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Morphological study of nematode on Norfolk Island Pine (*Araucaria heterophylla*) reveal close relatedness with *Deladenus uteropinusus* and is the first record in Chiang Mai, Thailand

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Abstract

The nematodes, *Deladenus* sp. obtained from withering pine trees, *Araucaria heterophylla*, at Chiang Mai, Thailand during April 2016 were morphologically studied. The nematodes were pinned with 70% warm ethanol and cleared by glycerin and morphologically classified according to the method of Deman (1876). The amphimictic nematodes were characterized mainly by the presence of 6 lateral incisures, a cephalic region low, the lip region offset from body, well-developed stylet with its shaft longer than a cone and with single guild ring, excretory pore positioning behind the hemizonid and six lateral incisures in both sexes. The mycetophagous female was 1,133.3±34.4 (1,100-1,200) µm in body length, 41±2.8 (36-46) µm in body width and 11.5±0.8 (10-13) µm in stylet length. Moreover, a large vulva was found with horizontal scratches on the posterior body, protuberant lip without covers, and post-uterine sac. The female tail was cylindrical, rounded, and hyaline. The male was 850±49.3 (850-920) µm in body length, 29.8±4.9 (24-40) µm in body width, 10.8±0.8 (10-11.5) µm in stylet length, and 22.8±2.7 (15.3-26.9) µm in spicule length. Furthermore, a presence of Y-shaped of tylenchoid type spicules was arcuate with pointed spicule tip and conoid tail with lateral membrane bursa. Based on the morphological characters, these nematodes, Deladenus uteropinusus were identified as entomophagous and mycetophagous nematodes. This study was useful for those who are interested in controlling wilting disease of pine trees and other plants.

Key words – *Araucaria heterophylla* – *Deladenus uteropinusus* – Entomophagous nematode – Morphology of nematode – mycetophagous nematode

Introduction

Araucaria heterophylla is an evergreen conifers gymnosperm plant that has evolved from a primeval plant. It is not a local pine tree in Thailand, but most imported to plant as ornamental plants. Pine is a native tree in North America. It is generally found in the southern hemisphere, Australia, New Guinea, Hawaii and the Philippines. Pine rubber can heal wounds and relieve pain. Moreover,

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pine trees can be used for pharmaceutical products and planted to absorb toxins. Most importantly, pinewood, with its numerous economic uses, are mainly industrially utilized in many countries (Ntima 1968, Bittencourt 2007, Abdel-Sattar et al. 2009). However, the pine wilt disease is a severe problem in temperate countries where pine trees are widely grown, such as the United States, Canada, Norway, Australia, West Germany, Finland, China, Korea, Japan, and Taiwan. The problem occurs when stem boring grubs, Monochamus in the family Cerambycidae, carriers of nematodes, *Bursaphelenchus xylophilus*, attack the trees cause a withering outbreak. The nematodes feed on the Blue Stain fungi, which live together with insects (Mota et al. 1999, Mota & Vieira 2008). In addition, pine pests are also found, such as the wood wasps in the Siricidae and Ichneumonida family (Slippers et al. 2015), as well as bark beetles in the family Scolytidae, Curculionidae, Cerambycidae and Buprestidae (Linsley 1961, Ikeda et al. 1980, Kelsey & Joseph 2003, Costello et al. 2013). These insects are associated with the nematodes in the genus *Deladenus* sp. of the family Neotylenchidae (Cobb 1922, Bedding 1972, Yeates et al. 1993). The *Deladenus* sp. nematode adults have 4 different body shapes: male nematodes, mycophagous female, infective female nematodes, and parasitic female. The nematodes that feed on fungi are called mycetophagous nematodes. The female nematodes have two transformations due to their two life cycles. The female that feeds on fungi is larger than the male, has a stylet, and establishes a parasitic relationship with a found insect. The female enlarges its size and shape of the stylet to penetrate the body of the insect (Yu et al. 2013, Siddiqi 2000). The *Deladenus* sp. nematodes are found in the Poaceae, Fagaceae, Salicaceae, Araceae, Fabaceae, Rhamnaceae, Moraceae and Malvaceae family (Wood 1973, Chitambar 1991, Shahina & Maqbool 1992, Bajaj 2015, Nasira et al. 2013, Tomar et al. 2015, Miraeiz et al. 2017). According to the survey of plant diseases in Chiang Mai, Thailand during April 2016, pine trees showed signs of wilt and blue stain. Moreover, the nematodes found on the blue stain are associated with insects and *Leptographium* spp. fungi that destroy pine trees. Therefore, this study aimed to identify the types of *Deladenus* nematodes by studying their morphology, and this report will be the first discovery in Thailand.

