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Uncovering *Cercospora* species affecting sugar beet in Iran with rapid and accurate detection of *C. beticola* using LAMP assay

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Cercospora leaf spot (CLS) is a major disease impacting global sugar beet cultivation and yield. This study investigated the potential diversity of *Cercospora* species causing CLS in Iranian sugar beet. Fungal isolates were characterized using integrated morphological and multi-gene sequence analyses. Subsequently, the possibility of rapid and specific diagnosis of the dominant pathogen using Loop-Mediated Isothermal Amplification (LAMP) was assessed. Infected leaves were collected from Ardabil, West Azerbaijan, Khorasan Razavi, Semnan, Mazandaran, Khuzestan, and Golestan provinces across the country. Genomic regions of *actA*, *cmdA*, *gapdh*, *his3* and *tef1* were amplified and sequenced. Phylogenetic results revealed that two species, *Cercospora beticola* and *Cercospora gamsiana*, are involved in causing cercospora leaf spot of sugar beet in Iran from which *C. beticola* was the dominant species. The LAMP-specific primers designed based on the *gapdh* gene region successfully discriminated *C. beticola* from *C. gamsiana* and other *Cercospora* species, as well as from some other fungal genera such as, *Alternaria*, *Cladosporium*, *Curvularia*, *Ramularia* and *Stemphylium*. The LAMP assay in this study demonstrated a detection limit of 50 fg μL^{-1}. This study found *C. beticola* to be the dominant species in Iranian sugar beet fields. The LAMP technique proved effective for rapid, accurate diagnosis, aiding optimized disease management and control strategy selection.

Keyword *Beta vulgaris* L., *Cercospora beticola*, Dominant species, Fast diagnostic kit, Multigene approach

Sugar beet (*Beta vulgaris* L.), a member of the family *Amaranthaceae*, is a globally significant crop cultivated primarily for its high sucrose content in the roots[1]. Sugar beet contributes substantially to the sugar industry, providing nearly 30% of global sugar production[2]. In Iran, sugar beet is a strategic agricultural commodity, with a cultivated area of 132,185 hectares under irrigation, producing 7,466,913 tons annually[3]. However, its productivity is threatened by various pathogens, including fungi, bacteria, viruses, and nematodes. Among these, Cercospora leaf spot (CLS), caused by *Cercospora* spp., is one of the most devastating diseases, leading to significant yield and quality losses[4].

Cercospora is one of the 100 most cited fungal genera[5]. Traditionally, *Cercospora* taxonomy relied on morphological traits and host specificity, leading to over 3,000 described species[6,7]. Crous and Braun[6] reviewed *Cercospora* species based on morphology and recognised 659 species. However, molecular phylogenetics has since reshaped the classification of *Cercospora*, revealing that many species with similar hosts or morphology are in fact distinct, while some species can infect multiple hosts[8–10]. Studies using ITS, *tef1*, *act*, *cmd*, *his3, tub, rpb2* and *gapdh* gene regions revealed hidden diversity within *C. beticola* Sacc.[8–10]. Recent research identified additional species (e.g., *C. americana* Vaghefi, S.J. Pethybridge & R.G. Shivas, *C. tecta* Vaghefi, S.J. Pethybridge & R.G. Shivas) associated with CLS[11]. In Iran, only six isolates have been genetically characterized, with five identified as *C. beticola* and one as *C. gamsiana* Bakhshi & Crous [10,12].

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Cercospora leaf spot is a polycyclic fungal disease that thrives under warm and humid conditions, causing necrotic leaf spots that reduce photosynthetic efficiency, root yield, and sugar content[5,13,14]. The pathogen produces conidia within seven days after infection, facilitating rapid disease spread[15].Current management strategies to control the diseases are the use of certified seeds[16], crop rotation[17] and fungicide applications[18]. However, fungicide resistance in *C. beticola* populations has emerged due to overuse[19,20]. Traditional disease forecasting models, based on weather conditions, often lack accuracy[21,22], necessitating advanced molecular detection methods for timely intervention.

Accurate pathogen identification is crucial for disease management[23]. In sugar beet, *Alternaria alternata* (Fr.) Keissl., *Phoma betae* A.B. Frank, and *Ramularia beticola* Fautrey & Lambotte are fungal pathogens often confused with *C. beticola* when accurate diagnostics are lacking[24]. Conventional PCR-based methods are time-consuming and require specialized equipment. In contrast, other new techniques such as Loop-Mediated Isothermal Amplification (LAMP) offer high sensitivity and specificity[25]; rapid detection (< 1 h)[26] and visual results (colorimetric/fluorescence)[27]. Loop-Mediated Isothermal Amplification uses *Geobacillus stearothermophilus* (Bst) DNA polymerase and six primers targeting conserved regions[28], making it ideal for field diagnostics. Its application in detecting *C. beticola* could optimize disease management by enabling early fungicide application and reducing unnecessary chemical use.

This study aimed to characterize *Cercospora* isolates from major sugar beet growing regions in Iran using multi-locus phylogenetics and develop a LAMP-based assay for rapid and accurate detection of *C. beticola*. By integrating genomic tools and molecular diagnostics, this research will enhance CLS management, supporting sustainable sugar beet production.

