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Morpho-molecular Characterization of Carrot Soft Rot Incitant, *Pectobacterium carotovorum* subsp *carotovoroum*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Carrot is an important root vegetable which plays an important role in human health. Globally, Post harvest diseases are the major constraint in carrot production, especially soft rot which resulting in severe yield loss. Early diagnosis of these post-harvest diseases paves a way for reducing the economic losses. Carrot samples showing typical rotting symptoms were collected from markets of four different districts of Tamil Nadu and the pathogen involved were isolated. Severe carrot soft rot

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incidence (66.74%) was observed in samples collected from Ooty area of The Nilgiris district and the least disease incidence of (16.21%) was recorded in Perundurai of Erode district. Pathogenicity of soft rot pathogen were conducted and the virulent isolates were identified based on Percent Disease Index (PDI) showing >50% rotting falls under disease grade 9 using disease grade scale (0 to 9). The bacterial isolates (KPB-7 and OCB-5) causing soft rot were characterized using various biochemical assays where in they showed positive response for methyl red, H2S gas production, KOH and catalase tests besides showing negative response for gram's reaction. Furthermore, molecular characterization of 16s rRNA region revealed the soft rot isolate (KPB-7) as *Pectobacterium carotovorum* subsp *carotovorum* (with an accession number OR251119).

Keywords: Carrot; soft rot; morphological; molecular characterization.

1. INTRODUCTION

Carrot is a biennial flowering plant of Apiaceae family and cultivated worldwide for its fleshy edible root and its nutritional status. It is a rich source of alpha and beta carotene which also contain Vitamins (A, K and B6) and minerals helps to improve eye vision and widely used for culinary purposes. In India, it covers an area of 110 thousand hectares with a production of 386.39 thousand tonnes (APEDA, 2022) and Haryana is the leading producer of carrot. In Tamil Nadu, major carrot growing districts are Nilgiris, Dindigul and Krishnagiri (2022). Post harvest loss is a major constraint in carrot cultivation and around 20-60% postharvest losses was observed in vegetables Kitinoja et al. [1]. Even 50-100% economic losses were recorded due to post harvest infections from field to storage Bhat et al. [2]. Chances of infection on harvested products would be high during harvest, transportation and storage and spreads through wounds of damaged plants causing economic damage to fleshy vegetables Whitehead et al. [3]. In vegetables, greater loss occurs mainly due to the soft rot and sour rot diseases Bhat et al. [2]. Soft rot caused by *Pectobacterium carotovorum* subsp *carotovorum* produces cell wall degrading enzymes such as cellulase, pectinase and polygalacturonase which act as a virulent factor for disease development. Early days, identification of microbes were done based on phenotypic character and biochemical tests De Boer and Kelman [4]. In recent years, molecular characterization of organisms is essential for confirming their identity wherein PCR (Polymerase Chain reaction) has been used and it is based on the amplification of target DNA sequence *Kang* et al. [5]. *Pectobacterium* are necrotrophic plant pathogens responsible for inducing diseases like wilting, rotting, and blackleg in crucial agricultural crops, notably potatoes, carrots, tomatoes, onions, pineapples, corn, rice, hyacinths, chrysanthemums, and calla lilies. These infections lead to substantial reductions in crop yield, as documented in previous studies by Adeolu et al. [6] ; Charkowski [7]; Charkowski et al. [8]; Perombelon and Kelman [9]. Soft rot of carrot caused by *Pectobacterium* sp was previously reported by Michalik et al. [10]; Parthiban [11]; Tang et al. [12]; Wasendorf et al. [13]. Since, the current study is focused on isolation and characterization of soft rot pathogen *Pectobacterium carotovorum* subsp *carotovorum* infecting carrot through morphological and molecular level analysis.

2. MATERIALS AND METHODS

2.1 Sample Collection and Isolation of Pathogen

Diseased carrot root samples from major carrot growing areas of Ooty and local markets of Coimbatore were collected based on symptoms. The disease severity of the soft rot was observed by using Percent disease index (PDI) and calculated by using this formula given by Rose [14].