Materials & Methods

Isolates and morphology

The bark samples were collected from the withered Norfolk Island pine trees in Chiang Mai province, Thailand, GPS 18°48'06.3"N 98°57'11.7"E .The sample was prepared for stereo microscope examination as follows; cutting the bark samples into small pieces 1 cm width and 1 cm length, placing the barks in the petri dish, adding distilled water to remove the nematodes from the barks .The nematodes were heat-killing and fixed at 50ºC for overnight in 70% ethanol. For morphological studies, nematodes were cleared into pure glycerine at 12 hrs and mounted as this treatment causes the nematodes to straighten instantly and die avoiding distortion while using cold fixatives followed by Seinhor's method (1959). The mounted type materials of all nematodes were used for photomicrographs, drawings and measurements. Photomicrographs were taken with a microscope digital camera system and drawings were conducted using the drawing tube. All measurements were observed under the microscope and morphological characteristics identified using the methods followed by De Man (1876, 1880) modified and amended by Cobb (1914), Thorne (1949), Yu et al. (2017). They are often known as the De Man Formulae or the De Man Indices.

Results

From the investigation of pine wilt and blue-stain wood with moldy mycelial, male and female nematodes including juveniles were found. The amphimictic male and female nematodes were found in the mycetophagous stage (Fig. 1 and the measurements in Table 1).

Description

Deladenus uteropinusus

Female (mycetophagous stage) – The morphological study results of the nematodes constituting the genus *Deladenus* sp. found the obvious characteristics of the female mycetophagous nematodes (Figs 2, 3). To begin with, the body is cylindroid and straight, with 1,133.3 ± 34.4 (1,100-1,200) µm in body length and 41 ± 2.8 (36-46) µm in body width. The cuticle is smooth and the abdomen is ventrally arcuate. When exposed to heat, the body sometimes curves into a C-shape. The annulated cuticle has 6 distinct lateral fields found in the middle of the body. The cervical papillae or deirids are not clear on the head. The head shape is non offset and truncate low and has a cephalic region low. The lips are squarish-round and slightly narrow, and they do not protrude from the body. The stylet is small and well developed. The basal knobs are rounded, of which shaft is longer than cone shaft. There is one guild ring surrounded. The stylet is 11.5 ± 0.8 (10-13) µm long. The dorsal gland orifice is from the base of the stylet until the end of the basal knobs. The sub ventral glands are mid-point between the basal knob and pharynx. The pharynx is connected to the intestine. The position of the pharynx is behind the nerve ring, which is present and easily identified. The esophagus looks like a fusiform slight swelling, with a small chamber in the middle. The esophagus is not muscular. The median bulb which looks like a cylindrical tube is not found. The esophagus consists of 3 glands: 1 dorsal gland and 2 sub-ventral glands. Both organs overlap with the anterior intestine. The size of the sub-ventral gland is longer than the dorsal gland. The dorsal gland cell has clear nuclei. The excretory pore is located behind the organ which looks like a convex lens penetrated under the hemizonid. The reproductive system of the female nematode is well developed. The vulva at the back of the body is positioned at 91% or more of the body length. The vulva is wide and has the protuberance but no cover. The well-developed uterus is found at the end of the body, but the post-uterine sac is not found. The uterus and vulva are connected. The uterus has a thick wall. The front of the vulva is X-shaped. Sometimes, 1 egg was found inside the vulva. One ovary is called the monodelphic, which is located in the front before the vulva or the prodelphic position. The ovary outstretches but does not overlap with the dorsal gland. Next, the characteristics of the oocytes consist of the arrangement of 1-2 rows to several rows in the multiplication zones and the oocytes in the growth zone. The oviduct is narrow, like a very long tube. The long, round to oval (ovoid) spermatheca has a thin wall filled with large, round sperms. The sperm has an unclear nucleus. Cells with their unique characteristics that secrete the egg membrane (crustaformeria) have 6 columns. The anus is found muscular, connected with the intestine. The anus has a dome-like shape. The tail has a cylindrical shape. The tip of the tail is rounded and hyaline.