Materials and methods

Sampling

Field surveys were conducted across sugar beet cultivations in multiple provinces of Iran; Khuzestan (Dezful, Shush and Andimeshk), West Azerbaijan (Khoy), Razavi Khorasan (Joveyn) Semnan (Meyami and Shahroud), Mazandaran (Behshahr), Ardabil (Moghan) and Golestan (Kordkuy and Kalaleh) during 2021–2023 to collect fungal isolates (Fig. 1). Leaves exhibiting symptoms of *Cercospora*-like leaf spots were sampled, placed in labeled paper bags and transported to the laboratory.

Fungal isolation, purification and morphological examinations

Samples were examined using a stereomicroscope Zeiss Stemi 305 (Oberkochen, Germany) for *Cercospora*-specific conidiophores and conidia. Single-spore isolation was performed directly from lesions as explained in Bakhshi et al.[29], briefly; Malt Extract Agar (MEA, Merck, Germany) plates were slanted, supplemented with 10 mL sterile water, and conidial masses were transferred into the water phase, homogenized, and incubated overnight. Excess water was removed after 24 h, and germinated conidia were transferred to fresh MEA plates under sterile conditions. Shape and size of morphological structures of all fungal isolates including stromata, conidiophores, and conidia extracted from lesions examined using a Nikon Eclipse 80i light microscope (Tokyo, Japan). Pure cultures were maintained on MEA slants and sterile distilled water at 4–6 °C. Representative isolates were deposited in the Iranian Fungal Culture Collection (IRAN …C; Table 1).

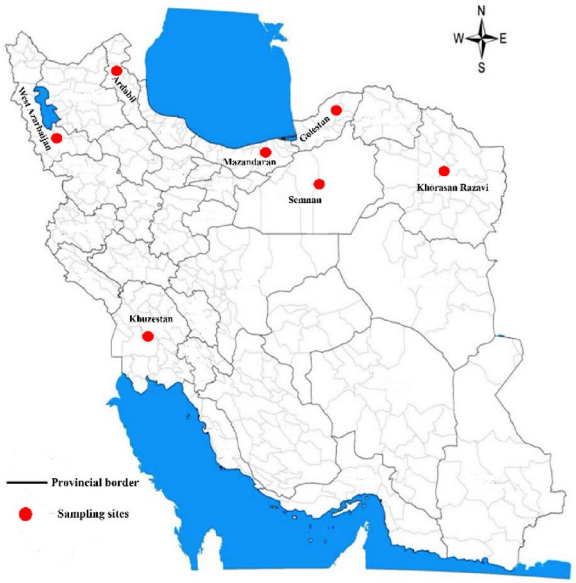

Fig. 1. Schematic representation of sampling locations included in this study.

