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Molecular identification of fungal species infesting tomato (*Lycopersicon esculentum*) at postharvest phase in Kwanar Gafan, Kano State

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ABSTRACT

Purpose: Fungal infections have become the most common problem to cause postharvest loss in tomato enterprise across Nigeria. Morphology-based identification of fungal species usually provides the inconclusive results with several species remain unidentified. Molecular identification method was used to supplement morphology-based techniques to identify the fungal species. **Research Method:** A Deoxyribonucleic Acid (DNA) through 5.8S-ITS (Internal Transcribed Spacer) region of the ribosomal DNA (rDNA) was used to identify 180 infested tomato specimens. The samples were collected from farm, retail and wholesale points in Kwanar Gafan located at Kura LGA of Kano State. **Findings:** Eight haplotypes have been detected from the total fungal specimens examined, A 65.6% of fungal specimens from wholesales and farms constituted haplotype-1 and identified as *Pichia kudriavzevii* (with 99.54% similarity) as BLAST in National Centre for Biotechnology Information (NCBI) database. The remaining seven haplotypes were exclusively found in the retailing points and largely constitutes *Aspergillus* spp., *Mucor fragilis*, *Russula atroglauca, Ganoderma* sp., *Alternaria* spp., *Exserohilum rostratum*, *Colletotrichum boninense*, *Naganishia* sp. and *Cladosporium* spp. **Research limitations:** Further research on molecular identification from other parts of kano is required for better understanding the fungi associated with postharvest loss in the state. **Originality/Value:** *Pichia kudriavzevii* is a single and only dominant fungal species that infest tomatoes in both farms and wholesale points (65.6%). However, 34.4% of the diverse fungal species have been found in retailing points which is related to the rapid infestation of tomatoes.

INTRODUCTION

Postharvest loss of fruits and vegetables is significant in developing countries where lack of appropriate storage facilities are the main challenges (Kassa & Senay, 2019). As a result, highly perishable fruits and vegetables undergo the greatest proportion of postharvest losses. Almost half of all fruits and vegetables produced are lost and wasted along the agri-food supply chain (Tiong et al., 2018).

Tomato (*Lycopersicon esculentum*) belongs to the family Solanaceae which is regarded as one of the most important, cultivated and consumed vegetable in the world (Lamidi et al., 2020; Maurya et al., 2022). Monte et al. (2013) reported that about 146 million tons of fresh tomato fruits are produced annually throughout the world. Tomatoes are consumed in large quantities directly in salads, cooked into soups or processed into juice, ketchup, whole peeled tomato and paste (Adedeji et al., 2006; Shankara et al., 2015). Tomatoes are rich in vitamins (particularly A and C), minerals, sugars, essential amino acids, iron, dietary fibres and phosphorus (Ayendiji & Adeniyi, 2011). It also contains high amount of β-carotene, vitamin C and lycopene, a carotenoid with antioxidant properties (Britt & Kristin, 2011; Dandago et al., 2018; Rani & Khetarpaul, 2009). In Nigeria, tomato fruits consumption accounts for about 18% of the daily consumption of vegetables (Lamidi et al., 2020). Nigeria is the second largest producer of tomatoes in Africa and 13th largest in the world (FAOSTAT, 2014). In Nigeria, tomatoes are majorly produced in the Northern part and conveyed to the other parts of the country using different types of vehicles (Etebu et al., 2013). Owing to lack of information on appropriate postharvest technology, packaging and storage conditions, the fruits not only lose their quality but also encounter substantial postharvest loss. Kutama et al. (2007) showed that the estimated postharvest loss of tomatoes in Nigeria to be around 60% resulting in a serious economic loss (Dandago et al., 2017). Kitinoja et al. (2019) reported postharvest loss in tomatoes of 1-18% in India, 10-40% in Nigeria, and 50-60% in Rwanda. These losses are particularly higher at the wholesale and retail stages (Aghadi et al., 2019).

Postharvest loss often develops on wounds, bruised tissues and during fruit softening. Therefore, sound tomatoes can be inoculated by plant pathogens via cross contamination. Tomato fruits are susceptible to numerous decays from field through postharvest handling. Fungi are the most important pathogens infecting tomatoes causing important losses during harvesting, transportation and storage (Dandago et al., 2018). The sources can be diseased fruits, dirty harvest containers and poorly sanitized handling systems. Fungal decays of tomatoes include black rot (*Alternaria alternata*), fusarium rot (*Fusarium* spp.), grey mould rot (*Botrytis cinerea*), mucor rot (*Mucor muledo*), Rhizopus rot (*Rhizopus stolonifer* and *Rhizopus oryzae*) and watery soft rot (*Sclerotonia minor* and *S. sclerotiorum*) (Sargent & Moretti, 2004).