Male (mycetophagous stage) – The characteristics of male nematodes in the mycetophagous stage are shown in Figs 2, 3. The male nematodes have a smaller body size than the female nematodes .When pinned in heat, the straightening body bends into a ventrally arcuate shape. There are 6 lateral fields with distinct features. The cuticle is annulated, each of which is 1 µm interval. The head shape is offset with the cephalic region low. The slightly narrow lips do not emerge from the body. The stylet is small and well developed. The basal knobs are distinctly round. The stylet has a more elongate shaft than the cone shaft and has 1 guild ring surrounded. The esophagus consists of the dorsal gland opening at the base of the stylet and the sub ventral gland in the middle of the body, opposite the base of the stylet. The pharynx connects the intestine which is behind the nerve ring. The structure of the esophagus in a male nematode is similar to a female nematode. The excretory pore located behind the organ looks like a convex lens penetrated in the skin, called the hemizonid. The reproductive system of male nematodes is well developed and different from female nematodes. The testis has only one monarchic set positioning on the right side of the intestine. The forward position extends to the esophagus. The spermatocytes arrange in 2 rows in the front, but they arrange in a single row at the back when fully developed. Sperms are on the back or at the end of the testicle, with two different forms: a large amoeboid spermatozoon and a small, round size with a clear nucleus. The vas deferens visible consists of rounded cells. The spicules are tylenchoid, consisting of three parts: a manubrium which is also called a cylindrical head, calomus, or a tube-like shaft and a flattened blade (lamina). The male reproductive organs are called spicules. The spicules are Y-shaped, arcuate, pointed tail, conoid shaped, and membrane bursa.

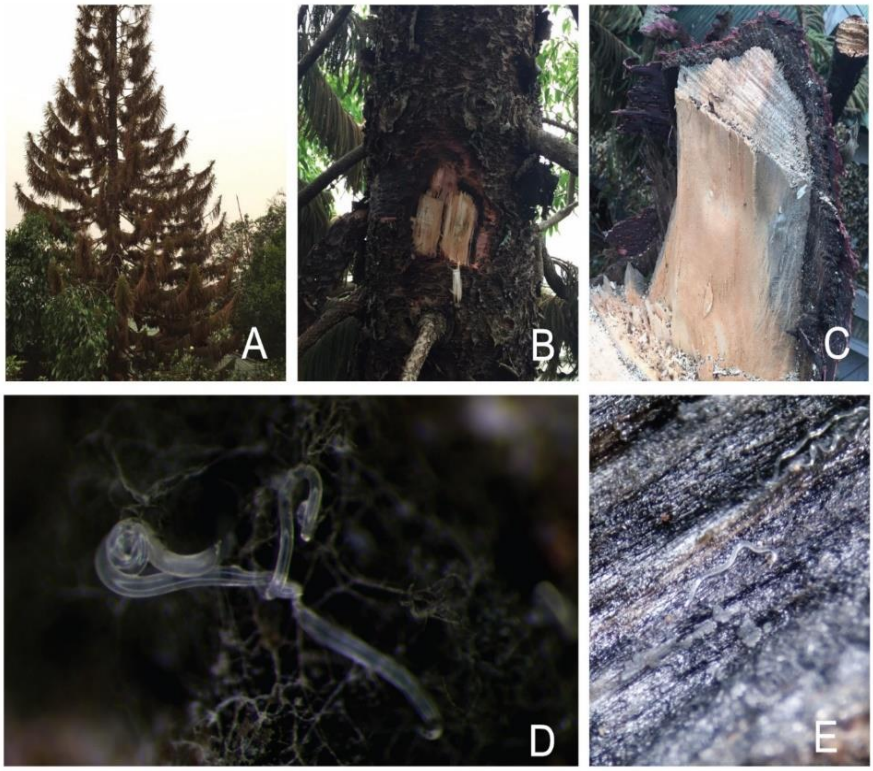

Fig. 1 – Wilt syndrome of Norfolk Island pine (*Araucaria heterophylla*). A-C blue-stain disease in pine. D-E Nematodes associated with fungal hyphae in bark.