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of individual ratings} \times 100}{\text{Total number of plants/ leaves observed} \times \text{Maximum disease grade}}$$

Grading the root vegetable by using 0-9 scale TNAU [15] and assessing the disease severity.

Chart 1. List of grade scale and its description

Grade scale	Description
0	No infection
1	Less than 1% lesion covering the root vegetable
3	1-10%lesion covering the root vegetable
5	11-25% lesion covering the root vegetable
7	26-50% lesion covering the root vegetable
9	Lesion covering more than 50% of root vegetable

The infected portion were cut from the carrot root and macerated in sterile pestle and mortar using 1-2 ml sterile distilled water and kept for 15 mins for oozing of bacterial cells. Then the bacterial suspension was serially diluted upto10^{-6} dilution and plated on Nutrient Agar medium using pour plate method in order to get uniform colonies. The single colonies were picked and streaked on NA medium and incubated at 28^0C for 48 hours. The isolates were also streaked on CVP (Crystal violet pectate) a selective medium for *Erwinia* species bacteria which formed cavities or deep pits Cupples and Kelman [16]. The isolates were named based on location and serially numbered as GPB-1, MPB-2, UDB-3, PPB-4, OCB-5, GMB-6, KPB-7, GKB-8, OTB-9, ITB-10, SMB-11 and PDB-12.

2.2 Morphological Characterization

The isolates of soft rot pathogen were characterized using various biochemical assays. The size, shape and arrangement of bacterial cells were identified through Gram staining Schaad et al*.* [17]. Other biochemical tests such as Methyl red test Mc Devitt [18], Catalase test Hayward [19], Gelatin hydrolysis test Clarke [20], KOH test Schaad et al*.* [17], H2S gas production Sendilvel et al*.* [21] and Potato soft rot test Muturi et al*.* [22] were also performed [23].

2.3 Pathogenicity Tests

The pathogenicity of soft rot pathogen was confirmed in two different ways as furnished below. In the first method, surface disinfected healthy carrots were cut into slices of 5mm thickness and placed in petri plates. A volume of 150-200 µl bacterial suspension (1×10^8cfu/ml) was inoculated by injecting them onto the slices Dadasogolu and Kotan [24]. In the second method, 200-300 µl of bacterial suspension (1×10^8cfu/ml) was injected on disinfected healthy whole carrots using sterile syringe, later covered with wet cotton and placed in polythene bags Chandrashekar et al*.* [25]. Then the inoculated carrots were incubated for 3-4 days at 28^0C for symptom expression and sterile distilled water is served as control in both the methods.

2.4 Molecular Characterization

Bacterial DNA was isolated from the virulent isolates using lysis method Chen et al*.* [26] and they were amplified using 16s rRNA gene of bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR reaction was carried out using 10 µl reaction mixture containing 1 µl template DNA, 5 µl Smart Prime 2X PCR Master Mix, 1 µl forward primer, 1 µl reverse primer and 2 µl sterile water. DNA Amplification parameters were fixed as follows: initial denaturation 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, initial extension at 72°C for 1 min and final extension at 72°C for 5 min. The amplified DNA was quantified through 1.2% agarose gel electrophoresis along with 1 Kb ladder. Resolved gel was documented in gel documentation unit (Bio rad) and the PCR products were partially sequenced and submitted in NCBI GenBank Lazaro et al*.* [27].

2.5 Statistical Analysis

Experimental data were analyzed statistically using Analysis of Variance (ANOVA) and the mean difference of all the treatment in Duncan's Multiple Range Test at 5% level of significance Gomez and Gomez [28]. All the data were analyzed using SPSS software (version 16) and interpreted.