Therefore, it is necessary to identify these microscopic pathogens especially those affecting humans to reduce the risk of contamination and future infection. In fact, there was a limited knowledge regarding the stage (harvest, retail and wholesale) at which the fungal contamination of tomato begins. Also, little is known concerning the fungal species affecting tomatoes at each stage. Therefore, understanding the susceptible stage of contamination and species involved would be a milestone for aiding the stakeholders to take the appropriate management decisions and strategies.

Conventional cultivation and microscopic identification have been widely practiced for fungal studies based on mycelia (colour, size and shape) and morphological characteristics (Hasan & Zanuddin, 2020). Earlier studies on isolation and identification of postharvest fungi from tomato fruits particularly in Nigeria were based on conventional methods (Dandago et al., 2018; Kutama et al., 2007; Mailafia et al., 2017; Yahaya & Ahmad, 2008; Yusuf & Okunsanya, 2007). These methods are challenging especially for *Alternaria* spp. and could misled the final

conclusions (Gherbawy et al., 2018). Alternatively, molecular identification has been recently used and provides a higher resolution of the fungal species of vegetable fruits including tomato (Akbar et al., 2018; Gherbawy et al., 2018). Therefore, the aim of this research is to use molecular techniques to identify postharvest fungi associated with tomato fruits decay at three different handling stages in Kura axis of Kano State.

MATERIALS AND METHODS

Source of materials and sample collection

Sixty tomato fruits (UC18) were collected from three different level of handling units (farm, whole sale and retail) located at Kwanar Gafan of Kano State, each from Unit (20 samples per farm × 3), Unit-2 (20 samples per retail-point × 3) and Unit-3 (20 samples per wholesale-points × 3) collected from November 2020 (Fig. 1). In total, One Hundred and Eighty (180) rotten tomato samples were randomly collected from nine sub-units. Tomato fruits samples were rinsed with sterile distilled water and were immediately arranged in the sterilized containers, labelled appropriately, and transported to Food Microbiology Laboratory of Kano University of Science and Technology for fungal isolation.

Isolation and identification of fungi

The pathogens from the tomato samples were isolated and identified via the method adopted by Mustapha and Yahaya (2006). A small dissect (4 mm thick) of the infected part of the tomato was cut with a sterilized scalpel and placed aseptically on a sterilized and dried Potato Dextrose Agar (PDA). The inoculated petri dishes were incubated at room temperature for a period of 5 days. The colonies on each plate were counted and recorded. Each type of fungal colony was sub-cultured onto fresh medium PDA to obtain pure culture in PDA slants. The fungal isolates were identified using the illustrated genera of imperfect fungi using Collins and Lyne (2004). All glass wares and working environment were properly cleaned and sterilized throughout the experimental period to avoid any external contaminants.

Molecular identification

DNA extraction, polymerase chain reaction (PCR) amplification and Sequencing of ITS region

Genomic DNA was extracted from cultures (purified fungi) using a modified protocol of plant genomic DNA kit (TIANGEN BIOTECH CO., LTD, Beijing, China). The 5.8S-ITS region of the ribosomal rDNA was amplified by PCR with universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCGG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Liu et al., 2007). The PCR was performed using Veriti 96 Well Thermal Cycler (Applied Biosystem, California, USA) in 25 µL reaction volume containing 18.2 µL sterile distilled water, 2.5 µL Taq buffer, 2.0 µL dNTP Mix (2.5 mM), 0.5 µL of each primer (10 µM), 0.3 µL of 5 unit/µL Taq polymerase (TaKaRa) and 1 µL template DNA (1-50 ng/µL). The PCR started with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 60 s and then a final extension at 72 °C for 5 min.

The success of the PCR amplification was checked through 1.5% of agarose gel in 1.0 × TBE using FloroSafe DNA Stain. The expected sizes of the PCR products were confirmed by including 2 µL 100-bp DNA ladder in the gel run (Elpis Biotech, Daejeon, Korea). Electrophoresis was run at 100 volts for 1 hour, photographed and visualized using Luminescent Image Analyzer LAS-1000plus v.2.0 (Fuji Photo Film Co. Ltd., Tokyo, Japan). Successful amplificons were sequenced by inqaba biotec.