Table 1 Morphometrics of *Deladenus uteropinusus*. All measurements are in µm and in the form ± standard deviation (rang)

Characters/ratios [a]	**Measurements of mycetophagous type**	
	Female	**Male**
N	12	13
L	1133.3±34.4 (1100-1200)	850±49.3 (850-920)
A	26.6±2.4 (22.1-29.5)	29.2±5.1 (21-36.7)
B	7.3±1.0 (5.8-9.0)	9.9±1.5 (8-12.6)
C	32.1±2.6 (28.9-38.6)	21.1±1.2 (19-23)
C’	1.6±0.2 (1.1-2)	1.8±0.2 (1.3-2.1)
G1 or T	69.8±7.2 (50-75.7)	67.2±6.2 (56.6-30)
V	85.2±42.2 (80.3-92.7)	-
Stylet	10±0 (10-10)	5±0 (5-5)
Body diam.	41±2.8 (36-46)	29.8±4.9 (24-40)
Excretory pore to anterior end	125±22.7 (95-153)	139±9.4 (130-160)
Hemizonid to Excretory pore	27.5±3.8 (20-30)	24.2±4 (20-30)
Hemizonid to anterior end	121.9±22.7 (92-150)	124±5.9 (110-130)

Table 1 Continued.

Characters/ratios [a]	Measurements of mycetophagous type	
	Female	**Male**
Opening of the dorsal gland to the anterior end	47.8±8.5 (25-46)	121±19.9 (105-180)
Subventral gland orifice to anterior end	57.5±7.2 (40-57.5)	140±20.4 (120-188)
Tail length	35.4±2.4 (30.5-38)	40.3±0.7 (40-42)
Spicules length	-	22.8±2.7 (15.3-26.9)

Note: [a] Abbreviations: N = total specimens measured, L = length of the body, A = maximum body width, B = pharyngeal length, C = body tail length of the tail C'; body width at anus; V, (distance from the anterior end to the vulva) x 100; T, (distance from cloacal aperture to anterior end of testis/body length) x 100; G1, (distance from anus aperture to anterior end of female gonad/ body length) x 100.