3. RESULTS AND DISCUSSION

3.1 Sample Collection and Isolation of the Pathogen

The diseased samples of carrot were collected from carrot growing areas of Ooty and from different local markets in Coimbatore, Erode and Dindigul districts. Percent disease index was

calculated for the randomly selected carrots (100) based on the symptoms observed and characterized using disease score chart (0 to 9). Maximum disease incidence of soft rot incidence (66.74%) was noticed in Kettipalada of The Nilgiris district followed by Ottanchatrum of Dindigul district (45.55%) and the least disease incidence of 16.21 per cent was recorded in Perundurai of Erode district (Table 1). Carrot showing water soaked lesions with depressed and discoloured symptoms were isolated and the colonies with creamy white, slimy appearance were streaked in Nutrient Agar (NA) Medium. Similar type of results were obtained by Rahman et al. [29] observed the symptoms started with water soaked lesions later developing soft, watery and decay. Snehalatharani and Khan [30] reported that bacterial colonies with cream to white raised colonies with mucoid. Muturi et al. [22] isolated *Pectobacterium* from infected potato tubers and observed creamy, white colonies with mucoid.

3.2 Morphological Characterization

Colony morphology studies of the bacterial soft rot pathogen revealed that the bacteria produced raised, mucoid, cream to white coloured colonies (Table 2, Fig. 1). Furthermore, biochemical characterization revealed that the bacterial isolates OCB-5 and KPB-7 recorded a positive reaction for Methyl red, Growth at 36-37°C, H_2S gas production, Gelatin liquefaction, Catalase test, KOH test and Potato soft rot test and negative for gram's reaction (Table 3). Gram staining indicate that pink colour bacterial cells results shows that the isolates were gram negative. Maximum growth of bacteria was recorded after 48 hrs incubation at 37^0C in the isolate KPB-7 and MPB-2 (OD value @ 620 nm 1.733 and 1.627) respectively. De Boer and Kelman,2001 also reported that *Pectobacterium carotovorum* can grow at 37^oC. Potato soft rot test showing softening of the tissue with rotting symptom Muturi et al. [22]. From the antibiotic resistance tests, inhibition zone was observed around the paper disc containing streptomycin and no such zone in paper disc containing erythromycin and found that the isolates were resistant to erythromycin and susceptible to streptomycin antibiotic. Akbar et al. [31] reported that *Pectobacterium carotovorum* isolates resistant to erythromycin and susceptible to streptomycin. The results of biochemical characterization are in agreement with similar results of Rahman et al. [29] and Ragavi [32]. In addition, Muturi et al. [22] found that the pathogen showed pectolytic activity to degrade the plant cell wall.

3.3 Pathogenicity Test

Pathogenicity test were established for all the 12 isolates and the carrot inoculated artificially with each isolates with three replications. The pathogen able to produce symptoms was reisolated and proving the Koch's postulates. Intensity of disease was calculated by using Percent Disease Index (PDI) under disease grade scale (0 to 9). All the tested 12 isolates showed typical rotting symptoms within 1-3 days. The isolates, OCB-5 and KPB-7 were found to be the virulent isolates which showed severe infection of > 50% rotting under grade scale 9 (Table 4, Fig. 2). Tang et al. [12] also reported that the artificially inoculated carrot showing soft rot symptoms. Chandrashekar et al. [25] reported that bacterial suspension artificially inoculated into whole carrot showing water soaked lesions after 24hrs and complete rotting after 72hrs of incubation. In carrot slices, after 24hrs of incubation showing water soaked lesions and extending complete rotting.