Species	Isolate	Province	County	host	Collection date	*actA*	*cmdA*	*gapdh*	*his3*	*tef1*
Cercospora beticola	AJ1L2I1	Razavi Khorasan	Joveyn	*Beta vulgaris*	2021	PX104696	PX104873	PX104932	PX104814	PX104755
C. beticola	AJ1L4	Razavi Khorasan	Joveyn	*B. vulgaris*	2021	PX104661	PX104838	PX104897	PX104779	PX104720
C. beticola	AJ1L6I1	Razavi Khorasan	Joveyn	*B. vulgaris*	2021	PX104665	PX104842	PX104901	PX104783	PX104724
C. gamsiana	IRAN 5143C	Razavi Khorasan	Joveyn	*B. vulgaris*	2021	PX104702	PX104879	PX104938	PX104820	PX104761
C. beticola	AJ2L2S1	Razavi Khorasan	Joveyn	*B. vulgaris*	2021	PX104666	PX104843	PX104902	PX104784	PX104725
C. gamsiana	AJ2L5S2	Razavi Khorasan	Joveyn	*B. vulgaris*	2021	PX104719	PX104896	PX104955	PX104837	PX104778
C. gamsiana	AJ2L6S3	Razavi Khorasan	Joveyn	*B. vulgaris*	2021	PX104710	PX104887	PX104946	PX104828	PX104769
C.gamsiana	AJ2L7S1	Razavi Khorasan	Joveyn	*B. vulgaris*	2021	PX104703	PX104880	PX104939	PX104821	PX104762
C. beticola	BM1L2	Semnan	Miamei	*B. vulgaris*	2021	PX104669	PX104846	PX104905	PX104787	PX104728
C. beticola	BM1L3	Semnan	Miamei	*B. vulgaris*	2021	PX104690	PX104867	PX104926	PX104808	PX104749
C. beticola	IRAN 5144C	Semnan	Miamei	*B. vulgaris*	2021	PX104667	PX104844	PX104903	PX104785	PX104726
C. beticola	BM1L6	Semnan	Miamei	*B. vulgaris*	2021	PX104691	PX104868	PX104927	PX104809	PX104750
C. beticola	BM2L3	Semnan	Miamei	*B. vulgaris*	2021	PX104668	PX104845	PX104904	PX104786	PX104727
C. beticola	BM2L9	Semnan	Miamei	*B. vulgaris*	2021	PX104693	PX104870	PX104929	PX104811	PX104752
C. beticola	BM3L1I2	Semnan	Miamei	*B. vulgaris*	2021	PX104700	PX104877	PX104936	PX104818	PX104759
C. beticola	BM3L11I3	Semnan	Miamei	*B. vulgaris*	2021	PX104701	PX104878	PX104937	PX104819	PX104760
C. beticola	FD1L5	West Azerbaijan	Khoy, Dizaj-Herik	*B. vulgaris*	2021	PX104697	PX104874	PX104933	PX104815	PX104756
C. gamsiana	FK1L1	West Azerbaijan	Khoy	*B. vulgaris*	2021	PX104707	PX104884	PX104943	PX104825	PX104766
C. gamsiana	CC2	Golestan	Kordkuy	*B. vulgaris*	2021	PX104711	PX104888	PX104947	PX104829	PX104770
C. beticola	CC10	Golestan	Kordkuy	*B. vulgaris*	2021	PX104683	PX104860	PX104919	PX104801	PX104742
C. gamsiana	CC13	Golestan	Kordkuy	*B. vulgaris*	2021	PX104712	PX104889	PX104948	PX104830	PX104771
C. beticola	CC15	Golestan	Kordkuy	*B. vulgaris*	2021	PX104684	PX104861	PX104920	PX104802	PX104743
C. gamsiana	CC17	Golestan	Kordkuy	*B. vulgaris*	2021	PX104718	PX104895	PX104954	PX104836	PX104777
C. beticola	IRAN 5145C	Golestan	Kordkuy	*B. vulgaris*	2021	PX104663	PX104840	PX104899	PX104781	PX104722
C. gamsiana	IRAN 5146C	Golestan	Kalaleh	*B. vulgaris*	2021	PX104704	PX104881	PX104940	PX104822	PX104763
C. gamsiana	FK1L4	West Azerbaijan	Khoy	*B. vulgaris*	2021	PX104708	PX104885	PX104944	PX104826	PX104767
C. gamsiana	IRAN 5147C	Golestan	Kalaleh	*B. vulgaris*	2021	PX104706	PX104883	PX104942	PX104824	PX104765
C. gamsiana	IRAN 5148C	Golestan	Kalaleh	*B. vulgaris*	2021	PX104705	PX104882	PX104941	PX104823	PX104764
C. beticola	CK5L5	Golestan	Kalaleh	*B. vulgaris*	2021	PX104662	PX104839	PX104898	PX104780	PX104721
C. beticola	IRAN 5149C	Mazandaran	Behshahr	*B. vulgaris*	2021	PX104673	PX104850	PX104909	PX104791	PX104732
C. gamsiana	DB1L5S1	Mazandaran	Behshahr	*B. vulgaris*	2021	PX104713	PX104890	PX104949	PX104831	PX104772
C. beticola	IRAN 5150C	Mazandaran	Behshahr	*B. vulgaris*	2021	PX104676	PX104853	PX104912	PX104794	PX104735
C. gamsiana	DB1L6S1	Mazandaran	Behshahr	*B. vulgaris*	2021	PX104714	PX104891	PX104950	PX104832	PX104773
C. beticola	IRAN 5151C	Mazandaran	Behshahr	*B. vulgaris*	2021	PX104670	PX104847	PX104906	PX104788	PX104729
C. gamsiana	DB1L7I2	Mazandaran	Behshahr	*B. vulgaris*	2021	PX104715	PX104892	PX104951	PX104833	PX104774
C. beticola	IRAN 5152C	Mazandaran	Behshahr	*B. vulgaris*	2021	PX104674	PX104851	PX104910	PX104792	PX104733
C. beticola	DB2	Mazandaran	Behshahr	*B. vulgaris*	2021	PX104680	PX104857	PX104916	PX104798	PX104739
C. gamsiana	FK2L2	West Azerbaijan	Khoy	*B. vulgaris*	2021	PX104709	PX104886	PX104945	PX104827	PX104768
C. beticola	EM1L5S1	Ardabil	Moghan	*B. vulgaris*	2021	PX104685	PX104862	PX104921	PX104803	PX104744
C. gamsiana	EM1L8S1	Ardabil	Moghan	*B. vulgaris*	2021	PX104717	PX104894	PX104953	PX104835	PX104776
C. beticola	EM2L3S1	Ardabil	Moghan	*B. vulgaris*	2021	PX104698	PX104875	PX104934	PX104816	PX104757
C. gamsiana	EM3L4S1	Ardabil	Moghan	*B. vulgaris*	2021	PX104716	PX104893	PX104952	PX104834	PX104775
C. beticola	EM4L5	Ardabil	Moghan	*B. vulgaris*	2021	PX104695	PX104872	PX104931	PX104813	PX104754
C. beticola	EM5L1	Ardabil	Moghan	*B. vulgaris*	2021	PX104694	PX104871	PX104930	PX104812	PX104753
C. beticola	IRAN 5153C	Ardabil	Moghan	*B. vulgaris*	2021	PX104671	PX104848	PX104907	PX104789	PX104730
C. beticola	IRAN 5154C	Khuzestan	Dezful	*B. vulgaris*	2022	PX104664	PX104841	PX104900	PX104782	PX104723
C. beticola	K1L6I2	Khuzestan	Shush	*B. vulgaris*	2022	PX104679	PX104856	PX104915	PX104797	PX104738
C. beticola	K1L9	Khuzestan	Andimeshk	*B. vulgaris*	2022	PX104681	PX104858	PX104917	PX104799	PX104740
C. beticola	K1L11S1	Khuzestan	Hamidabad, Shush	*B. vulgaris*	2022	PX104678	PX104855	PX104914	PX104796	PX104737
C. beticola	IRAN 5155C	Khuzestan	Hamidabad, Shush	*B. vulgaris*	2022	PX104699	PX104876	PX104935	PX104817	PX104758
C. beticola	K1L11S4	Khuzestan	Hamidabad, Shush	*B. vulgaris*	2022	PX104672	PX104849	PX104908	PX104790	PX104731
C. beticola	K1L11S5	Khuzestan	Hamidabad, Shush	*B. vulgaris*	2022	PX104692	PX104869	PX104928	PX104810	PX104751
C. beticola	K2L5	Khuzestan	Karoon	*B. vulgaris*	2022	PX104682	PX104859	PX104918	PX104800	PX104741
C. beticola	IRAN 5156C	Khuzestan	Dezful	*B. vulgaris*	2022	PX104675	PX104852	PX104911	PX104793	PX104734
C. beticola	IRAN 5157C	Khuzestan	Dezful	*B. vulgaris*	2022	PX104677	PX104854	PX104913	PX104795	PX104736
C. beticola	K3L6S1	Khuzestan	Shush	*B. vulgaris*	2022	PX104686	PX104863	PX104922	PX104804	PX104745