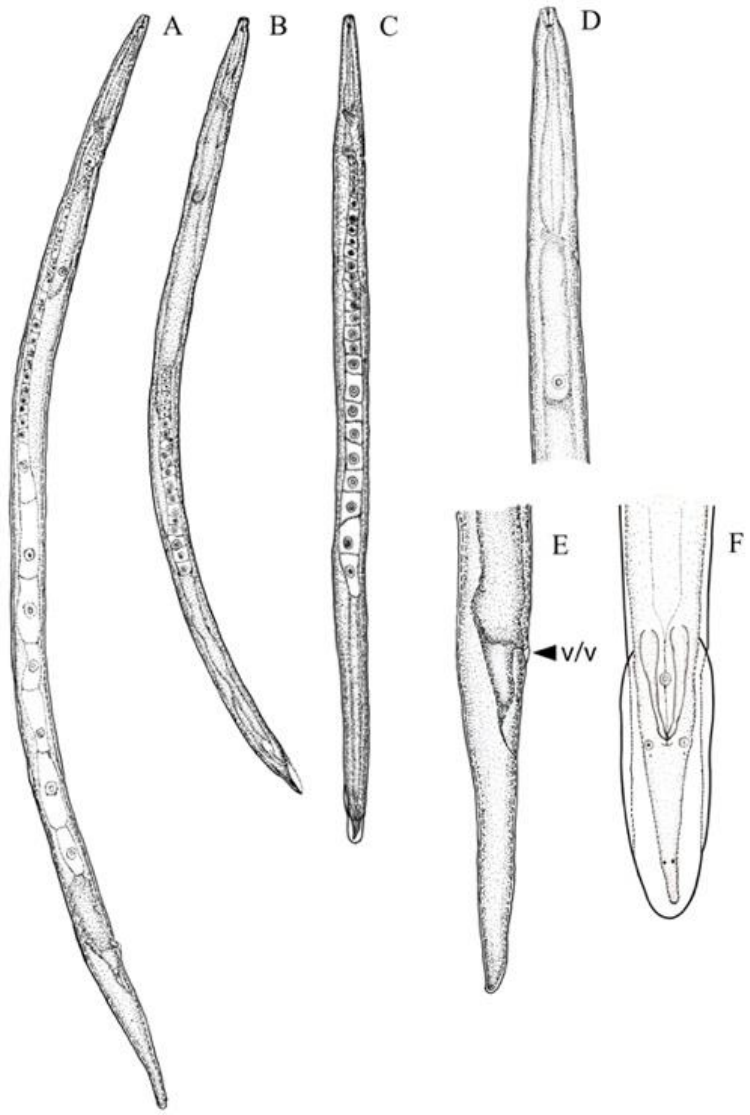

Fig. 2 – Line drawing of holotype *Deladenus uteropinusus.* A-B Entire body of mycetophagous female and male in lateral view. C Entire body of a mycetophagous male in ventral view. D Anterior region in lateral view, the position of hemizonid and excretory pore. E Posterior region of the female

tail in lateral view. F Posterior region of male tail in ventral view with bursa. Abbreviations: v/v = vagina and vulva. Scale bars: A-C = 50µm, D-F = 20µm.

