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Phyllosticta on tea plants in Iran: an update to species recognition

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Abstract: The tea plant (*Camellia sinensis*) is an economically important shrub cultivated in Northern Iran. Various fungal genera, including *Phyllosticta* spp., are associated with leaf spots on tea plants. Several species of *Phyllosticta* exhibit similar morphological and, in some cases, molecular characteristics. Therefore, precise species identification requires integrating all available data, including morphological features, molecular sequences, host-fungus relationships, pathogenicity tests, etc. To clarify the taxonomic position of *Phyllosticta* species associated with tea plants in Iran, a multi-faceted approach was employed, incorporating morphology, field observation, ITS, *tef-1α*, and *ACT* sequences, along with clustering analysis. This comprehensive study revealed that the previously identified *P. theacearum* shares characteristics with *P. capitalensis*, which is primarily recognized for its endophytic nature. This marks the first report of *P. capitalensis* being associated with tea plants in Iran.

Keywords: *Phyllosticataceae*, Multi-gene analysis, Cluster analysis, Biodiversity, *Camellia sinensis*.

INTRODUCTION

Phyllosticta species (*Botryosphaeriales*, *Ascomycota*) are important and widespread plant pathogenic fungi that cause serious diseases in numerous plant species from various plant families. Before molecular studies, the fungus-host relationship was used to distinguish species within the genus *Phyllosticta*, classifying many morphologically similar species into different species linked to various plants. To find a solution for the classification of the genus *Phyllosticta*, valuable studies using multi-gene analyses (such as the internal transcribed spacer region and 28S of the nuclear rDNA, Actin, glyceraldehyde-3-phosphate dehydrogenase, and translation elongation factor 1-α) have been conducted in recent years (Glienke 2011, Wikee et al. 2011, 2013, Zhang et al. 2015). The findings of these studies have led to the introduction of many new species supported by molecular evidence. However, it is also known that many previously identified species from various hosts fall within the species *P. capitalensis.* One of these sets of controversial species has been assigned to tea plants, including *Phyllosticta theacearum* Aa, *P. theae* Speschnew, *P. theicola* Curzi, and *P. theaefolia* Hara (Aa 1973, Aa and Vanev 2002, Lingyun et al. 2020). *Phyllosticta theacearum* is usually attributed to the brown spot of the tea plant, which has been reported in various tea-growing regions. This species is also difficult to distinguish from similar species found in unrelated plant families. *Phyllosticta theacearum* has been reported from Iran (Khodaparast et al. 1996, Darsaraei et al. 2016). However, these researchers have identified it based on morphology (Khodaparast et al. 1996) or single gene sequencing (Darsaraei et al. 2016). There is limited scientific literature regarding *Phyllosticta* spp. on tea plants. One was published by Jin (2011), who examined the production of pycnidia and conidia in four species, including *P. theacearum*. However, no valuable morphological data (except for conidium) or DNA sequence analysis was provided. Despite the excellent and comprehensive work of Wikee et al. (2013), tea isolates have not been included in these studies. Information about other species on the tea plant is even more scarce, and they are mostly listed by name only in fungal databases (Species Fungorum, https://www.speciesfungorum.org, and Mycobank, https://www.mycobank.org). Recently, Cheng et al. (2019) conducted a multilocus phylogeny using ITS, *tef1*, and *ACT* sequences and reported that *Phyllosticta* on tea plants shares 99% homology with *P. capitalensis*. They identified *P. capitalensis* as the causing agent of leaf spot disease on tea leaves in China and confirmed its pathogenicity (Cheng et al. 2019). Baayen et al. (2002) treated *P. theacearum* as a synonym of *P. capitalensis*, but this claim lacks robust evidence and requires further investigation, especially sequencing of type material. *Phyllosticta capitalensis*, as a common endophytic species, exhibits a wider host range (Sui et al. 2023). Host specialization in *Phyllosticta* may correlate with lifestyle, as endophytic fungi are generally less host-specific than pathogenic species. To understand the nature of *Phyllosticta* in tea plants in Iran, we conducted this study using the sequence of ITS-rDNA, *tef-1α*, and *ACT*. We designed a three-step

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survey using morphological, molecular, and cluster analyses to address this issue.

