A Survey of *Phytophthora* spp. in Midwest Nurseries, Greenhouses, and Landscapes

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Abstract

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A survey of nurseries, greenhouses, and landscapes was conducted from 2006 to 2008 in order to determine the prevalence and diversity of *Phytophthora* spp. From sites in Iowa, Michigan, Ohio, and, predominantly, Indiana, 121 *Phytophthora* isolates were obtained from 1,657 host samples spanning 32 host genera. Based on sequence of the internal transcribed spacer (ITS) region of the ribosomal DNA, 11 *Phytophthora* spp. and two hybrid species were identified. A majority of the isolates were *P. citricola* (35.9%) or *P. citrophthora* (27.4%). Six

isolates were confirmed as hybrids (four of *P. cactorum* × *hedraiaandra* and two of *P. nicotianae* × *cactorum*) by cloning and sequencing the ITS region. Three *P. cactorum* × *hedraiaandra* isolates were obtained from the same site, from three *Rhododendron* spp., which are known hosts to the parental species. The fourth isolate, however, was recovered out of a different location in a *Dicentra* sp., which is not a known host to either parental species, suggesting an expansion of host range of the hybrid isolate as compared with either parental species.

Phytophthora spp. are important pathogens in many agricultural and ecological systems (5,9,11,17,27) and are major threats to the floriculture and nursery industries, which grow a diversity of species in close proximity to each other in nurseries and greenhouses (9,11,27,32). In these situations, *Phytophthora* spp. can cause significant disease outbreaks, including root rots, crown rots, leaf blights, cankers, and stem dieback (11), jeopardizing the horticultural industry's \$11.7 billion sales value (31).

Phytophthora spp. are also of an ecological concern. *Phytophthora ramorum*, the causal organism of sudden oak death (SOD), has caused significant mortality of oak (*Quercus* spp.) and tanoak (*Lithocarpus* spp.), disrupting the forest systems (26). Since its introduction to the United States in 1994, *P. ramorum* has been detected in forests and nurseries in 14 coastal counties of northern California and 1 county in southern Oregon (26). *P. ramorum* has a wide host range consisting of 45 proven plant species and 91 associated plant species (30) and is thought to have a high potential to devastate eastern U.S. forests if ever introduced to these systems (15). There is a significant risk of this disease spreading throughout the United States due to the extraordinary amount of interstate trade of nursery and greenhouse plant species (29). An increase in public concern for invasive plant pathogens has prompted many researchers to survey for native and exotic species of *Phytophthora* and to investigate the risks they impose (5,9,11,27,32). A comprehensive survey of native and endemic *Phytophthora* spp. present in an area will provide tools to more rapidly identify new threats as they develop and, possibly, prevent unnecessary quarantines from being imposed against endemic *Phytophthora* spp. (27).

In order to be successful, surveys of *Phytophthora* spp. require accurate and efficient identification methods. In the past, identification was based solely on morphological traits (23,28,33). However, the variability of morphological characteristics that occur within species, the inconsistency of these structures in culture, and the similarity of characteristics between species can often lead to erroneous identifications (20). Therefore, numerous studies have

used molecular techniques such as restriction fragment length polymorphisms (3), amplified fragment length polymorphisms (3), isozyme analysis (6), single-strand conformation polymorphisms (16), and DNA sequence analysis (6,7,20,21) to aid in identification.

Many of these techniques rely upon sequence variations within the internal transcribed spacers (ITS) of the ribosomal genes, a part of the nuclear DNA that does not code for a protein (34). Mutations in this region have no apparent fitness penalties and, therefore, tend to accumulate quickly (34), which leads to highly variable sequences useful for identification of many *Phytophthora* spp. (3,20). However, Cooke et al. (6) stated that *P. citricola* and *P. inflata* have identical internal transcribed spacer (ITS) sequences, making analysis of other genes necessary to accurately distinguish between these two species. Nevertheless, the ITS region is still an incredibly useful identification tool because the sequence of the ITS region is readily discerned and, as a result, a large number of sequences are available from several public databases (14,18).