Data analysis

The ITS sequences (current study) were aligned and edited using ClustalW multiple sequence alignment program in MEGA v.7 (Kumar et al., 2016). DnaSP v.6 software was used to determine the variable sites and haplotypes among the sequences (Rozas et al., 2017). To trace the identity of fungal species, the detected haplotypes in the present study were queried using basic local alignment search tool (BLAST) in National Centre for Biotechnology Information (NCBI) nucleotide database. The highest species matching had been identified to a sequence similarity of at least > 92% to avoid false positives. Fungal haplotypes obtained in the current study were used to construct a phylogenetic tree using Maximum Likelihood method in MEGA v.7 to analyse the relationship.

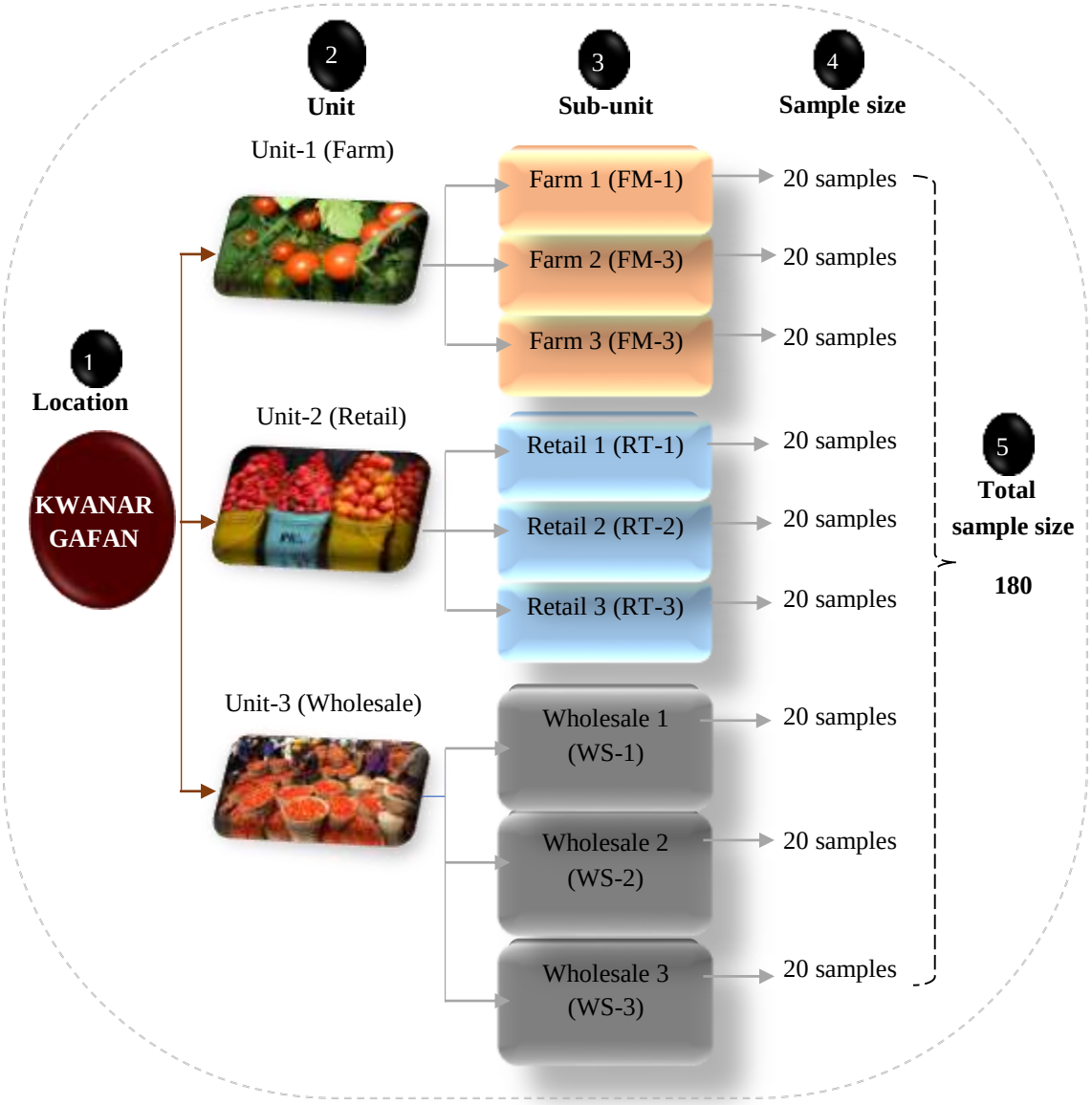

Fig. 1. Description of infected tomatoes sampling location, units, sub-units, sample sizes and total sample sizes used in current study.

