Downy mildew (Peronosporaceae; Oomycota) is an obligate biotrophic group that infects a wide range of monocotyledonous and dicotyledonous plants, including many economically relevant crops [1]. The genus *Pseudoperonospora* is a small group comprising only six species but includes two notoriously pathogenic species, *Pseudoperonospora cubensis* and *Pseudoperonospora humuli*. *P. cubensis* infects many cucurbitaceous crops, such as cucumber, gourd, melon, pumpkin, and watermelon [2,3], and more than 60 host plants have been listed [4,5]. *P. humuli* is one of the most critical threats to the cultivation of hops (Cannabaceae) [6,7]. Given their association with high economic losses, many recent studies have focused on the biology, host specificity, population structure, detection, and control of *Pseudoperonospora* species [3,8–13] as well as their taxonomy and phylogeny [14,15].

To date, five *Pseudoperonospora* species have been reported in Korea, namely *P. cannabina*, *P. cubensis*, *P. humuli* [15–18], *P. celtidis* [16], and *P. urticae* [19]. Among them, *P. cubensis* is the most destructive pathogen on both wild and cultivated Cucurbitaceae in Korea, including *Citrullus vulgaris*, *Cucumis melo*, *Cucumis sativus*, *Cucurbita moschata* [17], *Lagenaria siceraria* [20], and *Trichosanthes kirilowii* [18]. Particularly, *P. cubensis* raises major economic concerns in the cultivation of *C. melo*, *C. sativus*, and *C. moschata*.

Recently, two phylogenetic clades within *P. cubensis*, Clades 1 and 2, were described by molecular sequence analysis [14]. Although the clustering showed no clear relevance with the pathotype or geographic distribution [13], Clade 1 seems to be associated with the recent severe epidemics of cucurbit downy mildew in Europe and the United States, while Clade 2 includes pre-epidemic samples originating from East Asia. Interestingly, both clades were often detected in the Czech Republic, Germany, and the USA [13,14]. To date, however, there has been no research to ascertain whether the cucurbit downy mildews in East Asia are caused by two clades, similar to the cases reported in the above countries, or by a particular group, and the identity of the predominant clade remains unclear.

Oriental pickling melon (*C. melo* var. *conomon*) is a minor crop cultivated in the temperate climate zone of East Asia, including China, Korea, and Japan. Young fruits are harvested and processed to make salty pickles by putting them in sake lees. From April to June 2020, typical symptoms of downy mildew were observed on the leaves of...
oriental pickling melon (C. melo var. conomon) growing in Gunsan, Korea (Figure 1). The disease incidence was high, reaching 30–50% from April to May, but was approximately 90% in June. This study aimed to identify the causal agent of downy mildew on oriental pickling melon in Korea and to determine the causal clade among two phylogenetic clades of P. cubensis, using detailed morphological and multi-locus phylogenetic investigations.

On oriental pickling melon, downy mildew infection resulted in discoloration of the leaf tissues, with yellowish to brownish spots on the upper leaf surfaces (Figure 2(A,B,D,E)). The lesions were water-soaked, polyangular, and clearly delimited by the leaf veins (Figure 2(G,H)). A distinctive gray to dark brown, dense oomycete growth was observed on the corresponding abaxial leaf surface (Figure 2(J,K)). As the disease progressed, the spots turned blackish and often merged to cover larger areas. Several leaves showing downy mildew symptoms were collected within each of four melon plots (116, 164, 780, and 800 m²). Figure 1 displays the site map and a close view of sample locations, which were programmatically marked on Google Earth. All samples were deposited in the Kunsan National University Herbarium (KSNUH), and information on the dried herbarium samples has been provided in Table 1.

For a detailed microscopic examination, sporangiophores and sporangia formed underneath the infected leaves were transferred to a drop of lactic acid on a glass slide, covered with a coverslip, and gently warmed using an alcohol lamp. The microscope measurements were examined under the BX53F DIC-light microscope (Olympus, Tokyo, Japan) with the DigiRetina 16M digital camera (Tucson, Fuzhou, China) or under the Zeiss Imager M2 AX10 microscope (Carl Zeiss, Jena, Germany) with the AxioCam 512 camera (Carl Zeiss, Jena, Germany). Measurements were reported as follows; (minimum)–standard deviation toward the minimum – mean – standard deviation toward the maximum (–maximum). Sporangiophores emerging through the stomata were tree-like, hyaline, straight, or slightly curved, (202.6–256.3–355.3(–421.9)(av. 305.8) × 4.7–35.9–8.2(–10.2)(av. 7.1)(n= 100), and monopodially branched in 3–4 orders (Figure 2(C,F)). Ultimate branchlets were in pairs, straight to slightly curved (3.6–6.3–11.6(–15.6)(av. 9.0) μm long, (1.0–)1.3–1.9(–2.5)(av. 1.69) μm wide at the base (n = 100), with a truncate or rarely swollen tip. Sporangia were brownish, ovoidal, or lemon-shaped,
measured $(19.9 - 22.6 - 27.0 (-29.8) \text{av. } 24.8) \times (14.3 - 15.4 - 17.9 (-19.6) \text{av. } 16.7) \mu m$, with a length/width ratio of $(1.30 - 1.41 - 1.57 (-1.66) \text{av. } 1.49)(n = 100)$ (Figure 2(I,L)). Resting organs were not observed. Morphological observations revealed that this oomycete unequivocally belonged to the