3.4 Molecular Characterization

The DNA extracted from the virulent bacterial isolates (KPB-7) produced DNA fragments corresponding to the 16S region of the rRNA gene when subjected to PCR amplification with 16S rRNA universal primers. From the gel electrophoresis, it is evident that the isolates produced DNA fragments at the amplicon size of 1500 bp (Fig. 3). The partial sequences of the isolates (KPB-7) were obtained and they were submitted in NCBI GeneBank with an accession number (OR251119). The isolate is identified as *Pectobacterium carotovorum* subsp *carotovorum* through NCBI BLAST search which showed 99% identity with other isolates of *P. carotovorum* subsp *carotovorum* in NCBI Database. Caruso et al. [33] reported that the isolates from tomato belongs to *Pectobacterium carotovorum* subsp *carotovorum* and *P.carotovorum* subsp *brasiliensis*. In a similar study , Muturi et al.*.,*2018 stated that the analysis of 16S rRNA gene sequence revealed that the strain KPM17 was *Pectobacterium carotovorum* and 98% identity with *P.carotovorum* strain cc303. Ragavi [32] reported that rhizome rot of banana was *Pectobacterium carotovorum* subsp *carotovorum* had 99% similarity with existing isolates. Wasendorf [13] reported that the analysis of 16S region of the rRNA gene sequence was *Pectobacterium* strains isolated from carrot.