RESULTS

Fungal isolates and haplotypes

A total of 13 unidentified fungi were successfully isolated from 180 tomato samples collected in the current study (Fig. 2). The sequences of 5.8S-ITS region of the ribosomal rDNA generated have a total sequence length of 502 base pairs after deleting low quality nucleotides of both primers ends. The sequences yielded eight haplotypes that are highly distinct and distributed (Table 1). Entire samples from farm and wholesale made-up Hap-1 (accept two samples from WS-3). Other haplotypes (Hap-2 to Hap-8) were largely from to the retail units. The BLAST search results for eight haplotype sequences match displayed high percentage identity with various sequences of fungal species as shown in Table 2. Hap-1, Hap-3 and Hap-4 showed the highest sequence similarity with only one NCBI sequence each. While the remaining haplotypes matched with more than one NCBI sequences with the same % similarity.

Table 1. Fungal haplotypes and their percentage distribution isolated from tomatoes in Kwanar Gafan.

	Sampling units										
	Farm			Retail			Wholesale				
Haplotype	FM-1	FM-2	FM-3	RT-1	RT-2	RT-3	WS-1	WS-2	WS-3	N	%N
Hap-1	20	20	20	—	—	—	20	20	18	118.00	65.60
Hap-2	—	—	—	4	7	9	—	—	—	20.00	11.10
Hap-3	—	—	—	3	2	1	—	—	—	6.00	3.30
Hap-4	—	—	—	6	4	1	—	—	—	11.00	6.10
Hap-5	—	—	—	2	1	5	—	—	2	10.00	5.60
Hap-6	—	—	—	3	2	1	—	—	—	6.00	3.30
Hap-7	—	—	—	1	2	1	—	—	—	7.00	3.90
Hap-8	—	—	—	1	2	2	—	—	—	6.00	3.30
Total	20	20	20	20	20	20	20	20	20	180.00	100.00%

N: number of samples.

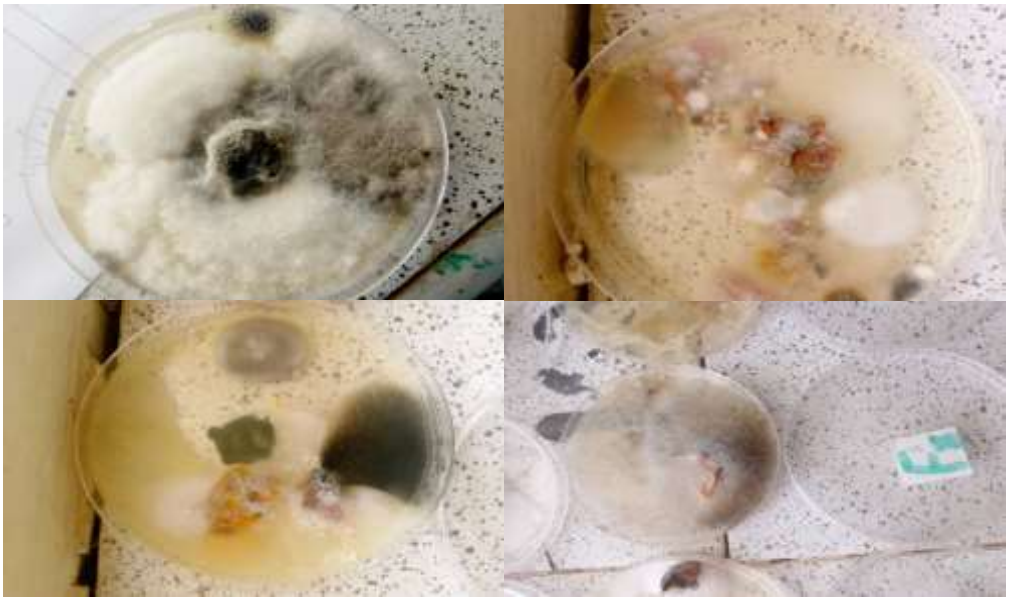

Fig. 2. Varieties of unidentified cultures of fungi.