Continued

Species	Isolate	Province	County	host	Collection date	*actA*	*cmdA*	*gapdh*	*his3*	*tef1*
C. beticola	K3L8S2	Khuzestan	Shush	*B. vulgaris*	2022	PX104687	PX104864	PX104923	PX104805	PX104746
C. beticola	GS 2	Khuzestan	Safiabad, Dezful	*B. vulgaris*	2023	PX104688	PX104865	PX104924	PX104806	PX104747
C. beticola	GS 3	Khuzestan	Safiabad, Dezful	*B. vulgaris*	2023	PX104689	PX104866	PX104925	PX104807	PX104748

Table 1. Fungal isolates used in this study.

Primer	Sequence (5′ to 3′)	Tm°	Dimer	2ndry	GC (%)
FIP (F1c + F2)	GCCGTCGACCTCGATCTTGC-GACATGTCCTCCAGGCGTA	83	weak	0	61
BIP (B1c + B2)	AACCAGGGCCTGATCGTCAAC-CTCACCCCATGGAATGGC	82	weak	0	59
F3	ATGGAGAGCTGCTCGGCT	58	0	0	61
B3	GGATTCGACGATGTACTCGG	60	0	0	55
LF	GTGGAGTCGTACTTCAGCATG	61	0	0	52
LB	CAAGAAGATCCGCTTCTACATGGAG	66	0	0	48

Table 2. LAMP primer set designed in this study.

DNA extraction, BOX fingerprinting and sequencing

DNA was extracted from all isolates using the method described by Möller et al.[30]. DNA quality was assessed by electrophoresis on a 1% agarose gel, while DNA quantity was measured using a NanoDrop spectrophotometer (Thermo Scientific, USA). The initial concentration of all samples was adjusted to 50 ng μL^{-1}. The BOX region was amplified for each isolate with the BOXA1R primer (5′- CTACGGCAAGGCGACGCTGACG-3′)[31], following the PCR protocol and conditions outlined by Bakhshi et al.[32]. Initial classification of the recovered isolates was based on BOX-PCR banding patterns, supplemented by morphological characteristics and isolate origin. Representative isolates were then selected for further sequencing.

PCR and sequencing

Multiple genomic regions including actin (*actA*), calmodulin (*cmdA*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), histone (*his3*), and translation elongation factor 1-alpha (*tef1*) were amplified using specific primers provided in Supplementary Table 1[33–35]. PCR amplification of the *actA*, *gapdh*, *his3*, *cmdA*, and *tef1* genomic regions was performed in 25 µL reaction volumes containing 5–10 ng of genomic DNA, 1 × reaction buffer, 2 mM MgCl2, 40 µM of each dNTP, 0.7 µL DMSO, 0.2 µM of each primer, 0.4 units of Taq DNA polymerase, and sterile deionized water, using a Bio-Rad thermocycler (North Carolina, USA). Thermal cycles consisted of an initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 45 s with a final extension at 72 °C for 5 min[12]. The *gapdh* gene was amplified using touchdown PCR conditions: initial denaturation at 94 °C for 5 min; 5 cycles of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 2 min; 5 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 2 min; 30 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 2 min; with a final extension at 72 °C for 8 min[11]. PCR products were electrophoresed on 1% agarose gels containing 0.5 µg mL^{-1} ethidium bromide (1% v/v). Following electrophoresis, DNA fragments were sized using a GeneRuler™ 1 kb DNA ladder (Sinacolon, Iran). PCR products were sequenced by Microsynth AG Company (Balgach, Switzerland).