MATERIALS AND METHODS

Morphological overview and field observation

To fulfill the morphological approach and field observations, samples were collected during the summer and autumn of 2017 from tea gardens in Guilan Province, Iran. All tea leaves were then examined for pycnidia of *Phyllosticta*. When pycnidia were detected, small sections of leaves (0.5 × 0.5 cm) were prepared from the boundary between the infected and healthy tissue. The sections were submerged in a 0.5% sodium hypochlorite solution for approximately five minutes, rinsed in sterile distilled water, and placed on sterile filter paper to dry. They were then transferred onto Potato-Dextrose-Agar (PDA) culture medium at 20 °C with a 12-hour photoperiod (van der Aa 1973). Additionally, to compare the morphology of the colonies with available descriptions on the Pine-Needle-Agar (PNA) culture medium, all isolates were cultivated on this medium and exposed to near-UV light at 27 °C with a 12-hour photoperiod. After 14 days, microscopic slides were prepared, and a comparison with *P. capitalensis* was conducted performed.

Fig. 1. Symptoms of *Phyllosticta capitalensis* on leaves of *Camellia sinensis*.

Molecular analysis

Genomic DNA was extracted from mycelia (on PDA) using Chelex 100 medium (Walsh et al. 1991, Darsaraei et al. 2016). The total reaction volume for the polymerase chain reaction (PCR) was 25 µl, which was composed of 12.5 µl of mastermix (Ampliqon, Denmark), 1 µl of each primer, 7.5 µl of deionized water, and 3 µl of genomic DNA. The primer paires ITS1 and ITS4 (White et al. 1990) were used to amplify the nuclear ribosomal DNA's internal transcribed spacer region (ITS). The PCR cycle conditions were 2 min at 95ºC, followed by 30 cycles of 95 ºC for 30 s, 52 ºC for 30 s, 72 ºC for 30 s, and a final elongation step at 72 ºC for 5 min. The primers EF1-728F (Carbone and Kohn 1999) and EF2 (O'Donnell et al. 1998) were used to amplify a part of the translation elongation factor 1-α gene (*tef-1α*). The PCR cycle conditions were 5 min at 94ºC, followed by 35 cycles of 94ºC for 45 s, 52ºC for 30 s, 72ºC for 90 s, and a final elongation step at 72ºC for 6 min. The primers ACT-512F and ACT-783R (Carbone and Kohn 1999) were used to amplify a part of the actin gene (*ACT*). The PCR cycle conditions were 5 min at 94ºC, followed by 35 cycles of 94ºC for 60 s, 64ºC for 45 s, 72ºC for 45 s, and a final elongation step at 72ºC for 10 min.

Amplicons were then sent to Bioneer Company, South Korea (https://eng.bioneer.com) to be sequenced using forward PCR primers. The sequences were aligned using MEGA software v7 (Kumar et al. 2016). A snapshot of the alignments for quick look and comparison was captured using GENEDOC software (Nicholas 1997).

The phylogeny was inferred using Bayesian analysis in the program BEAST version 2 (Bouckaert et al. 2019) using a Yule tree prior (Gernhard 2008) and a strict molecular clock. A single MCMC (Markov chain Monte Carlo) chain was run, with a burn-in of 10%. Posterior probabilities were calculated from the remaining 9,000 sampled trees. A maximum clade credibility tree was produced using TreeAnnotator (part of the BEAST package). The resulting tree was visualized using FigTree version 1.4.4 (Rambaut 2018).

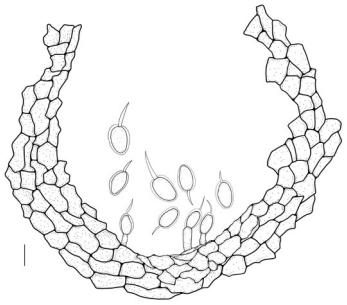

Fig. 2. Conidia and conidiogenous cells of *Phyllosticta capitalensis* on leaves of *Camellia sinensis*. Scale bar= 10 µm.

Cluster analysis

To perform cluster analysis, 61 *tef-1α* sequences were retrieved from NCBI at https://www.ncbi.nlm.nih.gov/ (access date: 2018). A fasta file was then prepared with these sequences with 5 *tef-1α* sequences from *C. sinensis* that originated in this study. A title file was created with information including sequence ID, GB accession number, and species name.