Table 2. Haplotype sequence BLAST showing different percentage identity between fungal species obtained in the current study and those from the NCBI.

Hap. No.	Acces. No.	Organism	% I.D	Reference
Hap-1 ∞	MN310532	*Pichia kudriavzevii*	99.54%	(*Vu et al., 2016)
Hap-2 ‡	MT529928	*Aspergillus flavus*	99.80%	(*Becker 2022)
	ON819577	*Aspergillus tamari*	99.80%	(*Niu, 2022)
Hap-3 ‡	KY345406	*Mucor fragilis*	93.30%	(*Villanueva-Ibanez et al., 2016)
Hap-4 ‡	MW850413	*Russula atroglauca*	92.50%	(*Shi, 2021)
Hap-5 †	ON208264	*Aspergillus niger*	90.23%	(*Lu et al., 2022)
	MK530080	*Aspergillus welwitschiae*	90.23%	(*Sanjotha et al., 2019)
Hap-6 ‡	MT534034	*Ganoderma* sp.	99.60%	(*Singh and Kumar, 2020)
	MN856333	*Alternaria eichhorniae*	99.60%	(*Feng, 2019)
	MN599626	*Exserohilum rostratum*	99.60%	(*Chand and Korra, 2019)
	MF495402	*Colletotrichum boninense*	99.60%	(*Mejia et al., 2017)
	MK793207	*Alternaria citri*	99.60%	(*Yarahmadi et al., 2019)
	MK158222	*Alternaria brassicicola*	99.60%	(*Gill and Vasundhara, 2018)
	OK011826	*Alternaria alstroemeriae*	99.60%	(*Lei et al., 2021)
Hap-7 ‡	MK518426	*Alternaria tenuissima*	99.39%	(*Kiranmayee et al., 2019)
	MK226308	*Alternaria tomato*	99.39%	(Poudel et al., 2018)
	MH560053	*Alternaria burnsii*	99.39%	(Poudel et al., 2018)
	KX156938	*Alternaria alternata*	99.39%	(*Gowrishankari, et al., 2016)
	KU639594	*Alternaria longipes*	99.39%	(*Gowrishankari, et al., 2016)
	HM204456	*Alternaria porri*	99.39%	(*UdayaShankar et al., 2010)
	HM003687	*Alternaria arborescens*	99.39%	(*Visalakchi et al., 2010)
Hap-8 ‡	ON712254	*Cladosporium crousii*	100%	(*Nageen et al., 2023)
	MT529231	*Cladosporium ramotenellum*	100%	(*Li, 2020)
	MT378422	*Cladosporium colombiae*	100%	(*Yue, 2020)
	MT378416	*Cladosporium austroafricanum*	100%	(*Yue, 2020)
	MT367262	*Cladosporium cladosporioides*	100%	(*Shi, 2020)
	MN947589	*Cladosporium coralloides*	100%	(*Poli and Varese, 2020)
	MN886551	*Cladosporium pseudocladosporioides*	100%	(*Choi, and Lee, 2019)
	MN857898	*Cladosporium anthropophilum*	100%	(*Silva-Rojas et al., 2019)

* Direct submission; ‡ farms and wholesale; ∞ retail; † retail and wholesale.

DISCUSSION

Tomato is regarded as an essential vegetable cultivated for human consumption (Sibomana et al., 2015). In Nigeria, the tomato farming and marketing activities are largely controlled and practiced in northern part of the country (Etebu et al., 2013). Tomato farming and enterprises provides huge support and helps the locals for their daily needs as well as boosting the economy of the country. Despite these benefits, the enterprise usually suffers the great losses from the harvest to retails and wholesales units, largely caused by fungal infections. To address the problem, we analysed the scenario in Kwanar Gafan by identifying the fungal species involved throughout the units using molecular identification method. Kwanar Gafan is in Garun Malam LGA of Kano State, < 7 km away from giant tomato processing factory own by Dangote Farms Limited Kadawa. We chose the area to conduct this study as its highly recognised for large tomato production and marketing centre in the state. Recently, the local tomato farmers in the area were benefited from the World Bank grant supported to boost the tomato production through Agro-Processing Productivity Enhancement and Livelihood Improvement Support (APPEALS) Project (2018-2022).