Another threat that *Phytophthora* spp. pose is the formation of new and hybrid species. Various studies describe new *Phytophthora* spp. and hybrids, which likely arose from formerly geographically distant pathogen species that lack robust reproductive barriers coming into contact in the same area (4,14,20,21,27). There is a high probability of different *Phytophthora* spp. being moved to close proximity to one another as international plant trade continues to result in the shipment of latently infected plants long distances. This increases exposure of plant production systems and natural ecosystems to invasive pathogens and creates the opportunity for hybridization between previously geographically separated species (5). For example, naturally occurring *P. cactorum* × *nicotianae* (3,22) and *P. cactorum* × *hedraiaandra* (21) hybrids were recently described and characterized as being morphologically similar to *P. cactorum* but with noticeably different genotypes and host ranges. The *P. cactorum* × *hedraiaandra* hybrid was reported to be pathogenic to the plant genera *Allium*, *Idesia*, and *Penstemon*, none of which was a host to either parental *Phytophthora* spp. (21). *P. alni* subsp. *alni* is a naturally occurring hybrid species with a different host range that may have resulted from the multiple hybridization events between *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis* (4,23). *P. alni* subsp. *alni* is the first *Phytophthora* sp. reported to be pathogenic on alder (*Alnus* spp.) (4) and is a major threat to European riparian forest systems (13). Due to the diversity and hybridization of *Phy-*

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tophthora spp., it is clear that knowledge of this genus is incomplete (3,4,14,21,23).

The objective of this study was to identify conclusively *Phytophthora* isolates collected as part of the Indiana SOD survey. Identification of these organisms to the species level was accomplished through DNA sequence analysis of the ribosomal DNA (rDNA) ITS region, including hybrid isolates. This survey provides an important description of the diversity of *Phytophthora* spp. and hybrids infecting common ornamental plants, updates host range information that may assist in identifying new species hybridizations, and supplies a baseline of *Phytophthora* spp. for comparison with future survey results.

Materials and Methods

Isolate collection. The majority of isolates were recovered from samples collected by Indiana Department of Natural Resources inspectors participating in the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) National SOD Nursery Survey from 2006 to 2008. These samples were tested for the presence of *Phytophthora* spp. using an Agdia Pathoscreen double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Agdia Inc.). Plant samples which tested positive for *Phytophthora* spp. via ELISA were divided into three subsamples. One subsample was sent to a USDA-APHIS-approved *P. ramorum*-certified National Plant Diagnostic Network laboratory for confirmation of *P. ramorum*. Another subsample was placed into long-term storage. The last subsample was used to isolate the *Phytophthora* spp. using selective P₁₀VP medium (cornmeal agar [17 g/liter], pimarin [10 µg/ml], vancomycin [200 µg/ml], and pentachloronitrobenzene [PCNB; 100 µg/ml]) or P₁₀ARP (cornmeal agar [17 g/liter], pimarin [10 µg/ml], ampicillin [250 µg/ml], rifampicin [10 µg/ml], and PCNB [100 µg/ml]) (12). Additional *Phytophthora* isolates were taken from samples of symptomatic host plants using P₁₀VP or P₁₀ARP medium and were received by the Purdue Plant and Pest Diagnostic Laboratory from various nurseries, greenhouses, and landscape plantings from Indiana, Ohio, Michigan, and Iowa.

To eliminate bacterial contamination, a small 2- to 3-mm sliver from the growing edge of the culture was placed in an empty petri dish and then a round section of clarified V8 (cV8) agar amended with P₁₀ARP of the same diameter as the petri dish was placed on top (23). Pure cultures were obtained by taking a hyphal tip subcultures from the cV8-P₁₀ARP overlay cultures. Isolates were maintained on cornmeal agar (CMA; 17 g/liter). To confirm that a *Phytophthora* sp. was isolated from the plant sample, a 6-mm cork borer was used to take three plugs from the edge of a culture growing on CMA. The plugs were placed in an empty, sterile petri dish and 1% nonsterile aqueous soil extract was added to the petri plate. Then, 2 to 3 days later, the plugs were viewed for the presence of *Phytophthora* sporangia. For long-term storage, isolates were placed on cV8 agar slants, allowed to grow for 3 to

4 days, covered with sterile mineral oil, and stored at room temperature.

