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DNA sequence data confirms the presence of two closely related cypress-feeding aphid species on African cypress (*Widdringtonia* spp.) in South Africa

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Aphids in the genus *Cinara* (Hemiptera: Aphididae) are pests of coniferous trees globally. Some of these aphids have become invasive in various parts of the world and have led to significant economic and environmental damage. During surveys conducted as part of a sentinel plant project, severe aphid infestations were observed on *Widdringtonia* trees in the Kirstenbosch National Botanical Garden, Cape Town, South Africa. In addition, planted *Widdringtonia wallichii* Endl. ex Carrière trees within their natural range of the Cederberg Wilderness Area, Western Cape, South Africa were found infested with aphids. In this study, we investigated the species identity of the aphids using DNA sequence data for the mitochondrial cytochrome c oxidase (COI) gene. The results revealed the presence of two closely related aphid species, the cypress aphid, *Cinara cupressi* (Buckton) and the cypress pine aphid, *Cinara tujafilina* (Del Guercio) infesting *Widdringtonia* spp. in South Africa. Both aphid species are alien to South Africa. While this is not the first report for either species in the country, the current study provides evidence of impact, with severe infestations leading to branch dieback and tree death. This finding supports the regulation of *Cinara* spp. in South Africa and highlights that management is urgently needed.

Keywords: botanical gardens, *Cinara cupressi*, *Cinara tujafilina*, conifers, cypress aphid, sentinel plants

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Introduction

The genus *Cinara* (Hemiptera: Aphididae) includes approximately 200 species, making it one of the largest aphid genera (Blackman and Eastop 2020). These insects feed on conifers in the Cupressaceae and Pinaceae (Eastop 1972), with the genus containing a number of important pests of commercial and ornamental trees (van Rensburg 1979; Watson et al. 1999). Morphological similarities between *Cinara* spp. have occasionally led to identification challenges (Foottit and Mackauer 1990; Watson et al. 1999; Favret and Voegtlin 2004). Phylogenetic studies using the mitochondrial cytochrome c oxidase (COI) gene have assisted in identifying closely related species (El Mutjar et al. 2009; Akyildirim Begen and Gorur 2019).

The cypress aphid, *Cinara cupressi* (Buckton), has emerged as a particularly important pest of Cupressaceae in invaded regions, exemplified by its inclusion in the Global Invasive Species Database (2023) list of 100 of the world's worst invasive alien species (Lowe et al. 2000). It is currently accepted that *C. cupressi* represents a species complex, with the CABI Invasive Species Compendium (2019) considering them all under *C. cupressi 'sensu lato'*.

In Africa, *C. cupressi* has caused significant economic losses and environmental damage in east, central and southern African countries (Chilima 1991; Ciesla 1991; Missanjo and Kamanga-Thole 2015; Demeke 2020). It was first reported in 1986 in Malawi (Ciesla 1991) and subsequently spread to several countries on the continent including Burundi, the Democratic Republic of Congo, Ethiopia, Kenya, Libya, Morocco, Rwanda, Tanzania, South Africa, Uganda and Zimbabwe (Chilima 1991; Chilima 1995; Missanjo and Kamanga-Thole 2015; Demeke 2020; Kebede and Mulugeta 2021).

Millar's (1994) catalogue of aphids of sub-Saharan Africa includes South Africa in the distribution of *C. cupressi*, however, the exact date of its establishment in this country is unknown. Considering its high impact in other invaded regions, this aphid has been listed as a category 1b species (invasive species that must be controlled) in the South African National Environmental Management: Biodiversity Act (NEM:BA, Act 10 of 2004) Alien and Invasive Species Regulations (NEM:BA A&IS Regulations; Department of Environment, Forestry and Fisheries 2020a, b). The congeneric and morphologically

similar species *C. tujafilina* has also been reported in South Africa on *Callitris*, *Chamaecyparis*, *Cupressus*, *Platycladus*, *Thuja* and *Widdringtonia,* with the first report of its presence in the country in 1914 (as *Lachniella thujafolia*) (Millar 1990).