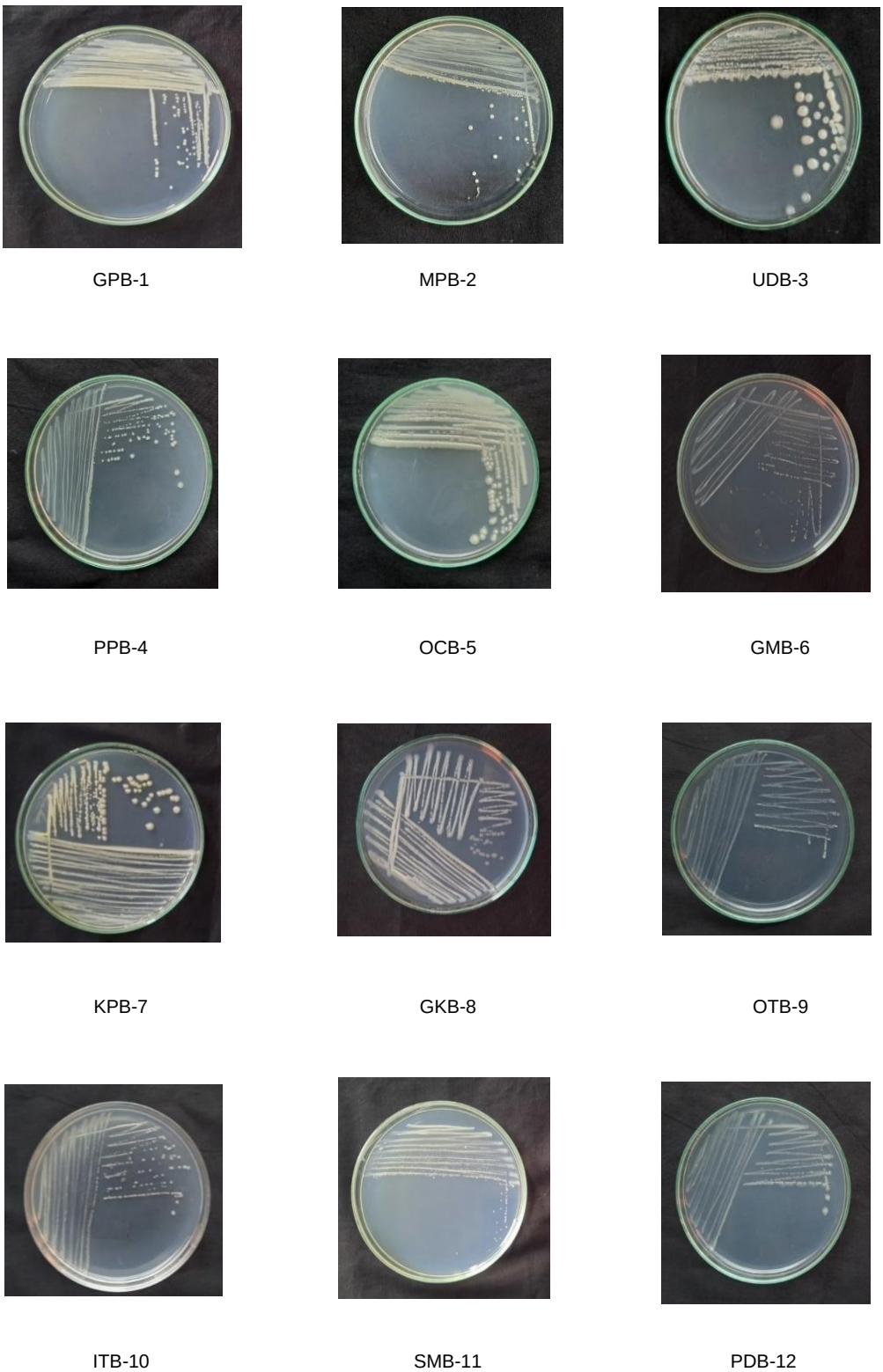

Fig. 1. Pure cultures of different isolates of soft rot pathogen associated with carrot

Table 1. Collection of isolates from different district of Tamil Nadu infecting carrot

S.No.	Isolate No	Location	District	Latitude (^{0}N)-Longitude(^{0}E)	Percent Disease Index (PDI)* Soft rot
1	GPB-1	Gandhipuram	Coimbatore	"11.01-76.96"	42.57^{c} (40.70)
2	MPB-2	Mettupalayam		"11.30-76.93"	38.27^{bc} (38.14)
3	UDB-3	Ukkadam		"10.99-76.96"	27.52^{abc} (31.35)
4	PPB-4	Periyanaickenpalayam		"11.14-76.94"	18.27^{ab} (24.83)
5	OCB-5	Ottanchatrum	Dindigul	"10.36-77.96"	45.55^{cd} (42.41)
6	GMB-6	Gandhi Market		"10.48- 77.75"	34.77^{abc} (35.96)
7	KPB-7	Kethipalada	The Nilgiris	"11.35-76.73"	66.74^{d} (55.57)
8	GKB-8	Gandhikandi		"11.30-76.62"	40.23^{c} (39.12)
9	OTB-9	Ooty		"11.41-76.69"	36.64^{abc} (37.13)
10	IRB-10	Ithalar		"11.30-76.65"	24.47^{abc} (29.16)
11	SMB-11	Sathyamangalam	Erode	"11.50-77.23"	41.63^{c} (39.94)
12	PDB-12	Perundurai		"11.27-77.58"	16.21^{a} (23.66)

**Mean of three replications and the mean followed by a common letter as superscript does not differ significantly at 5% level by DMRT. Values in parentheses are arc sine transformed value*

Table 2. Cultural characteristics of different isolates of soft rot pathogen associated with carrot

Isolate No	Colony colour	Appearance
GPB-1, UDB-3,GMB-6 and IRB-10	White	Slimy
MPB-2 ,OCB-5, GKB-8, IPD-12	Creamy white	Slimy
PPB-4, KPB-7	Yellowish white	Slimy, Raised colonies
ISM-11	Yellowish white	Slimy
OTB-9	Creamy white	Slimy, Raised colonies

A- Control , B- Inoculated carrot showing soft rot symptoms

Fig. 2. Pathogenicity test
Soft rot pathogen: Pectobacterium carotovorum subsp carotovorum

Table 3. Biochemical characterization of different isolates of soft rot pathogen associated with carrot

S. No.	Biochemical test	GPB-1	MPB-2	UDB-3	PPB-4	OCB-5	GMB-6	KPB-7	GKB-8	OTB-9	IRB-10	SMB-11	PDB-12
1.	Gram staining	-	-	-	-	-	-	-	-	-	-	-	-
2.	H2S Production	+	-	+	-	+	-	+	-	-	+	-	-
3.	Catalase test	-	+	-	+	+	+	+	-	+	-	+	-
4.	KOH test	-	+	+	-	+	+	+	-	+	-	-	+
5.	Gelatin hydrolysis	-	-	-	+	+	-	+	+	-	+	-	+
6.	Growth at 36-37^{o}C	+	+	+	+	+	+	+	+	+	+	+	+
7.	Potato soft rot test	+	+	+	-	+	+	+	-	+	+	+	-
8.	Pits formation in CVP Medium	+	+	-	-	+	-	+	+	-	-	-	-
9.	Methyl red test	-	+	-	-	+	+	+	-	+	+	-	+
10.	Erythromycin sensitivity test	-	-	-	+	+	-	+	+	+	+	-	-