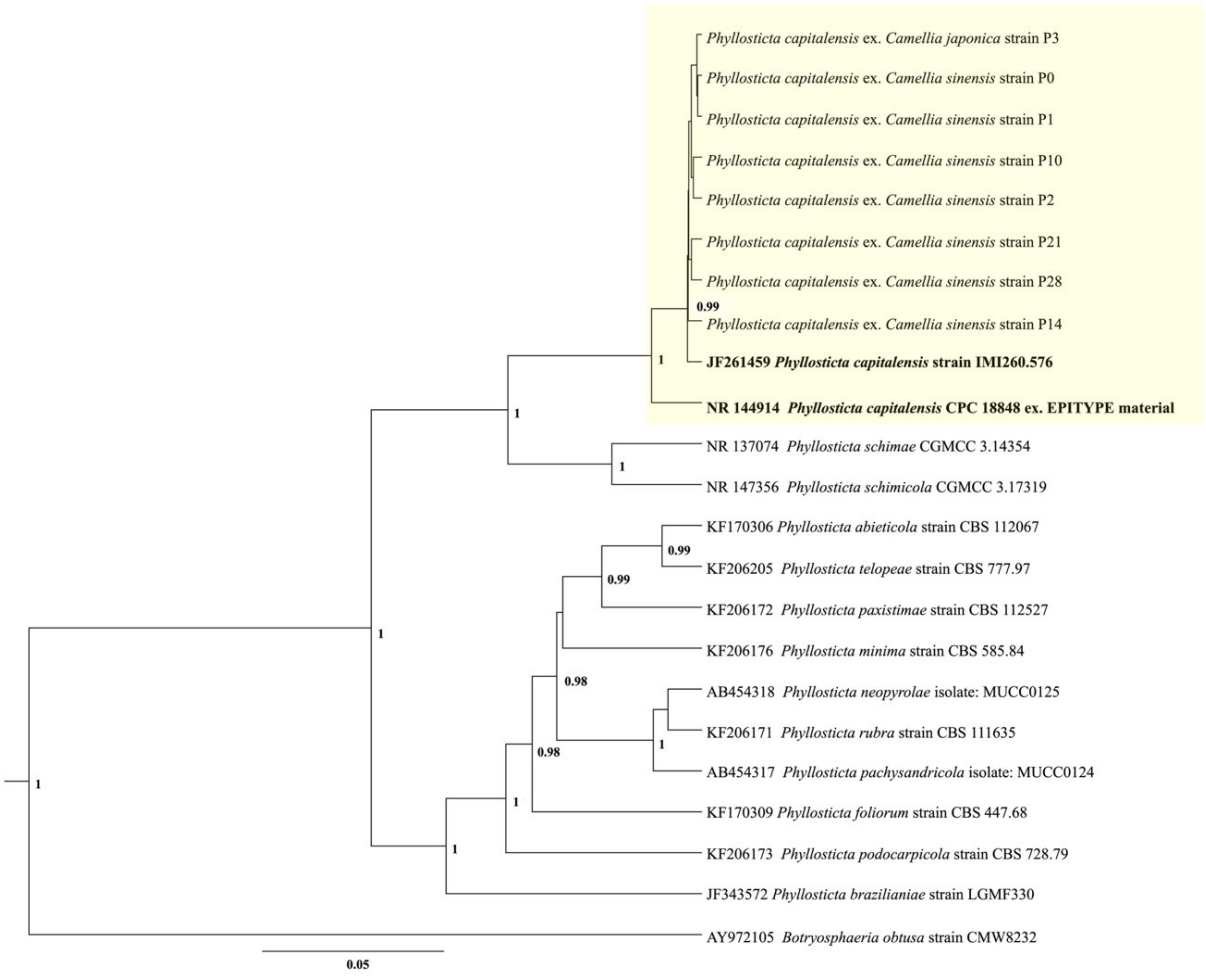

Fig. 3. Bayesian maximum clade credibility tree of sequences from the combined ITS, *ACT*, and *tef-1α* regions in BEAST software. Posterior probabilities > 0.9 are displayed.

Afterward, the cluster analysis was done by fMLC software based on the Connected Component-Based Clustering (Ccbc) algorithm (Vu et al. 2017). The software will compare all sequences and place those essentially the same, which should have similar names, into a single group (cluster). The output will consist of a table and a 3D image. The 3D coordinates of the sequences were visualized via the LargeViz tool embedded in fMLC software.