Severe aphid infestations were observed on *Widdringtonia* spp. in the Kirstenbosch National Botanical Gardens (Kirstenbosch NBG), Cape Town, Western Cape, South Africa during plant health surveys conducted in 2019 and 2020. These surveys were undertaken as part of the Sentinel Plant Project (https://www.fabinet.up.ac.za/index.php/sentinel-plant-network) funded by the South African National Biodiversity Institute (SANBI), which uses plant collections in botanical gardens to identify new and emerging pest risks. Additional samples were collected from planted stands of the Cape cedar *Widdringtonia wallichii,* within its natural range of the Cederberg Wilderness Area, Western Cape, South Africa.

The aim of this study was to describe the damage caused by *Cinara* spp. and to confirm the species identity of South African collections using DNA sequence data. The South African collections were also compared with specimens collected in other African countries.

Materials and methods

Aphid collection

Aphid samples were collected from *W. nodiflora*, *W. schwarzii*, *W. wallichii* (formerly *W. cedarbergensis*) and *W. whytei* saplings and trees in the Kirstenbosch NBG nursery and managed estate in 2019 and 2020. In addition, samples were collected from planted *W. wallichii* trees in the De Rif plantation, Cederberg Wilderness Area, Western Cape, South Africa in 2021 (Table 1). For comparative purposes, aphid samples were collected from Kenya and Malawi in 2019, countries where severe outbreaks of the cypress aphid, *C. cupressi*, occurred on *Cupressus lusitanica* and *Widdringtonia* spp. in the 1980s and 1990s, respectively (Chilima 1991). In Kenya, collections were made from *Cupressus lusitanica* in 12 sites across Gatundu, Kikuyu, Kinangop and Lari sub-counties. Samples from Malawi were collected from *C. lusitanica* and *W. whytei* at nine sites across the Mzimba, Ntcheu and Zomba districts (Table 1). Aphids collected from different trees were kept as separate samples. All the samples were preserved in absolute ethanol and stored frozen at –20 °C until use.

Aphid identification

DNA extraction, amplification and sequencing

Multiple specimens were randomly selected and sequenced from each tree-based sample from South Africa, whereas a single randomly selected aphid specimen was sequenced from each tree-based sample from Kenya and Malawi. DNA was extracted from the thorax of randomly selected aphids representing the various tree-based samples. The preserved insects were rinsed with sterile distilled water to remove the ethanol and total genomic DNA was extracted using prepGEM™ Insect DNA extraction kit (ZyGEM) following the manufacturer's protocol (MicroGEM, West Sussex, UK). The barcoding region of the mitochondrial cytochrome c oxidase I (COI) gene was amplified using the universal primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: '5-TAAACTTCAGGGTGACCAAAAAATCA-3' (Simon et al. 1994).

Amplification reactions were performed in a 25 µL reaction solution. The solution was made up of 16.2 µL ultrapure water, 3 µL concentrated (10x) PCR reaction buffer mixed with 20 mM $MgCl_2$ (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 µL dNTP mix (10 mM; 2.5 mM each), 1 µL of each primer (10 mM) (White-Sci), 0.3 µL of FastStart Taq DNA polymerase (5 U μL^{-1}) (Roche Diagnostics GmbH, Mannheim, Germany) and 1 µL of cleaned insect genomic DNA (100 ng μL^{-1}). The thermocycling reactions were run at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 sec, 47 °C for 1 min and 72 °C for 30 sec and final extension at 72 °C for 10 min in a Bio-Rad iCycler thermocycler (BIO-RAD, Hercules, CA, USA). The amplicons were cleaned using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The cleaned products were sequenced in the forward and reverse directions at the Sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria using an ABI PRISM™ 3100 DNA Analyser (Thermo Fisher Scientific, Waltham, MA, USA).