Genomic DNA extraction. The mycelia of each isolate were grown in a sterile petri dish with cV8 broth at 17°C for 4 to 5 days. The mycelial mat was washed with sterile distilled water and approximately 100 mg of mycelia were placed in a 2-ml screw cap tube with two 5-mm sterile glass beads. The mycelia were stored at -20°C until DNA was extracted. Tubes (2-ml size) with mycelia were placed in liquid nitrogen for 30 s and then milled in a BioSpec Mini-Beadbeater (BioSpec Products, Inc.) at the lowest speed for 30 s, two to three times. The DNA was extracted using a Qiagen DNeasy Plant mini kit (Qiagen Inc.) following the manufacturer's protocols. DNA was stored at -20°C.

ITS region amplification. A 1:10 dilution of total DNA was used to amplify the ITS region with either primer sets ITS1 and ITS4 (34) or ITS6 (6) and ITS4. Failure to amplify the ITS region with ITS1 and ITS4 in some of the isolates prompted the use of primer set ITS6 and ITS4. All PCR reactions were performed in an Eppendorf Mastercycler (Eppendorf North America) with the following parameters: 95°C for 2 min; 34 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. Amplified rDNA products were purified using the QIAquick PCR purification kit (Qiagen Inc.). DNA concentration was determined with a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). Direct sequencing of the purified PCR product was carried out at the Purdue Genomics Core Facility, Purdue University, West Lafayette, IN on an Applied Biosystems ABI 3730XL sequencer (Applied Biosystems Inc.) using the same primer set that was used to amplify the DNA.

Cloning of putative hybrid species rDNA. An isolate was considered a potential hybrid species if the sequence analysis of its rDNA ITS region resulted in the identification of heterozygous nucleotide signals. The ITS regions from the potential hybrid isolates were then cloned using the Invitrogen TOPO TA Cloning kit (Life Technologies) and transformed into Invitrogen One Shot TOP10 *Escherichia coli* cells (Life Technologies) following the manufacturer's protocols. Eight clones with putative inserts were selected at random from each isolate. Plasmids were extracted using the QIAquick Miniprep spin kit (Qiagen Inc.) and were then sequenced by the Purdue Genomics Core Facility on an Applied Biosystems ABI 3730XL sequencer.

Species determination and sequence alignment. Electropherogram files from the ABI 3730XL sequencer were viewed for quality of reaction and sequence reactions were repeated if necessary. Consensus sequences were created using both forward and reverse sequence data with BioEdit v7.0.9 (North Carolina State University, Raleigh). Consensus sequences were compared against the National Center for Biotechnology Information (NCBI) GenBank database and the *Phytophthora* Database (2,25) for species identification and were identified as the same species. Hybrid species were confirmed by comparing the eight cloned ITS sequences

Table 1. Sources of the nucleotide sequences of the validated reference cultures were obtained through the National Center for Biotechnology Information GenBank (2)^a

Reference <i>Phytophthora</i> spp.	GenBank accession number	Culture collection ^b	Culture number
<i>Pythium ultimum</i>	GU259285.1	WPC	15967D740
<i>Phytophthora cactorum</i>	GU259314.1	WPC	6625D1304
<i>P. cambivora</i>	GU259493.1	WPC	6360A418
<i>P. capsici</i>	FJ801852.3	WPC	3605B1733
<i>P. citricola</i>	AF266788.1	IMI	IMI031372
<i>P. citrophthora</i>	AF266785.1	IMI	IMI332632
<i>P. cryptogea</i>	GU259336.1	WPC	1810D1579
<i>P. drechsleri</i>	GU259322.1	WPC	7016D286
<i>P. hedraiaandra</i>	GU259092.1	WPC	11678B1115
<i>P. nicotianae</i>	EU331089.1	ATCC	MYA-4036
<i>P. palmivora</i>	GU259469.1	WPC	15828D143
<i>P. syringae</i>	FJ801802.1	WPC	2004A1357

^a *Phytophthora* spp., GenBank accession number, culture collection, and culture number are listed in this table.

