Ranunculus (Ranunculus asiaticus L., Persian buttercup) is an important ornamental crop throughout temperate regions. In Japan, ranunculus is grown for commercial production of potted or cut flowers. Ranunculus production in Japan is affected by two diseases, shoot rot caused by Pseudomonas marginalis pv. marginalis (Brown) Stevens and mottle caused by a potyvirus. Some varieties of ranunculus have been known to develop the symptom proliferation or phyllody in Kanagawa Prefecture, Japan since the late 1980's. The disease has not been consistently associated with nutritional and environmental factors, nor have fungal or bacterial pathogens have not been isolated from the affected plants. The authors, therefore, suspected a phytoplasma to be the causal agent. Characteristic symptoms of this disease become apparent at the flowering stage of plant development.

For the detection and identification of phytoplasmas, polymerase chain reaction (PCR) techniques have been increasingly employed. Ahrens and Seemüller reported that the 16S rRNA gene of phytoplasmas could be amplified by PCR using specific primers. Namba et al. described the detection of phytoplasmas and those phylogeny by PCR analysis. We report the results of electron microscopic observations and molecular detection of the pathogen causing proliferation or phyllody of Ranunculus asiaticus in Japan.

**Plant materials and electron microscopy**

Leaves, petioles and flowers from ranunculus plants bearing proliferation or phyllody symptoms and as well as from asymptomatic plants were collected from greenhouses in Gunma, Kanagawa and Chiba Prefectures in November 1994 to March 1995 and March 1996 (Table 1).

For transmission electron microscopy (TEM), small segments were cut out from leaves, stalks and flowers of diseased and asymptomatic plants and immersed immediately in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, on ice. After 2-16 hr, samples were rinsed in the buffer, postfixed 2 hr in 1% buffered OsO4, dehydrated in a graded series of ethanol and embedded in epoxy resin. The resin blocks were polymerized at 80°C for 8 hr. Ultrathin sections were cut with a diamond knife set on a LKB ultramicrotome, type V, stained in 2% uranylacetate solution and then counterstained in lead citrate solution. The sections were examined with a JEM model 1200E transmission electron microscope at 80 kV.

Characteristic symptoms of ranunculus phyllody are shown in Plate I-1. At flowering stage the stigmas of diseased plants developed into proliferation and petals became green and leaf-like. Sometimes all petals changed into leaf-like structures. More than 75% of the plants were symptomatic at each location (Table 1). No significant differences were not observed between healthy and diseased plants during the vegetative growing stage. No bacterium or fungus was consistently isolated from either diseased or healthy plants. Examination of ultrathin sections revealed phytoplasmas in sieve elements from stems of diseased plants (Plate I-2). The phytoplasmas varied in shape, ranged from 60 to 800 nm in diameter and were bound by unit membranes. In some preparations, only a few sieve elements were infected. Phytoplasmas were not observed in tissues from asymptomatic plants.

**Nucleic acid isolation and PCR amplification**

Total nucleic acids used in the PCR were isolated from leaves, stems and flowers of symptomatic and asymptomatic R. asiaticus using the CTAB procedure. Nucleic acid concentration was determined both spectrophotometrically (GeneQuant, Pharmacia) and in 1% agarose gels in 0.5×TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) using lambda DNA as a standard. The nucleic acid was either used without dilution or with dilutions ranging from 1:10 to 1:100 before being set in the PCR. After flowering, plant bulbs were also examined by PCR analysis. The universal primer pair used to screen all ranunculus samples was SN910502 and SN910601, which amplified approximately 1.37 kbp of the 16S rRNA gene. A second set of primers was SN910601 and SN920204 which specifically amplified 0.75 kbp of the 16S rRNA gene of phytoplasmas. Amplified DNA fragments were electrophoresed in 1.2% agarose gels, stained with ethidium bromide and visualized by UV illumination.
Using the universal primer and phytoplasma-specific primer sets, about 1.3 and 0.75 kbp DNA fragments were amplified in diseased plants from each location (Plate I-3), but not in the samples from asymptomatic plants (data not shown). In some samples, phytoplasmas were detected even when diluted 100 fold with sterilized distilled water (data not shown). Template DNA isolated from diseased fresh bulbs were also amplified with each 1.3 and 0.75 kbp DNA fragments.

Bertaccini et al. (1988)2) first reported phyllody and virescence disease of ranunculus in Italy. Stunting or rosette-like symptoms were also observed, but the vector was not described. Kato et al. (1989)6) first reported a phytoplasma associated with anemone witches' broom in Japan. They found that the phytoplasma was transmitted to healthy anemone and Ranunculus sceleatus L. by the leafhopper, Macrosteles atriifrons Anufriev. But the symptoms which appeared in R. asiaticus and R. sceleatus are distinct from the ranunculus phyllody in our study.

Our results strongly suggest that the symptoms of ranunculus found in Japan is caused by a phytoplasma. Our present report is the first report of ranunculus phyllody from Japan. We must compare this Japanese strain with Italian strain of ranunculus phyllody phytoplasma. Insect transmission and molecular biology studies are now under way.

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Literature cited

和文要旨
業平 勉、堀越紀夫、山本裕子、篠原正行：ラナンキュラス薬化病（新称）の発生とphytoplasmaの検出
1994年から1996年、神奈川、群馬、千葉の各県で栽培されていたラナンキュラス（Ranunculus asiaticus L.）で薬化症状を示す株の発生を認めた。薬化生長期には外観上薬化株と差はなく、開花期以降に柱頭が突出し、花器中心部から花器全体を緑色となり、薬化状を呈した。病株の花茎他各の試料を電子顕微鏡観察し、花柱の生長抑制機構についても観察した。Namba et al. (1993)のプライマーセットを用い、PCR增幅したところ病株からの試料で1.3と0.75 kbpのDNAが増幅された。これからのことから、本症候はphytoplasmaによるものと考え、ラナンキュラス薬化病（新称）を提案した。

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Plate I. Symptoms of ranunculus phyllody, morphology of the phytoplasma and PCR amplificaton of 16S rDNA sequences from the phytoplasma.

1: Phyllody in *Ranunculus asiaticus* L. (sample NU96025).
2: Transmission electron micrograph of phytoplasmas observed in a phloem sieve element from peduncle of diseased *Ranunculus asiaticus* L. (sample NU95029). Scale bar=200 nm.
3: PCR amplification of 16S rDNA sequences from ranunculus phyllody phytoplasma using two pairs of primers, SN910601 and SN920204 (lanes 1, 2 and 3) and SN910502 and SN910601 (lanes 4, 5 and 6). PCR products were separated by electrophoresis through a 1.2% agarose gel. DNA templates for PCR were derived from affected ranunculus samples NU95029 (lanes 1 and 4), NU96024 (lanes 2 and 5) and NU96025 (lanes 3 and 6). Lane M is a 1 kb DNA Ladder (GIBCO-BLR; upper, middle and lower arrow-head indicate 1636, 1018 and 571 bp, respectively).