Phylogenetic analyses

The sequence data were edited and consensus sequences of the forward and reverse sequences were generated using Biological Sequence Alignment Editor (BioEdit) software (Hall 1999) version 7.0.9. A search was conducted in BLASTn on the online NCBI database (https://www.ncbi.nlm.nih.gov/) using the consensus sequences obtained from the current study. Sequences of closely related species were downloaded from GenBank and included in the phylogenetic analysis (Table 2). A sequence of the Asian Woolly Hackberry Aphid, *Shivaphis celti* (MH820857) was included as an outgroup. Only representative sequences were included in the phylogenetic analysis to keep the phylogenetic tree concise. All the sequences were then aligned using the online Multiple Sequence Alignment Program (MAFFT) version 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013). The MAFFT-aligned sequences were further edited and trimmed in BioEdit. Maximum likelihood (ML) analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Tamura et al. 2021) with the default Tamura-Nei substitution model, uniform rates among sites and 1 000 bootstraps.

Results

Browning of the needles was obvious on infested *Widdringtonia* trees and saplings at Kirstenbosch NBG and the De Rif plantation (Figure 1a). In severe cases, branch dieback and whole tree death was observed in Kirstenbosch NBG (Figure 1b and c). Close inspection of the trees revealed the presence of well-camouflaged aphids (Figure 1d and e). Sooty mould had developed on some of the severely affected saplings in the nursery (Figure 1F).

Aphid samples were collected from a total of 40 trees (Kenya = 19, Malawi = 9 and South Africa = 12), each tree-based sample containing multiple aphid specimens (Table 1). A total of 68 specimens (Kenya = 19, Malawi = 9 and South Africa = 40) were sequenced. Sequences of 526 bp were generated from the amplicons of the representative

Table 1: Details of aphid sampling sites across three African countries, tree species sampled, and number of insects sequenced

Collection year	Sampling site (locality)	GPS coordinates		Host tree species	No. trees sampled	Number of insects sequenced
		Lat (S)	Long (E)			
			Kenya			
2019	Fly over	0.86416	36.58333	*Cupressus lusitanica*	1	1
	Gitiha	1.06027	36.67777	*Cupressus lusitanica*	2	2
	Kamae	0.84805	36.62694	*Cupressus lusitanica*	2	2
	Kari Muguga	1.38666	36.63555	*Cupressus lusitanica*	3	3
	Kereita	0.97750	36.64194	*Cupressus lusitanica*	3	3
	Kieni	0.85388	36.67583	*Cupressus lusitanica*	1	1
	Kinale	0.92500	36.60583	*Cupressus lusitanica*	1	1
	Kwa Haraka	0.75222	36.60833	*Cupressus lusitanica*	1	1
	Magumu	0.85638	36.56333	*Cupressus lusitanica*	1	1
	Munyaka	0.68250	36.61750	*Cupressus lusitanica*	1	1
	Mwendando	0.80777	36.57750	*Cupressus lusitanica*	1	1
	Njabini	0.71444	36.64888	*Cupressus lusitanica*	1	1
	Uplands	1.05666	36.66055	*Cupressus lusitanica*	1	1
			Malawi			
2019	Ntcheu	14.82015	34.63924	*Cupressus lusitanica*	2	2
	Luwawa	12.10542	33.71359	*Widdringtonia whytei*	2	2
	Luwawa	12.11256	33.71916	*Cupressus lusitanica*	3	3
	Zomba	15.37780	35.32150	*Cupressus lusitanica*	2	2
			South Africa			
2019	Kirstenbosch NBG	33.98969	18.43065	*Widdringtonia nodiflora*	1	5
	Kirstenbosch NBG	33.98969	18.43065	*Widdringtonia wallichii*	1	5
2020	Kirstenbosch NBG	33.98561	18.43618	*Widdringtonia nodiflora*	4	12
	Kirstenbosch NBG	33.98561	18.43618	*Widdringtonia schwarzii*	1	5
	Kirstenbosch NBG	33.98561	18.43618	*Widdringtonia wallichii*	3	7
	Kirstenbosch NBG	33.98561	18.43618	*Widdringtonia whytei*	1	2
2021	Cederberg Wilderness Area	32.43909	19.23316	*Widdringtonia wallichii*	1	4
Total					40	68