Phylogeny

The resulting nucleotide sequences were blasted at the GenBank database and high-similarity sequences containing ex-type strains were downloaded and aligned with each other to make alignments. Individual gene alignments were performed using MAFFT v. 7[36] with manual editing in MEGA v. X [37] when necessary. For multi-gene analysis, individual gene alignments were concatenated using Mesquite v.3.81[38]. Bayesian inference was implemented in MrBayes v. 3.2.6[39] using Markov Chain Monte Carlo (MCMC) sampling. Four simultaneous chains were run from random starting trees with a heating parameter of 0.15 and default priors. For each dataset, two independent runs were executed, sampling every 1,000 generations until the average standard deviation of split frequencies reached 0.01. After discarding the initial 25% of sampled trees as burn-in, the remaining trees were used to generate a 50% majority-rule consensus phylogeny with posterior probability (PP) values. Resulting trees were visualized in Geneious v. 8.1.8[40] and finalized for publication using Adobe Illustrator 2024 Artwork v. 28.0. All new sequences were deposited in GenBank with accession numbers (Table 1).

LAMP primer design based on target genomic regions

For the development of LAMP-specific primers, nucleotide variation was initially analyzed in the amplified genomic regions (*gapdh*, *tef1*, *his3*, *cmd*, and *actA*) among fungal isolates through visual inspection using MEGA v. X software. Based on the observed nucleotide divergence between *Cercospora* species, the *gapdh* genomic region was selected for LAMP primer design. Primers were designed using the online tool Primer Explorer V5 (https://primerexplorer.jp/e/).

To assess the specificity of the designed LAMP primers (Table 2), reaction mixtures (Supplementary Table 2) were incubated at 65 °C for 45 min in a thermocycler. Sterile nuclease-free deionized water served as the negative

control. The DNA concentration of all reactions was adjusted at 50 ng μL^{-1}. For amplification verification, 5 µL of each reaction product was electrophoresed on 1% agarose gels containing 0.5 µg mL^{1} ethidium bromide. Following electrophoresis, amplification bands were visualized at 300 nm using a transilluminator and documented with a Gel Doc system (Bio-Rad). All reactions were performed in triplicate to ensure reproducibility; with band sizes determined using GeneRuler^{TM} 1 kb DNA ladders. To rigorously evaluate primer specificity, a cross-reactivity test against six non-target fungal genera including *Alternaria*, *Cladosporium*, *Curvularia*, *Ramularia*, and *Stemphylium* was performed. Furthermore, to test the limit of detection (LOD) of LAMP assay designed in this study, genomic DNA from *C. beticola* (strain IRAN 5155C) as the representative isolate was serially diluted tenfold and amplification was conducted under the LAMP condition as described earlier and followed by electrophoresis.

Results

Isolates and preliminary examinations

Extensive field surveys conducted across major sugar beet growing regions of Iran, including Khuzestan, West Azerbaijan, Golestan, Razavi Khorasan, Semnan, Ardabil, and Mazandaran provinces, revealed widespread incidence of CLS disease. The characteristic symptoms appeared as numerous circular lesions (2–5 mm diameter) with distinctive coloration patterns: brown-gray centers surrounded by purple-to-burgundy margins (Fig. 2). In severe infections, coalescing lesions resulted in extensive leaf blight and necrosis. From these symptomatic plants, 283 fungal isolates were successfully isolated and morphologically identified as *Cercospora* species. Following comprehensive analysis incorporating BOX-PCR genotyping, morphological characterization, and geographical distribution patterns, a total of 61 representative isolates (Table 1) were selected for DNA sequencing studies. This selection strategy ensured optimal representation of both genetic diversity and geographical distribution across all surveyed regions.

Molecular identification and phylogenetic analysis of *Cercospora* isolates from Iranian sugar beet fields

Phylogenetic analysis consisted of 105 *Cercospora* strains, including 46 reference sequences from NCBI and 59 isolates from this study, with *Cercospora sorghicola* CBS 136448 serving as the out-group. The final concatenated alignment comprised 1,974 characters, including alignment gaps. Model selection analysis determined GTR + G as the optimal substitution model for *cmdA*, HKY + G for *actA* and *tef1*, and GTR + I + G for *his3* and *gapdh* gene regions, all with Dirichlet base frequencies. Bayesian inference of the 1,974 character dataset identified 513 unique site patterns, generating 412 phylogenetic trees through MCMC sampling. After discarding the initial 25% as burn-in, the 50% majority-rule consensus tree and posterior probabilities were calculated from the remaining 310 trees (Fig. 3).

The phylogenetic analysis revealed two distinct *Cercospora* species clades among the 62 Iranian isolates with high posterior probability: *C. beticola* (71% prevalence) and *C. gamsiana* (29% prevalence). Geographic distribution patterns showed *C. beticola* as the exclusive species in Khuzestan and Semnan provinces, while both species coexisted in West Azerbaijan, Ardabil, Golestan, Khorasan, and Mazandaran. These results conclusively demonstrate *C. beticola* as the dominant *Cercospora* species affecting sugar beet production across Iran, with *C. gamsiana* showing regional distribution in northern and western growing areas (Figs. 1 and 3).