Figure 2. Downy mildew disease caused by *Pseudoperonospora cubensis* on oriental pickling melon (*Cucumis melo* var. *conomon*) in Korea. (A and B) Downy mildew outbreak in a field of oriental pickling melon; (D and E) Vein-limited spots above (D) and below (E) an infected leaf; (G and H) Close-up view of vein-limited downy mildew growth developing on the lower surface; (J and K) Dense sporangiophores with grayish, numerous sporangia; (C and F) Sporangiophere; (I and L) Sporangium. Scale bars: 100 $\mu m$ for sporangiophore; 10 $\mu m$ for sporangium.
Table 1. Information of Pseudoperonospora collections parasitic to oriental pickling melon (Cucumis melo var. conomon) used in this study.

| Plot number | Herbarium number | Geographic location | GPS         | Date       | DNA number     | Phylogenetic clade | GenBank Acc. No. (cox2) |
|-------------|------------------|---------------------|-------------|------------|----------------|-------------------|------------------------|
| 1 (ca. 800) | KSNUH621         | Chango-ri 88-2, Seongsan-myeon, Gunsan-si, Korea | 35°59'7.67"N 126°49'33.39"E | June 23 2020 | D822A 1        | 1                  | MW273007               |
|             |                  |                     |             |            | D822B 1        | 1                  | MW273021               |
|             |                  |                     |             |            | D822C 1        | 1                  | MW273027               |
|             |                  |                     |             |            | D822D 1        | 1                  | MW273025               |
|             |                  |                     |             |            | D822E 1        | 1                  | MW273026               |
|             |                  |                     |             |            | D822F 1        | 1                  | MW273020               |
|             |                  |                     |             |            | D822G 1        | 1                  | MW273017               |
| 2 (ca. 780) | KSNUH623         | Chango-ri 106-1, Seongsan-myeon, Gunsan-si, Korea | 35°59'14.0"N 126°49'41.4"E | June 23 2020 | D824A 1        | 1                  | MW273008               |
|             |                  |                     |             |            | D824B 1        | 1                  | MW273019               |
|             |                  |                     |             |            | D824C 1        | 1                  | MW273016               |
|             |                  |                     |             |            | D826A 1        | 1                  | MW273015               |
|             |                  |                     |             |            | D826B 2        | 2                  | MW273010               |
|             |                  |                     |             |            | D826C 2        | 2                  | MW273012               |
|             |                  |                     |             |            | D826D 1        | 1                  | MW273014               |
|             |                  |                     |             |            | D826E 2        | 2                  | MW273013               |
|             |                  |                     |             |            | D826F 2        | 2                  | MW273009               |
| 3 (ca. 164) | KSNUH625         | Chango-ri 103-1, Seongsan-myeon, Gunsan-si, Korea | 35°59'10.6"N 126°49'39.0"E | June 23 2020 | D827A 1        | 1                  | MW273018               |
|             |                  |                     |             |            | D827B 2        | 2                  | MW273022               |
|             |                  |                     |             |            | D827C 2        | 2                  | MW273006               |
|             |                  |                     |             |            | D827D 2        | 2                  | MW273023               |
|             |                  |                     |             |            | D827E 2        | 2                  | MW273011               |
|             |                  |                     |             |            | D827F 2        | 2                  | MW273024               |
| 4 (ca. 116) | KSNUH626         | Chango-ri 55-5, Seongsan-myeon, Gunsan-si, Korea | 35°59'18.0"N 126°49'36.7"E | June 23 2020 | D828A 1        | 1                  | MW273041               |
|             |                  |                     |             |            | D828B 2        | 2                  | MW273027               |
|             |                  |                     |             |            | D828C 2        | 2                  | MW273023               |
|             |                  |                     |             |            | D828D 2        | 2                  | MW273018               |
|             |                  |                     |             |            | D828E 2        | 2                  | MW273024               |

The genus Pseudoperonospora, and the characteristics were consistent with those described from P. cubensis (Berk. & Curt.) Rostov. [21].