NBG = National Botanical Garden

specimens. Thirty-seven of these sequences, chosen to represent the intraspecific genetic variation as well as different host species and countries, were included in the analysis, together with 16 reference sequences and one outgroup from the GenBank (Table 1, Table 2).

Sequences of specimens from South Africa grouped in two distinct clades, each supported by strong bootstrap values (Figure 2). Twenty-seven of the 40 sequences were identical and grouped with reference sequences of the cypress pine aphid *C. tujafilina* (Del Guercio) (MH821712, MH821713 and MH821714). The remaining thirteen sequences grouped with reference sequences of the cypress aphid *C. cupressi* (KR033001, HQ970762 and MN178367), of which eleven had identical sequences and were closest to the Lithuanian reference sequence (MN178367), while the other two sequences grouped with the Canadian and USA reference sequences (KR033001 and HQ970762).

C. cupressi was found on *W. nodiflora*, *W. schwarzii, W. wallichii* and *W. whytei*, while *C. tujafilina* was detected only on the first three *Widdringtonia* spp. (Table 2). In South Africa, both aphid species were found to be widespread in Kirstenbosch NBG, whereas in the Cederberg Wilderness Area, only *C. tujafilina* was found on planted *W. wallichii* trees (Table 2). Sequences of specimens from Kenya and Malawi, which were included in the study for comparative purposes, all grouped with *C. cupressi* sequences from the USA and Canada.

Discussion

This study confirmed the presence of two closely related aphid species, namely *C. cupressi* and *C. tujafilina,* on *Widdringtonia* spp. in South Africa. Both species had previously been reported in the country (Millar 1990; Millar 1994), however, our study has confirmed their presence using DNA sequence data. This study also confirms the presence of *C. cupressi* on *W. schwarzii, W. wallichii* and *W. whytei* in South Africa for the first time, as well as providing the first report of *C. tujafilina* on *W. schwarzii*. To the best of our knowledge, this is also the first report of *Cinara* infesting *W. wallichii* in its natural range.

Economic and environmental damage resulting from *C. cupressi* invasions in other African countries has been well documented. This includes impacts on commercial plantations of *Cupressus lusitanica*, an important exotic agroforestry species in the region, as well as indigenous Cupressaceae. For example, within four years of its arrival in Malawi, the pest caused over US$2.4 million in losses on the standing crop of cypress and cedar, with a further loss in growth increment of US$1 million (Chilima 1991). Substantial losses to *C. lusitanica* plantations were also reported from

Table 2: GenBank accession numbers for aphid specimens used in the phylogenetic analyses