Fig. 2. The symptoms of Cercospora leaf spot disease appeared as characteristic circular lesions on sugar beet (*Beta vulgaris* L.) leaves, progressing to extensive leaf blight in severe cases.

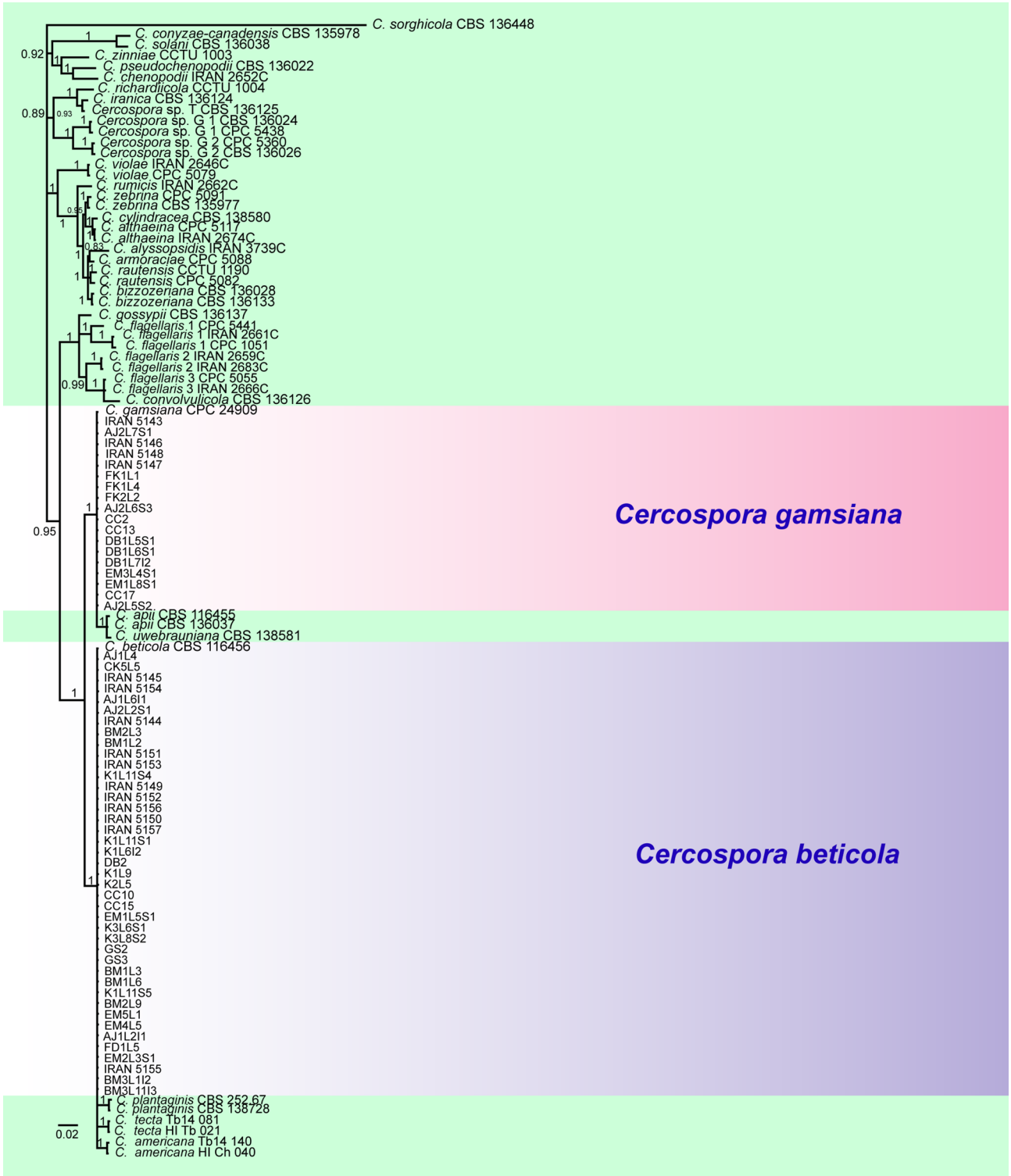

Fig. 3. Multilocus phylogenetic tree (based on *actA*, *cmdA*, *gapdh*, *his3*, and *tef1* gene regions) of *Cercospora* species identified in this study. The scale bar represents 0.02 expected substitutions per site. *Cercospora sorghicola* (CBS 136448) served as the out-group.