Identified fungal species

Eight haplotypes (Hap-1 to Hap-8) obtained in current study had clearly indicated that Kwanar Gafan have a diverse fungal species responsible for infesting the tomatoes after harvesting. Phylogenetic relationship using a Maximum Likelihood between the haplotypes had confirmed the huge fungal species diversity in the current study area (Fig. 3). Only one haplotype (Hap-1) was shared between the samples obtained from farms and wholesale units. It is possible to have a common haplotype between them, since most wholesalers bought the entire ripped tomato in the farm (at point of harvest), harvest and filled the baskets directly within the farm. Based on BLAST, Hap-1 was identified as *Pichia kudriavzevii* with highest percentage similarities of 99.54% (Table 1). Application of *P. kudriavzevii* have been recently reported for retarding the fungal decay rate, delay the colour change, and weight loss of different fruits including tomato (Liu et al., 2020). Appearance of *P. kudriavzevii* alone in farms and wholesale units may likely inhibit the growth of other fungal species. The *P. kudriavzevii* could possibly be a primary fungal species that strike tomato fruits immediately after harvest in the farm and wholesale units.

Aspergillus spp., *Mucor fragilis*, *Russula atroglauca*, *Ganoderma* sp., *Alternaria* spp., *Exserohilum rostratum*, *Colletotrichum boninense*, *Naganishia* sp. and *Cladosporium* spp. were the main fungal species identified are contributed to the deterioration of tomato fruits in the retail units studied. These species were previously reported for damaging tomato fruits from different markets in Nigeria (Etebu et al., 2013; Sinno et al., 2020). The number of fungal species identified in the retail unit was higher compared to the farms and wholesale units. Figure 4 described the general factors responsible for having more fungal species in retail section. Fully ripe tomatoes are commonly found in retail points which are more susceptible to be infected by fungal species. Also, tomatoes stay longer during postharvest period in retail units compare to farms and wholesale units. Therefore, tendencies of more fungal effect would be higher in retail units. For wholesale purpose sometimes, tomatoes were harvested partially ripped to decrease fungal activities as the fruit may be transported for more than 700 km without cooling facilities aid. In addition, fungal activities at farms may be reduced due to a residual effect of pesticides used during the production period. Tomatoes are packaged in separate baskets in both farms and wholesale points. Unlike retail points where multiple fruits were mixed which may result to the rapid transfer of fungal species from other fruits (Fig. 5).

Continuous usage of traditional weaved baskets and unclean surfaces where tomatoes are kept in retail units may also promote the infestation of tomato fruits (Etebu et al., 2013; Kutama et al., 2007). For decades, it has been reported that the microbes responsible for tomato spoilage originates or initiated from the contaminated baskets used for storing tomatoes (Snowdon, 1991). Lack of cooling conditions in most tomato retailing units favours the growth and development of fungal species especially during the period of hot season. Few years ago, a study was conducted in Kura Kano state to investigate the effects of packaging and storage conditions on storage life and quality of tomatoes (Dandago et al., 2017). The findings revealed that storing tomato in cool place (not freezing) and packaging in kraft paper bag was a better combination for maintaining the fruit quality and extending the storage period. However, most retailers could not maintain such conditions as it requires energy and extra storage facilities.