Species	Specimens	Host	Locality	GenBank accession number	Reference
Cinara cupressi	CNC#HEM033310		Canada	KR033001	Gwiazdowski et al. (2015)
	CNC#HEM069870		USA	HQ970762	iBOL
	Isolate 563		Lithuania	MN178367	Havelka et al. (2020)
	1383	*Cupressus lusitanica*	Kenya	PP275813	This study
	1382	*C. lusitanica*	Kenya	PP275814	This study
	1393	*C. lusitanica*	Kenya	PP275815	This study
	1082	*Widdringtonia nodiflora*	Kirstenbosch NBG	PP275816	This study
	1380	*C. lusitanica*	Kenya	PP275817	This study
	1389	*C. lusitanica*	Kenya	PP275818	This study
	1386	*C. lusitanica*	Kenya	PP275819	This study
	1396	*C. lustanica*	Kenya	PP275820	This study
	1400	*W. whytei*	Malawi	PP275821	This study
	1432	*W. whytei*	Kirstenbosch NBG	PP275822	This study
	1404	*C. lusitanica*	Malawi	PP275823	This study
	1390	*C. lusitanica*	Kenya	PP275824	This study
	1402	*C. lusitanica*	Malawi	PP275825	This study
	1084	*W. nodiflora*	Kirstenbosch NBG	PP275826	This study
	1086	*W. nodiflora*	Kirstenbosch NBG	PP275827	This study
	1407	*W. wallichii*	Kirstenbosch NBG	PP275828	This study
	1408	*W. wallichii*	Kirstenbosch NBG	PP275829	This study
	1430	*W. schwarzii*	Kirstenbosch NBG	PP275830	This study
	1409	*W. wallichii*	Kirstenbosch NBG	PP275831	This study
	1083	*W. nodiflora*	Kirstenbosch NBG	PP275832	This study
	1431	*W. schwarzii*	Kirstenbosch NBG	PP275833	This study
Cinara tujafilina	HLshujia526		China	MH821712	Li et al. (2020)
	HLshujia620		China	MH821713	Li et al. (2020)
	HLshujia64		China	MH821714	Li et al. (2020)
	1087	*W. wallichii*	Kirstenbosch NBG	PP275834	This study
	1410	*W. wallichii*	Kirstenbosch NBG	PP275835	This study
	1415	*W. nodiflora*	Kirstenbosch NBG	PP275836	This study
	1435	*W. wallichii*	Kirstenbosch NBG	PP275837	This study
	1438	*W. wallichii*	Cederberg Wilderness Area	PP275838	This study
	1090	*W. wallichii*	Kirstenbosch NBG	PP275839	This study
	1426	*W. schwarzii*	Kirstenbosch NBG	PP275840	This study
	1413	*W. wallichii*	Kirstenbosch NBG	PP275841	This study
	1423	*W. nodiflora*	Kirstenbosch NBG	PP275842	This study
	1427	*W. schwarzii*	Kirstenbosch NBG	PP275843	This study
	1437	*W. wallichii*	Cederberg Wilderness Area	PP275844	This study
	1417	*W. nodiflora*	Kirstenbosch NBG	PP275845	This study
	1088	*W. wallichii*	Kirstenbosch NBG	PP275846	This study
	1089	*W. wallichii*	Kirstenbosch NBG	PP275847	This study
	1436	*W. wallichii*	Cederberg Wilderness Area	PP275848	This study
	1091	*W. wallichii*	Kirstenbosch NBG	PP275849	This study
Cinara confinis	2800	*Abies cephalonica*		KF649385	Jousselin et al. (2013)
Cinara confinis				KR029876	GenBank
Cinara confinis	INRA CBGPACOE2466			KF639317	Coeur d'Acier et al. (2014)
Cinara fresai	2983			KF649480	GenBank
Cinara fresai	OAI415		Australia	MF462151	GenBank
Cinara piceae	ZMIOZ25387		China	JQ916799	Chen et al. (2012)
Cinara piceae	ZMIOZ25103		China	JQ916795	Chen et al. (2012)
Cinara juniperi	Isolate 755			MN178388	Havelka et al. (2020)
Cinara juniperi	Isolate 757			MN178390	Havelka et al. (2020)
Cinara juniperi	Isolate 756			MN178389	Havelka et al. (2020)
Shivaphis celti	HLshujia802		China	MH820857	Li et al. (2020)