Evaluation of primer specificity

The designed LAMP primers specifically amplified DNA from *C. beticola*, the predominant causal agent of Cercospora leaf spot in sugar beet, while successfully differentiating it from other *Cercospora* species (*C. gamsiana*, *C. apii*, *C.* cf. *flagellaris*, and *Cercospora* sp. G) and fungal genera (*Alternaria*, *Cladosporium*, *Curvularia*, *Ramularia* and *Stemphylium*). In triplicate testing, no amplification was observed in negative

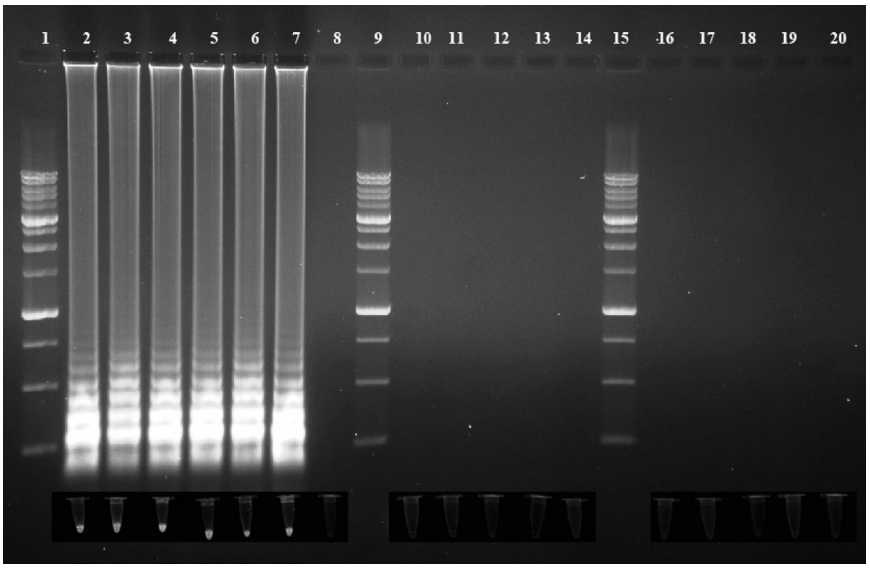

Fig. 4. Results of DNA amplification using LAMP primers specific to *C. beticola* showing: (1) GeneRuler™ 1 kb DNA ladder, (2) *C. beticola* IRAN 5144C, (3) *C. beticola* IRAN 5149C, (4) *C. beticola* IRAN 5153C, (5) *C. beticola* IRAN 5157C, (6) *C. beticola* IRAN 5145C, (7) *C. beticola* IRAN 5148C, (8) *C. gamsiana* IRAN 5143C, (9) GeneRuler™ 1 kb DNA ladder, (10) *C. gamsiana* IRAN 5148C, (11) *C. cf. flagellaris* IRAN 2720C, (12) *Cercospora* sp. G IRAN 4098C, (13) *Cercospora apii* IRAN 2655C, (14) *Alternaria atra* IRAN 4671C, (15) GeneRuler™ 1 kb DNA ladder, (16) *Stemphylium vesicarium* IRAN 4667C, (17) *Curvularia inaequalis* IRAN 4792C, (18) *Ramularia lamiigena* IRAN 3980C, (19) *Cladosporium macrocarpum* IRAN 4654C and (20) negative control. The results demonstrate exclusive amplification in *C. beticola* samples (lanes 2–7) with no cross-reactivity observed in other *Cercospora* species or fungal genera, confirming the high specificity of the designed primers. Negative control (lane 20) showed no amplification, validating the assay's reliability.

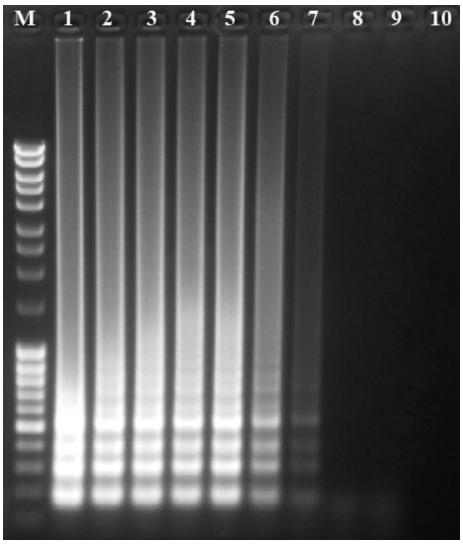

Fig. 5. Lanes 1–10 indicate the sensitivity of LAMP amplification using a ten-fold serial dilution of *C. beticola* IRAN 5155C genomic DNA, ranging from 50 ng μL^{-1}, 5 ng μL^{-1}, 500 pg μL^{-1}, 50 pg μL^{-1}, 5 pg μL^{-1}, 500 fg μL^{-1}, 50 fg μL^{-1}, 5 fg μL^{-1}, 500 ag μL^{-1}, and 50 ag μL^{-1}, respectively. Lane M contains the GeneRuler™ 1 kb DNA ladder (MBI Fermentans, Vilnius, Lithuania) as a molecular size marker.

control (Fig. 4). The turbidity of positive reactions, resulting from DNA amplification of *C. beticola* isolates, was visually observed, confirming successful detection of the target pathogen by the LAMP assay without the need for electrophoresis (Fig. 4). Furthermore, in the sensitivity test employing a serial dilution of genomic DNA extracted from *C. beticola* (IRAN 5155C) as a representative strain, the LAMP assay demonstrated a detection limit of 50 fg μL^{-1} in this study (Fig. 5).

Discussion

Cercospora leaf spot (CLS) demonstrates higher prevalence in regions with warm and humid climates during the sugar beet growing season[24]. In Iran, the disease shows significant incidence in several areas, particularly Khuzestan, Ardabil, and Golestan provinces. This pattern of distribution results from the confluence of three key factors: favorable environmental conditions, the persistent presence of pathogenic inoculums, and the widespread cultivation of susceptible cultivars across multiple growing seasons.