^b Reference cultures originated from the International Mycological Institute (IMI), the World *Phytophthora* Collection (WPC), or the American Type Culture Collection (ATCC).

against the NCBI GenBank and *Phytophthora* databases for a combination of two species. Neighbor-joining analysis with 1,000 bootstrap replicates was performed with the Clustal X 2.0 program (Conway Institute University College, Dublin, Ireland) (19) to confirm statistically that isolates were similar to known reference sequences. Reference sequences were of validated isolates from the International Mycological Institute, World *Phytophthora* Collection, or American Type Culture Collection obtained through the GenBank database (Table 1) (2). *Pythium ultimum* was used as an outgroup to root the neighbor-joining analysis.

Results

Species identification. In total, 106 *Phytophthora* isolates were obtained from 1,657 plant samples consisting of 32 host genera. The plant samples originated from 30 sites: 24 nurseries in 16 Indiana counties, 2 from Iowa counties, 2 from Ohio counties, and 1 from a Michigan county. Thirteen *Phytophthora* spp. were identified through ITS sequence comparison. *Phytophthora citricola* isolates, which comprised 35.9% of the identified isolates (Fig. 1), were obtained from 12 sites and were recovered from five host genera: *Forsythia*, *Juglans*, *Pieris*, *Rhododendron*, and *Syringa*. *P. citrophthora* accounted for 27.4% of the isolates, were obtained from 13 locations and found on eight host genera: *Abies*, *Calycanthus*, *Forsythia*, *Ilex*, *Kalmia*, *Pieris*, *Rhododendron*, and *Syringa*. The other identified *Phytophthora* spp. consisted of *P. cactorum*, *P. cactorum* × *hedraiaandra*, *P. cambivora*, *P. capsici*, *P. drechsleri*, *P. hedraiaandra*, *P. nicotianae*, *P. nicotianae* × *cactorum*, *P. palmivora*, and *P. syringae* (Fig. 1).

Using a neighbor-joining analysis, representative isolates were grouped with published reference sequences of the same species as identified by the BLAST sequence analysis (Fig. 2). The isolates grouped into 5 of the 10 established *Phytophthora* clades (18). The majority of the isolates fell into clade 2 (*P. capsici*, *P. citricola*, and *P. citrophthora*) and clade 1 (*P. cactorum*, *P. hedraiaandra*, and *P. nicotianae*). The other isolates belonged to clades 4, 7, and 8. This analysis revealed that isolates of *P. citricola*, a homothallic *Phytophthora* sp. (17), were grouped into two distinct groups of genetically similar individuals. Isolates of *P. nicotianae* and *P. citrophthora*, both heterothallic species, consist of genetically diverse isolates within their respective clades (Fig. 2).

Hybrid species. Six putative hybrid isolates were identified during the sequence analysis of the ITS region, identifying heterozygous nucleotides at positions that were previously reported to occur in hybrids (Fig. 3). Hybrids were recovered from three sites: two

Indiana locations and one Michigan location. Of 16 potential hybrid isolates, 10 were determined to be false positives. Four were found to be hybrids of *P. cactorum* and *P. hedraiaandra*. Three of the *P. cactorum* × *hedraiaandra* isolates originated from the St. Joseph County, IN site on the same host, *Rhododendron*. The fourth *P. cactorum* × *hedraiaandra* hybrid isolate was recovered from a *Dicentra* sp. collected from Ottawa County, MI. The two remaining hybrid isolates, *P. nicotianae* × *cactorum*, were recovered from *Rhododendron* plants from Howard County, IN.

Discussion

P. cactorum, *P. cactorum* × *hedraiaandra*, *P. cambivora*, *P. capsici*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. hedraiaandra*, *P. nicotianae*, *P. nicotianae* × *cactorum*, *P.*

Fig. 1. *Phytophthora* spp. recovered among 106 isolates from nurseries, greenhouses, and landscape plantings in the Midwest. The number of isolates of each species appears at the end of each bar.