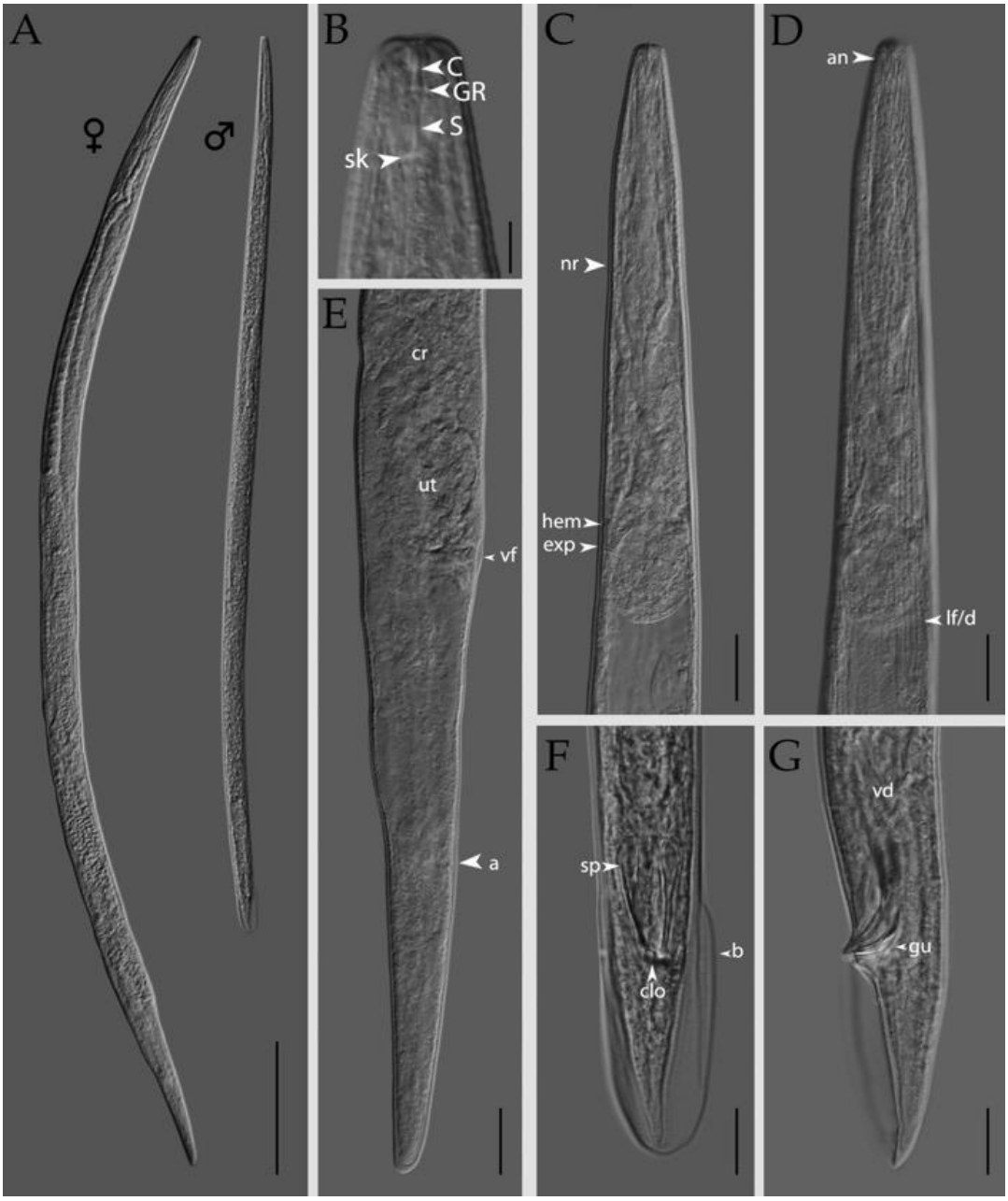

Fig. 3 – *Deladenus uteropinusus.* A Entire body of female and male. B Lip region. C Anterior region, lateral view showing the cephalic and hemizonid located behind excretory pore. D Anterior region, lateral view showing the lateral field. E Posterior region, lateral view of female showing the vulval. F Posterior region, ventral view of male tail showing the spicule shape. G Posterior region in lateral view of male tail showing spicule. Abbreviations: sk = stylet knobs, S = shaft, C = conus, GR = guild ring, nr = nerve ring, exp = excretory pore, an = annulation, lf/d = lateral field/deirid, vf = vulva flap, ut = uterine sac, cr = crustaformeria, a = anus, sp = spicule, clo = cloaca, b = bursa, gu = gubernaculum, vd = Vas deferens. Scale bars: A = 500µm, B = 10µm, C-G = 20µm.

Discussion

The mycetophagous stage of *Deladenus uteropinusus* was founded and identified under the bark of declining Norfolk Island trees in Chiang Mai, Thailand. This was the first record of a *D. uteropinusus* in Thailand. When comparing the nematode measurements with Thorne's research

(1949), Chitambar (1991), Bajaj (2015), Yu et al. (2017) it found that the nematodes have their body proportion and morphological characteristics similar to the research conducted by Bajaj (2015). Therefore, the classification of *D. uteropinusus* nematodes is considered the first findings in Thailand. Moreover, the nematodes are associated with insects and blue stain fungi (Slippers et al. 2015, Yu et al. 2013). In Japan, it is reported that the *Deladenus* spp. nematodes were found on the bark stained with blue stain fungi on red pine trees showing signs of wilt (Kanzaki et al. 2016).

The nematode had the same general characteristics as the nematodes in the genus *Deladenus* by having the pharynx-intestinal junction located immediately behind the nerve ring, V ratio greater than 90%, posterior-uterine sac absent. *D. uteropinusus* was distinguished from other *Deladenus* spp. nematodes in that they have 6 incisures in the lateral field and cylindrical tapered female tail and rounded tail tip with a terminal membrane. The nematodes can live off of the fungus that lives within the host pine tree and can switch over to living inside the woodwasps (family Siricidae) (Morris et al. 2013) and beetle (family Cerambycidae) to disperse to a new host substrate (Kanzaki et al. 2016). Both insect and fungi pathogens are associated with declining stands, normally attracted to dead or dying wood. The existence of *Deladenus* spp. in the epidemic indicates the fungal pathogen and may enable disseminating the pine tree and increasing occurrences.

The results of the morphological study of *Deladenus* sp. nematodes obtained from withering *Araucaria heterophylla* pine trees in Chiang Mai together with the classification of *D. uteropinusus* male and female nematodes during the mycetophagous stage are considered the first discovery in Thailand. Moreover, the study results can be used as a database for those interested in understanding and preventing wilt diseases in pine trees and other plants in the future.

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