Through comprehensive molecular and morphological analyses of 283 isolates collected from seven Iranian provinces, this study successfully identified and differentiated two fungal species, *C. beticola* and *C. gamsiana*, as the causal agents of CLS in sugar beet. Sequencing of five genomic regions and phylogenetic analysis of 62 selected isolates revealed that while these species share some morphological characteristics, they are genetically distinct (Fig. 3).

Cercospora beticola was identified as the dominant species with a frequency of 71% across all study regions whereas *C. gamsiana* was primarily observed in northern regions (particularly Ardabil, Golestan, Mazandaran, and West Azerbaijan provinces) with a frequency of 29%. Notably, no *C. gamsiana* isolates were detected in Khuzestan or Semnan provinces. This geographical distribution pattern likely reflects ecological and climatic differences between regions, with northern areas (characterized by higher relative humidity and more moderate temperatures) appearing more favorable for *C. gamsiana* growth and spread.

Recent studies have reported several other *Cercospora* species as causal agents of CLS worldwide, including *C. americana*, *C. apii* Fresen., C. cf. *flagellaris*, *Cercospora* sp. G, *C. tecta* and *C. zebrina* Pass.[11]. *Cercospora gamsiana* was first isolated and reported from weeds in northern Iran[10] and given that *C. beticola* has also been previously isolated from various broadleaf weeds[8,9,41], it appears that weeds play a crucial role in maintaining pathogenic inoculums and facilitating its transmission to sugar beet fields. This transmission may occur through wind, rain, or other means such as movement of agricultural equipment or farm labor.

The most significant and novel finding of this study is the first report of the relatively widespread presence and pathogenicity of *C. gamsiana* on sugar beet in Iran. This finding is particularly important as *C. beticola* was previously considered the sole significant causal agent of CLS on sugar beet. The identification of *C. gamsiana* as a prevalent pathogen on sugar beet may explain some failures in disease control programs in northern regions of the country, as this species may differ from *C. beticola* in terms of pathogenicity characteristics, host range, and sensitivity to fungicides. Consequently, further studies are needed to better understand the biological properties, damage potential, and responses to chemical and non-chemical control methods for *C. gamsiana*.

Molecular analysis of this study revealed the *gapdh* gene as the most variable between species, making it ideal for designing specific LAMP primers. Laboratory tests demonstrated these primers could distinguish *C. beticola* from *C. gamsiana* and other fungi with high sensitivity (detecting nanogram DNA amounts) within 45 min (Fig. 4). This represents a significant improvement over traditional morphological identification methods that take long time and are susceptible to growth conditions and observer bias. In the only study conducted to develop a LAMP assay for detecting *C. beticola* based on the ITS-rDNA and *CbCyp51* genes, *C. beticola* and its resistant isolates were successfully identified, respectively[24]. However, the ITS-rDNA region is unsuitable for discriminating *Cercospora* species in sugar beet due to its low genetic variability[8–10,42].

Our study achieved a detection limit of 50 fg μL^{-1} through LAMP primers targeting the *gapdh* gene, demonstrating both high sensitivity and specificity in discriminating *C. beticola* from *C. gamsiana* and other fungal genera without observable cross-reactivity (Fig. 5). While Shrestha et al.[24] similarly developed an effective LAMP assay using ITS-rDNA targets, their reported sensitivity of 100 fg μL^{-1} and the inherent conservation of ribosomal DNA sequences limited the assay's ability to differentiate between closely related *Cercospora* species. These findings hold particular significance for regions with complex *Cercospora* populations, where the *gapdh*-based LAMP assay emerges as a more robust diagnostic tool for accurate pathogen identification and subsequent disease management optimization.

Given the significant impact of Cercospora leaf spot on sugar beet yield and quality (with reported losses up to 50% in some cases), the findings of this study could substantially enhance integrated disease management strategies. The rapid and accurate identification of pathogenic species in different regions enables more targeted control approaches, including: (1) deployment of resistant cultivars, (2) optimized fungicide application schedules, and (3) selection of most effective chemical treatments. Furthermore, the ability to quickly monitor fungal populations during the growing season allows for better prediction of critical disease outbreak periods and facilitates timely intervention measures.

Data availability

All sequence data from this study (Table 1) are publicly available in GenBank (https://www.ncbi.nlm.nih.gov/genbank) and can also be obtained from the corresponding author upon reasonable request.

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Author contributions

Conceptualization, K.K. and M.B.; methodology, K.K., M.B., M.G. and MA.TG; software, M.B. and K.K.: validation, K.K., M.B., R.Z., H.J. and B.M.; formal analysis, K.K. and M.B.; writing original draft preparation, K.K. and M.B.; writing, review and editing, R.Z., H.J., MA.TG., M.G. and B.M.; project administration, K.K., M.B. and H.J.; funding acquisition, M.B. and H.J.; All authors have approved the final version of the manuscript.

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Declarations

Competing interests
The authors declare no conflicts of interest.

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