RESULTS

Morphological overview and field observation

Twenty-eight isolates of *Phyllosticta theacearum* from tea gardens in different regions of Guilan Province were selected for this study (Fig. 1). Based on the morphological characteristics, *P. theacearum* is indistinguishable from *P. capitalensis*, and only obscure differences were observed that are listed as follows: a) negligible difference in the pycnidia diameter; b) negligible difference in the length of the apical appendage; c) conidia are 10–13 µm which placed in *P. capitalensis* range (8–15 µm) (Fig. 2). Field observational showed that this species isolates from weak, previously infected plants (with other pathogens). Furthermore, this species is isolated from shrubs that were previously harvested.

Molecular analysis

The alignment comprising *P. capitalensis* and other fungal isolates from the *Theaceae* family revealed only one substitution in the rDNA ITS-rDNA and the *tef-1α* gene. In the case of *ACT* alignment, no substitution was observed. In the tree inferred from the combined sequences of ITS, *ACT*, and *tef-1α* regions, all sequences retrieved from *Camellia* spp. and *P. capitalensis* formed a fully supported clade (posterior probability = 1) (Fig. 3). Sequences from *Camellia* spp. were grouped with the type sequence of *Guignardia mangiferae* (the teleomorph of *P. capitalensis*, GB accession number: JF261459) and the epitype sequence of *P. capitalensis* (GB accession number: NR 144914).

Cluster analysis

Clustering results showed that all *tef-1α* sequences from *C. sinensis*. (blue dots) clustered with *P. capitalensis* (orange dots) (Fig. 4). Furthermore, the suggested name for these sequences is also *P. capitalensis* (Table 1).

DISCUSSION

As mentioned in the introduction, four *Phyllosticta* species have been assigned to tea plants. *P. theae* is a legitimate name in MycoBank database, with type specimen on living leaves of *C. sinensis*, in Georgia; but according to Aa and Vanev (2002), "this fungus is most likely a *Phomopsis* species with rather large pycnidia (up to 240 µm in diam.) and oblong-ellipsoidal, biguttulate, hyaline conidia, 6–8 × 1.5–2 µm. *Phomopsis theicola* Curzi has been described on leaves of the same host species in Italy, and has similar characters" (Aa and Vanev 2002). According to the MycoBank database, *P. theicola* is a legitimate name and its type specimen is on living leaves of *C. sinensis* (Location: Italy). Aa and Vanev (2002) excluded this species from *Phyllosticta* and hypothesized that "this is probably a small-spored *Phoma* species with short conidiogenous cells and cylindrical, biguttulate, hyaline conidia, 4–5 × 1.5–2 µm" (Aa and Vanev 2002). There is no reliable information about *P. theaefolia*.

In recent years, comprehensive studies have been conducted on the genus *Phyllosticta*, but type isolates from tea have been absent in these studies (Motohashi et al. 2009, Glienke 2011, Wikee et al. 2011, 2013,