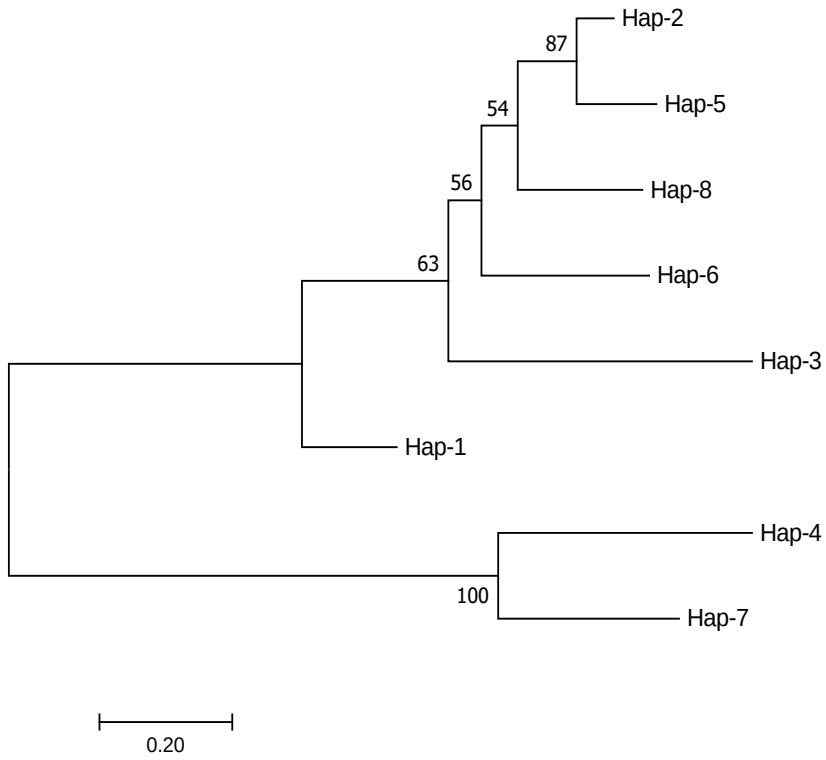

Fig. 3. Molecular Phylogenetic analysis inferred by Maximum Likelihood method based on the Kimura 2-parameter model of eight fungal haplotypes discovered from infested tomatoes collected in Kwanar Gafan.

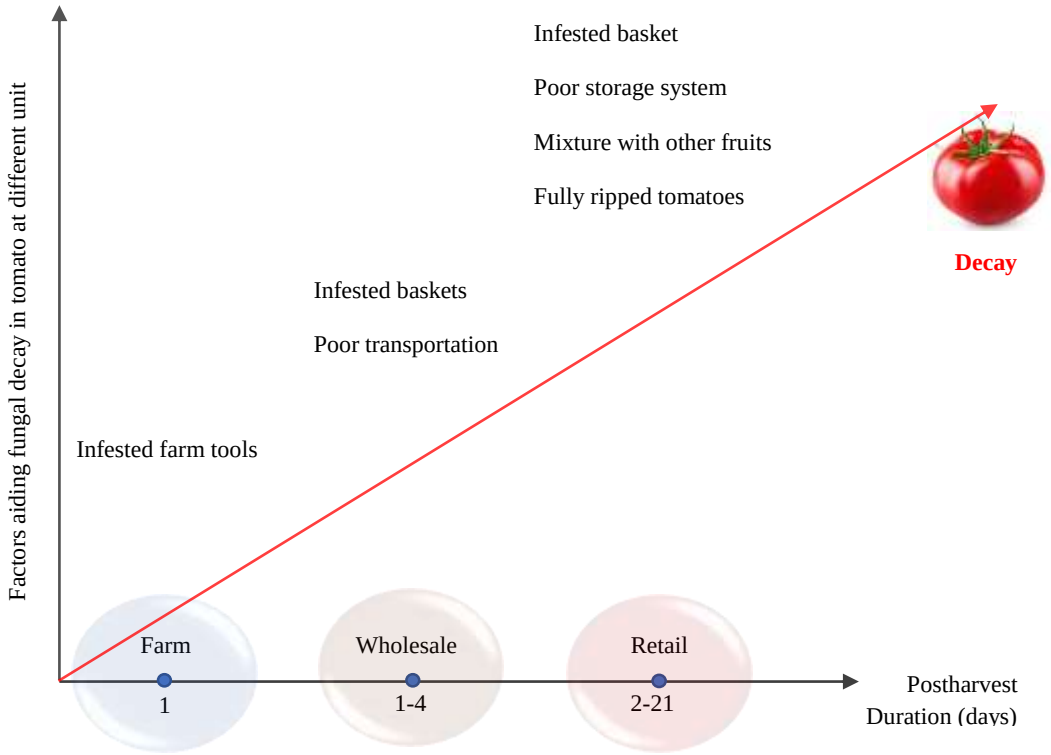

Fig. 4. Increased fungal decay of tomato in farm, wholesale, and retail with an increase of postharvest duration and factors aiding the fungal species.

Fig. 5. Possibility of fungal transfer in a retail unit where several vegetables are mixed together A, tomatoes; B, cucumber; C, lemon; D, cabbage; E, okra; F, pepper; G, hot pepper; H, onion.

CONCLUSION

Molecular identification was successfully used in identifying the fungal species collected from farms, wholesale, and retail units. In current study, *Pichia kudriavzevii* was a major fungal species identified in tomato samples collected from both farms and wholesale units. In retail units however, eight fungal genera were identified from retail unit collected. Large number of fungal species identified in retail units was attributed to several factors which includes long duration of tomatoes in retail market and poor storage conditions.

Conflict of interest

The authors declare that there is no conflict of interest.

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