+: Positive reaction
-: Negative reaction

Table 4. Severity level of different isolates of Carrot soft rot pathogen associated with carrot

Score chart/ Isolates	GPB-1	MPB-2	UDB-3	PPB-4	OCB-5	GMB-6	KPB-7	GKB-8	OTB-9	IRB-10	SMB-11	PDB-12	Severity
0	-	-	-	-	-	-	-	-	-	-	-	-	No infection
1	<1%	-	-	-	-	-	-	-	-	<1%	<1%	<1%	Water soaked lesion
3	-	-	1-10%	-	-	-	-	1-10%	1-10%	-	-	-	Water soaked lesion with initial rotting
5	-	11-25%	-	11-25%	-	-	-	-	-	-	-	-	25% rotting of the vegetable
7	-	-	-	-	26-50%	-	-	-	-	-	-	-	50% rotting of the vegetable
9	-	-	-	-	-	-	>50%	-	-	-	-	-	Complete rotting of the vegetable

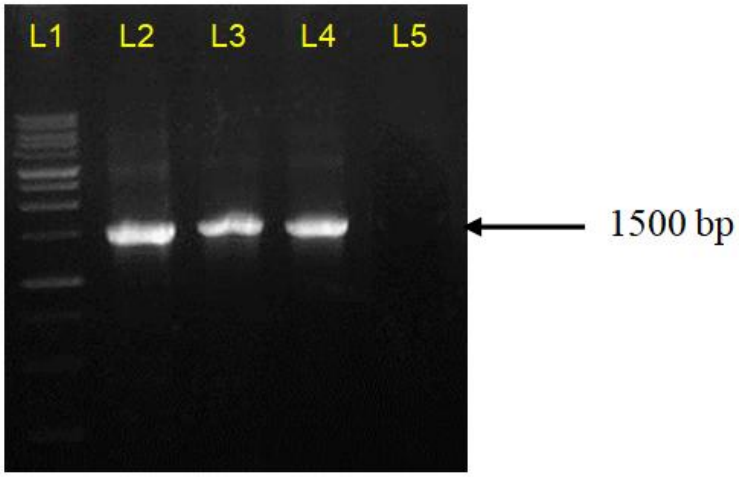

Fig. 3. Molecular characterization of *Pectobacterium carotovorum* subsp *carotovorum* (KPB-7 Isolate)

L1 : 1 Kb Ladder
L2 : GPB 1
L3 : OCB 5
L4 : KPB 7
L5 : Control

4. CONCLUSION

In this current study, soft rot pathogen infecting carrot were isolated and they were identified through morphological and molecular characterization. Highest Soft rot incidence (66.74%) was observed in samples collected from Ooty area of The Nilgiris district followed by Ottanchatrum of Dindigul district with 45.55% incidence and the least disease incidence of (16.21%) was observed in Perundurai of Erode district. All the isolates of soft rot pathogen expressed pathogenic nature calculated based on the Percent Disease Index (PDI) wherein OCB-5 and KPB-7 were found to be highly virulent by using disease grade chart (0 to 9) showing more than 50% rotting under disease grade 9. Biochemical characterization of 12 isolates revealed that the isolate OCB-5 and KPB-7 were positive for Catalase test, Methyl red, H_2S gas production, Gelatin liquefaction, KOH test, Growth at 36-37°C, and Potato soft rot test and negative for gram's reaction. The soft rot bacterial isolate (KPB-7) was identified as *Pectobacterium carotovorum* subsp *carotovorum* with an accession number (OR251119) showing 99% identity with other *Pectobacterium* isolates in NCBI Database. Earlier detecting of the pathogen through morpho-molecular characterization helps in effective management strategies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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