For molecular sequence analysis, genomic DNA was extracted from the infected plant tissue of the herbarium specimens using the MagListo 5M plant Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The mitochondrial cytochrome c oxidase II (cox2) gene was sequenced using primers cox2-F [22] and cox2-RC4 [23]. Amplicons were visualized on 1.2% agarose gel, purified using the AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea), and sequenced by a DNA sequencing service (Macrogen, Seoul, Korea), with the primers used for amplification. The resulting sequences were edited using the DNASTAR software package version 5.05 (DNASTAR, Madison, WI) and deposited in GenBank (see Table 1). In addition to the reference sequences of Pseudoperonospora species available in NCBI GenBank, they were aligned using MAFFT version 7 [24] with the Q-INS-I algorithm [25]. Minimum evolution (ME) and maximum likelihood trees were constructed with MEGA version 7.0 [26], using the Kimura 2-parameter model and performing 10,000 bootstrap replicates.

Phylogenetic structures of Pseudoperonospora species, including 22 Korean specimens of oriental pickling, were inferred by the ME and ML methods using cox2 mtDNA sequences, a barcoding locus of oomycetes. As the two trees were congruent, only one ME tree is shown in Figure 3. In both analyses, the Korean specimens were divided into two different clades, matching each of the two phylogenetic clades of P. cubensis. A sequence dissimilarity of 1.4% (7 out of 472 characters were different) was observed between the two clades. Clade 1 includes Pseudoperonospora species of Citrullus, Cucurbita, Cucumis, and Melothria spp. originating from the Czech Republic, Germany, Italy, Korea, the Philippines, and the USA, with a high bootstrapping value of 98/98%. Clade 2 includes Cucurbita, Cucumis, and Lagenaria specimens originating from the Czech Republic, Germany, Japan, Korea, the Netherlands, Spain, and the USA, with a moderate value of 73/79%. Within Clade 2, the Korean specimens formed a subgroup with a weak value of 65/64%. Of the four melon plots of the downy mildew collections, only Clade 1 was detected in plots 1 (n = 7) and 2 (n = 3), Clades 1 and 2 coexisted in the same number in plot 3 (n = 6), and Clade 2 was predominant in plot 4 (n = 6).

For the pathogenicity test, sporangia were harvested from each infected leaf of two clades (826A in Clade 1 and 826B in Clade 2) collected at site 3. They were suspended in sterile water (1.0 × 10⁶ sporangia/mL) and sprayed onto the lower leaf surface of five healthy C. melo var. conomon. Five control plants were sprayed with distilled water. All plants were incubated in a growth chamber at 22 °C under a relative humidity of 95%. After 7 d, both isolates (826A and 826B) developed downy mildew symptoms on the inoculated leaves. Since there is no difference in disease incidence and characteristics between the two isolates, only the results of the isolate 826A are shown in Figure 4. It formed yellow to green polygonal lesions on the leaves (Figure 4(A)), whereas the control plants remained symptomless (Figure 4(B)). White sporangiophores and dark greyish sporangia were observed on the vein-limited spots of the inoculated leaves (Figure
Their morphological characteristics (Figure 4(F,G)) and cox2 sequences were consistent with those initially investigated. Based on morphological characteristics, molecular sequencing data, and pathogenicity test, the pathogen was identified as *P. cubensis*. Previously, *P. cubensis*
cubensis has been recorded on C. melo var. conomon in Japan [27] and under laboratory conditions by Zitter et al. [28]. To our knowledge, this is the first report of downy mildew caused by P. cubensis on C. melo var. conomon in Korea. With the recent increase in the popularity of oriental pickling melons, the growing area of this crop in Korea is gradually expanding. However, the high disease severity and low resistance in melons pose a potential risk for oriental pickling melon production.

Beyond the geographic origin and distribution of the two clades of P. cubensis, it should be noted that both clades coexist within a few countries [13,14], including Korea. Additionally, this study has
demonstrated that two clades of \( P. \ cubensis \) can coexist even within a small crop plot at the same time. However, it remains unclear whether such a simultaneous coexistence is ubiquitous in other global cucurbit farms, and there is no research on the extent of damage caused by each clade and a disease management method specific to the clade. As the overlapping host ranges and indistinguishable morphological characteristics of the clades pose challenges for their detection and diagnosis in the field and laboratory, there is an urgent need to develop a clade-specific molecular diagnostic method and to study whether they can be distinguished at the species level.

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