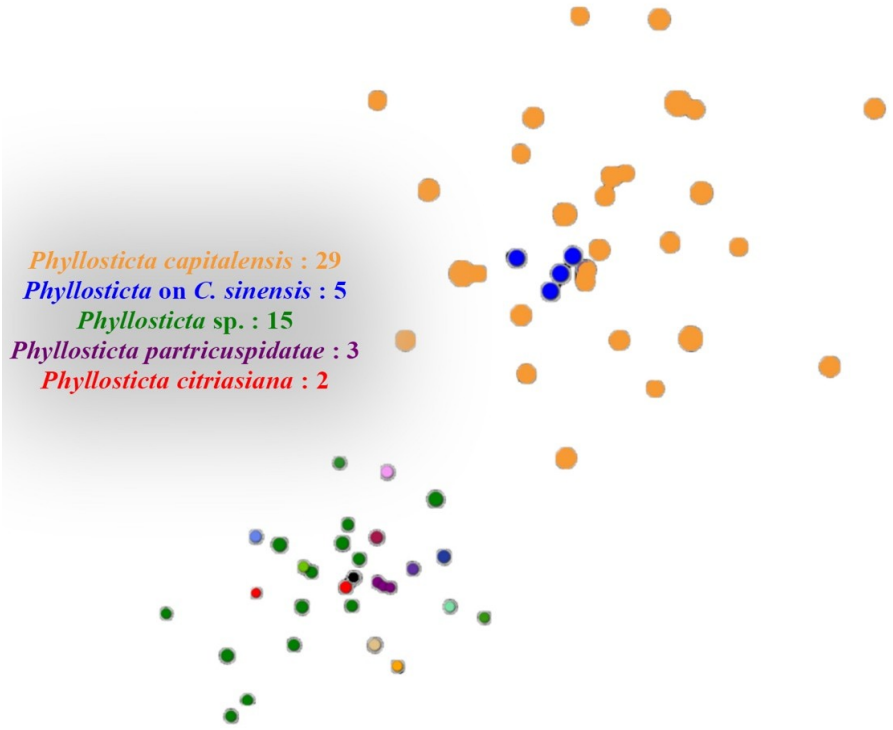

Fig. 4. The distributions of the *tef-1α* sequences retrieved from NCBI and those generated in the current study. The 3D coordinates of the sequences were computed using fMLC software. The sequences of the same taxonomic species share the same color.

Table 1. The output of fMLC software. Only rows refer to sequences generated from *Theaceae* and the name that the software suggested using are showing here.

Sequence ID	Sequence name	Reference name	Suggested name
1	1|NA140000|*Phyllosticta* ex. *Camellia*	*Phyllosticta* ex. *Camellia*	*P. capitalensis*
2	2|NA130000|*Phyllosticta* ex. *Camellia*	*Phyllosticta* ex. *Camellia*	*P. capitalensis*
3	3|NA120000|*Phyllosticta* ex. *Camellia*	*Phyllosticta* ex. *Camellia*	*P. capitalensis*
4	4|NA110000|*Phyllosticta* ex. *Camellia*	*Phyllosticta* ex. *Camellia*	*P. capitalensis*
5	5|NA100000|*Phyllosticta* ex. *Camellia*	*Phyllosticta* ex. *Camellia*	*P. capitalensis*

Zhang et al. 2015, Cheng et al. 2019). Nevertheless, with the help of these studies, the concept of the species *P. capitalensis* has been redefined using sequence analysis, and it is now possible to determine the relationship of obtained isolates to this species through multi-gene sequencing.

As a result, we found that *Phyllosticta* on tea plant in Iran aligns with the new concept *P. capitalensis*, and our isolates are classified within this species.

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REFERENCES

Aa, H.A. van der. 1973. Studies in *Phyllosticta*. Studies in Mycology 5: 1–110.

Aa, H.A. van der and Vanev S. 2002. A revision of the species described in *Phyllosticta*. CBS, Utrecht, The Netherlands.

Baayen, R.P., Bonants, P.J.M., Verkley, G., Carroll, G.C., Van Der Aa, et al. 2002. Nonpathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). Phytopathology92(5):464-477.

Bouckaert, R., Vaughan, T.G., Barido-Sottani, J., Duchêne, S., Fourment, M., et al. 2019. BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. PLoS computational biology, 15(4): e1006650.

Carbone, I. and Kohn, L.M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553-556.

Cheng, L. L., Thangaraj, K., Deng, C., Deng, W. W., and Zhang, Z. Z. 2019. *Phyllosticta capitalensis* causes leaf spot on tea plant (*Camellia sinensis*) in China. Plant Disease 103(11): 2964

Darsaraei, H., Khodaparast, S. A., and Zare, R. 2016. Study on the genus *Phyllosticta* (*Ascomycota*: *Phyllostictaceae*) from Guilan province (N. Iran). Rostaniha 17(1), 51-69.

Gernhard, T. 2008. The conditioned reconstructed process. Journal of theoretical biology 253: 769-778. doi: 10.1016/j.jtbi.2008.04.005

Glienke, C., Pereira, O., Stringari, D., Fabris, J., Kava-Cordeiro, V., et al. 2011. Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. Persoonia: Molecular Phylogeny Evolution of Fungi 26:47-56.

Jin, J. 2011. Conidial morphology changes in four *Phyllosticta* species. Mycotaxon 115:401-406.

Khodaparast, A. S., and Hedjaroude, G. A. 1996. Fungal pathogens of tea plant in Northern Iran. Iranian Journal of Plant Pathology 32: 233-243.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution 33:1870-1874.

Lingyun, Z., Yunfeng, L., Chunyan, J., Hongyan, L., Wei, L. et al. 2020. Identification of the pathogen responsible for tea white scab disease. Journal of Phytopathology 168(1): 28-35.

