to *Methylobacterium* sp. samples collected from Finland by Nissinen et al. (2023) is unsure. One potential explanation could be that AAP bacteria in Japan are adapted to higher temperatures, and consequently, the temperature used for bacterial cultivation was too low.

Furthermore, in laboratory experiments involving the isolation of bacteria from the phyllosphere, endosphere and branches, insufficient amount of isolation buffers were used, which might have affected the quantity of

bacterial isolation from the plant samples. In accordance with the method, leaf and branch samples should have been fully submerged in the isolation buffer. However, due to the lack of buffers, all samples were not fully submerged. Overall, sampling at Tokyo had some difficulties and error factors compared to sampling in Chiba as mentioned above, which might have affected the result.

However, further research is needed to determine the factors which influence the difference in AAP bacterial species and their prevalence, such as differences between host plant species, climate, temperature, humidity etc.

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Miyu Murai

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