Fig. 2. Neighbor-joining analysis of representative *Phytophthora* isolates from each group of species and published reference cultures. Reference sequences are indicated with species names and GenBank accession number and originate from validated isolates from the International Mycological Institute, World *Phytophthora* Collection, or the American Type Culture Collection (2). Numbers above the nodes are the number of the trees from bootstrap analysis (1,000 replications) that support the observed topology.

palmivora, and *P. syringae* were isolated from 24 nurseries, greenhouses, or landscape plantings in Indiana, two counties in Iowa, one county in Michigan, and two counties in Ohio and identified utilizing the ITS sequence. The two most commonly found *Phytophthora* spp. were *P. citricola* (35.9%) and *P. citrophthora* (27.4%). In a 2002 to 2005 survey of Minnesota nurseries, *P. citricola* (40.9%) was also found to be the most prevalent *Phytophthora* sp. isolated but *P. citrophthora* (19.9%) was found at a much lower frequency (27). In floriculture and nursery surveys of North Carolina, the most frequently isolated *Phytophthora* spp. were *P. nicotianae* (46.7%) and *P. drechsleri* (31.5%) (11,24,32). In the North Carolina survey, only 10.43% of the isolates recovered were *P. citricola*, and no isolates of *P. citrophthora* were recovered. In this study, *P. cactorum* was isolated only from *Rhododendron* spp. (7.5%). Although it is not uncommon for *P. cactorum* to cause disease in *Rhododendron* spp., other surveys of ornamentals found *P. cactorum* in diseased tissue from a variety of host genera (27). In Minnesota, *P. cactorum* was the most frequently identified *Phytophthora* sp., and it was isolated from 10 host genera (27). In a California ornamental nursery survey, *P. cactorum* was found to be less than 10% of the total isolates recovered from eight host genera (35) and, in a survey of North Carolina greenhouses, *P. nicotianae* was the primary *Phytophthora* sp. identified (11,24,32). This difference in the frequency and distribution of *Phytophthora* spp. identified in the four states may be the result of variability in production systems (greenhouse versus nursery), climate, host species, or number of isolates collected (Fig. 4), suggesting a regional aspect of distribution of *Phytophthora* spp. that is deserving of further study.

The preponderance of isolates belonging to clade 1 and clade 2 may be attributed to heavy sampling of woody ornamental nursery plants. This may also be the result of most of the propagative nursery stock material in the region originating from just a few suppliers. The two groups of *P. citricola* may represent two clonal lineages surviving multiple years. This would suggest that the *P. citricola* is coming from a few nurseries early in the crop cycle. Thus, it may be possible to significantly reduce the overall incidence of *Phytophthora* disease in Indiana by more stringent disease management practices during the production of a few host plants, in a limited number of production facilities. Conversely, the genetic variation of *P. nicotianae* and *P. citrophthora* isolates would suggest that multiple isolates of the species are being introduced throughout the crop cycle, and further population analysis would be necessary to determine the locations of these introductions, suggesting that control would be more challenging for these species.

In Indiana, *P. drechsleri* was isolated from *Rhododendron* and *Magnolia* leaves and *Heliopsis* crowns. *P. drechsleri* is mainly a root pathogen of a broad number of host plants (8). In Washington State, *P. drechsleri* was repeatedly recovered from soil cores taken from ornamental retail nurseries (8). Finding a root-infecting *Phytophthora* sp. on foliar tissue is not surprising because many nurseries and greenhouses use overhead watering, which may be contaminated with the pathogen's zoospores. Alternatively, rain and irrigation, which splash soil or potting media onto the foliage,

could place a root pathogen in contact with leaves. Similar findings have occurred with *P. citricola* in an Indiana nursery (1). There were no previous reports of *P. drechsleri* causing disease on either *Heliopsis* or *Magnolia* spp. (10).

In this study, *P. nicotianae* was found on three woody host genera: *Aesculus*, *Hamamelis*, and *Magnolia*. In Minnesota, *P. nicotianae* was isolated from *Euphorbia*, *Magnolia*, *Rhododendron*, and *Rhus* nursery stock (27). Although *P. nicotianae* has been reported from a multitude of woody hosts, no prior reports could be found where it caused disease on either *Aesculus* or *Hamamelis* (10).