Motohashi, K., Inaba, S., Anzai, K., Takamatsu, S., and Nakashima, C. 2009. Phylogenetic analyses of Japanese species of *Phyllosticta sensu stricto*. Mycoscience 50: 291-302.

Nicholas, K.B. Genedoc: a tool for editing and annotating multiple sequence alignments. http://wwwpscedu/biomed/genedoc. 1997.

O'Donnell, K., Kistler, H.C., Cigelnik, E., and Ploetz, R.C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences 95:2044-2049.

Rambaut, A. 2018. Figtree ver. 1.4.4. Available online at http://tree.bio.ed.ac.uk/software/figtree.

Sui, X. N., Guo, M. J., Zhou, H., and Hou, C. L. 2023. Four new species of *Phyllosticta* from China based on morphological and phylogenetic characterization. Mycology.14(3): 190-203.

Vu, D., Georgievska, S., Szoke, S., Kuzniar, A., and Robert, V. 2017. fMLC: fast multi-level clustering and visualization of large molecular datasets. Bioinformatics 34:1577-1579.

Walsh, P.S., Metzger, D.A., and Higuchi, R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10:506-513.

White, T.J., Bruns, T.D., Lee, S., Taylor, J. 1990. Amplification and directsequencing of fungal ribosomal RNA genes for phylogenetics. In:PCR protocols: aguide to methods and applications. (Innis M.A, D.H. Gelfand, J.J. Sninsky & T.J. White eds): 315–322 Academic Press, San Diego.

Wikee, S., Udayanga, D., Crous, P. W., Chukeatirote, E., McKenzie, E. H. et al. 2011. *Phyllosticta*—an overview of current status of species recognition. Fungal Diversity 51: 43-61.

Wikee, S., Lombard, L., Nakashima, C., Motohashi, K., Chukeatirote, E. et al. 2013. A phylogenetic re-evaluation of *Phyllosticta* (*Botryosphaeriales*). Studies in mycology 76(1): 1-29.

Zhang, K., Shivas, R.G., and Cai, L. 2015. Synopsis of *phyllosticta* in China. Mycology 6:50-75.

جنس *Phyllosticta* روی چای در ایران: به روز رسانی روش‌های تشخیص گونه

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چکیده: گیاه چای (*Camellia sinensis*) یک گیاه مهم از نظر اقتصادی است که در نواحی شمال ایران کشت می‌شود. جنس‌های مختلفی از جمله *Phyllosticta* به عنوان عامل ایجاد لکه قهوه‌ای چای برشمرده می‌شوند. پیش از این گونه‌ی *P. theacearum* به عنوان عامل لکه قهوه‌ای چای در ایران معرفی شده بود. این گونه امروزه مترادف گونه‌ی *P. capitalensis* است. گونه‌های اندوفیت *Phyllosticta* ویژگی‌های ریخت‌شناختی و حتی مولکولی مشابهی دارند، بنابراین تشخیص دقیق گونه به مجموعه‌ای از اطلاعات موجود از قبیل ریخت‌شناسی، توالی‌های مولکولی، رابطه‌ی قارچ-میزبان، آزمون بیماریزایی و ... نیاز دارد. اطلاعات مولکولی نمونه‌ی تیپ *P. theacearum* موجود نیست و تمامی رکوردهای پیشین این گونه بر اساس داده‌های ریخت‌شناختی تهیه شده‌اند. برای مشخص کردن موقعیت دقیق گونه‌ی *Phyllosticta* که با گیاه چای در ایران مرتبط است، روشی چند بعدی شامل ریخت‌شناسی، بررسی‌های میدانی، توالی نواحی ITS، *tef-1α* و *ACT*، همچنین آنالیز خوشه‌ای مورد استفاده قرار گرفت. نتایج نشان داد که آنچه پیش از این به عنوان *P. theacearum* معرفی شده بود، بسیار مشابه *P. capitalensis* است که مشهورترین گونه‌ی اندوفیت این جنس است. این اولین بار است که *P. capitalensis* به عنوان گونه‌ی مرتبط با گیاه چای در ایران معرفی می‌شود.

کلمات کلیدی: *Phyllosticataceae*، آنالیز چند ژنی، آنالیز خوشه‌بندی، تنوع زیستی، *Camellia sinensis*

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