Hybrid isolates of *P. cactorum* × *hedraiaandra* were found on *Rhododendron* and *Dicentra*. *Rhododendron* has been reported previously to be a host of this hybrid and its parents (21). However, *Dicentra* has never been reported to be a host of *P. cactorum*, *P. hedraiaandra*, or *P. cactorum* × *hedraiaandra*, which suggests an expansion of the host range due to species hybridization. The significance of this could easily be overlooked until one considers that the Midwest has three different species of native genus *Dicentra*: Squirrel-corn (*Dicentra canadensis*), Dutchman's' breeches (*D. cucullaria*), and wild bleeding heart (*D. eximia*). Wildflowers and other native plants throughout the Midwest are challenged with increasing threats from habitat loss, invasive weeds, illegal collecting, and now possibly newly developed hybrid *Phytophthora* spp. with expanded host ranges. A *Dicentra* plant infected in a nursery could be planted in a woodland setting and serve as a disease focus that expands into the native population. Further testing would be needed to determine whether the *P. nicotianae* × *cactorum* isolates had a change of host range due to hybridization. Finally, unlike the novel host range of *P. cactorum* × *hedraiaandra* isolates noted above, the *P. nicotianae* × *cactorum* isolates were recovered from

Fig. 4. Comparison of *Phytophthora* spp. survey of nursery and greenhouse industries from this study, Minnesota, and North Carolina (11,24,27,32).

Fig. 3. Electropherogram from a *Phytophthora cactorum* × *hedraiaandra* isolate showing heterozygous nucleotides (black arrows). This is the result of an isolate containing two distinctive alleles each originating from different parental species.

plants previously identified as hosts for the parental *Phytophthora* spp.

Hybridization of *Phytophthora* spp. represents a real and emerging threat to native ecosystems, as well as the nursery and floriculture industries, because hybridization could account for swift formation of new pathogens (18,22). Little is known about how species hybridize but it has been suggested that the greatest risk of hybridization occurs when different, geographically isolated species are moved into the vicinity of one another, allowing hybrids to form by zoospore fusion or interspecific mating (4,18,22). Due to the nature of their business, nurseries and greenhouses contribute to this threat by bringing together different *Phytophthora* spp. and a diversity of host plants under environmental conditions conducive to *Phytophthora* disease outbreaks (4,18). Ornamental plant producers can lower the risk of *Phytophthora* hybrids arising by only utilizing certified pathogen-free seed and plant material, limiting visitors' access, scouting thoroughly and regularly for pests and diseases, separating plants according to source of origin, and removing plant debris from previous crops (5). This would lower the risk that geographically distant *Phytophthora* spp. can meet, hybridize, and disseminate through the nursery and greenhouse industries. Surveys should be expanded to look for invasive *Phytophthora* spp., novel host-pathogen interactions, and new hybrid *Phytophthora* spp., which have the potential to be as devastating as introduced exotic pathogens (4,18).

In conclusion, we found a variety of *Phytophthora* spp. present in Midwest nurseries, greenhouses, and landscapes, most likely due to the large amount of plant material imported from throughout the country. This study provides a baseline of *Phytophthora* spp. present in the region's ornamental plant industry, with which future surveys may detect changes in the frequency of *Phytophthora* spp. in this area over time. *P. ramorum*, the causal agent of sudden oak death, was not found among any of the plant samples included in this survey. This survey concentrated on plant tissue exhibiting foliar symptoms of *Phytophthora* disease and, thus, likely underestimates the level of *Phytophthora* infestations in nurseries by under-representing samples from infected root systems. Disease symptoms may be suppressed due to prophylactic fungicide treatments or the natural lag period between root and crown rots and the development of foliar symptoms.

Additionally, we described potential new host-pathogen combinations for several of the *Phytophthora* spp. Four *P. cactorum* × *hedraiaandra* hybrid isolates were found on two hosts from two locations. Completion of Koch's postulates is needed to confirm the pathogenicity of the *P. cactorum* × *hedraiaandra* isolate found on *Dicentra* spp. and to confirm the putative expansion in host range compared with the parental species. Further analysis is needed to determine whether these isolates are the product of single or multiple hybridization events. It would also be beneficial to determine under what circumstances these *Phytophthora* hybrids arise. The information presented here emphasizes the need for continued surveys in this and other regions to provide a better understanding of how *Phytophthora* spp. are disseminated and to rapidly identify new hybrid species and their host ranges before they are introduced to new ecosystems.

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