iBOL = International Barcode of Life NBG = National Botanical Garden

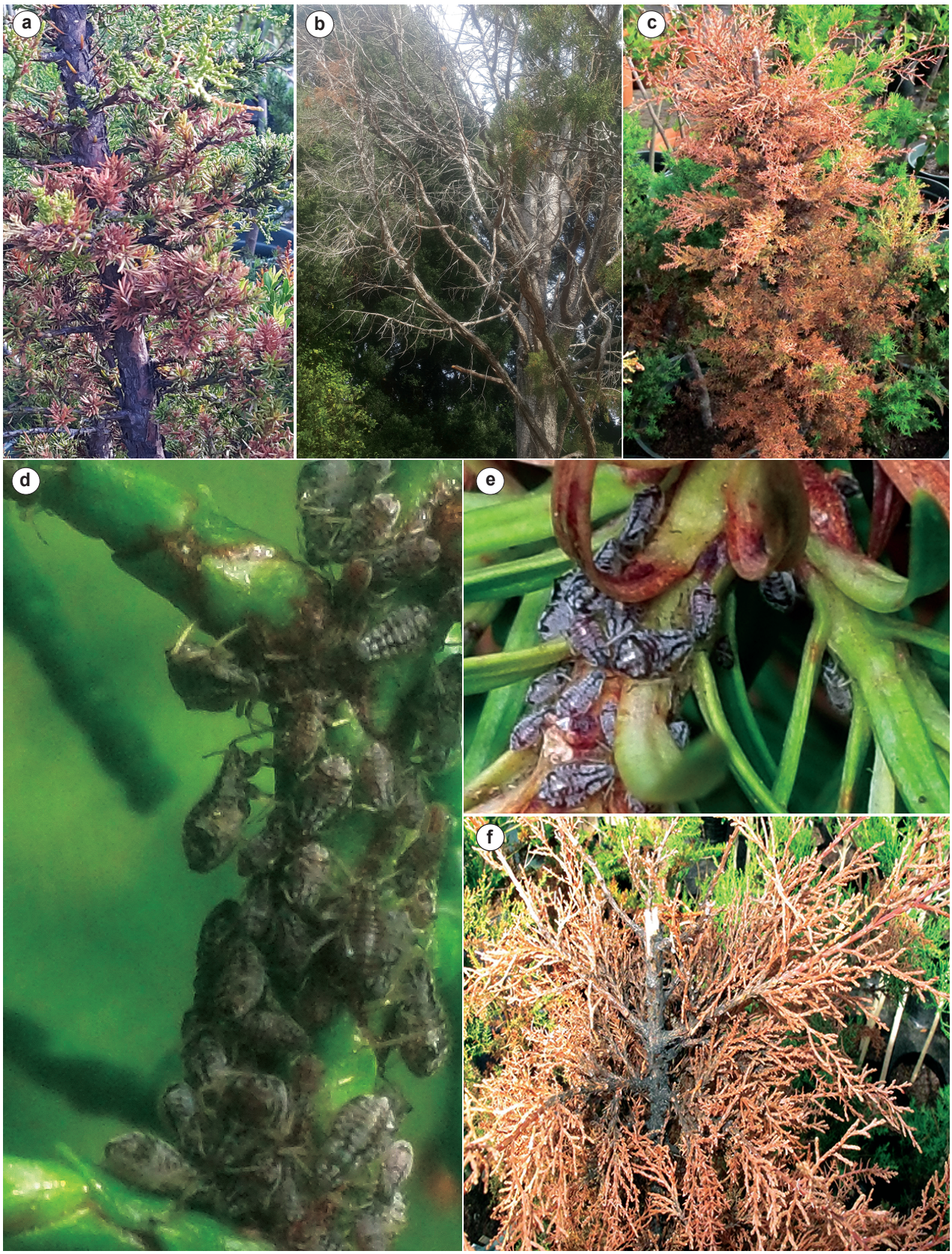

Figure 1: *Cinara cupressi* and *C. tujafilina* on *Widdringtonia* spp. in Kirstenbosch NBG and the damage they caused: (a) browning of foliage on *W. wallichii* sapling; (b) branch dieback on a *W. wallichii* tree; (c) *W. wallichii* sapling killed by severe aphid infestation; (d-e) camouflaged aphids in the canopy of *W. nodiflora* and *W. wallichii*; and (f) sooty mould developed on honeydew secreted by aphids on a *W. wallichii* sapling

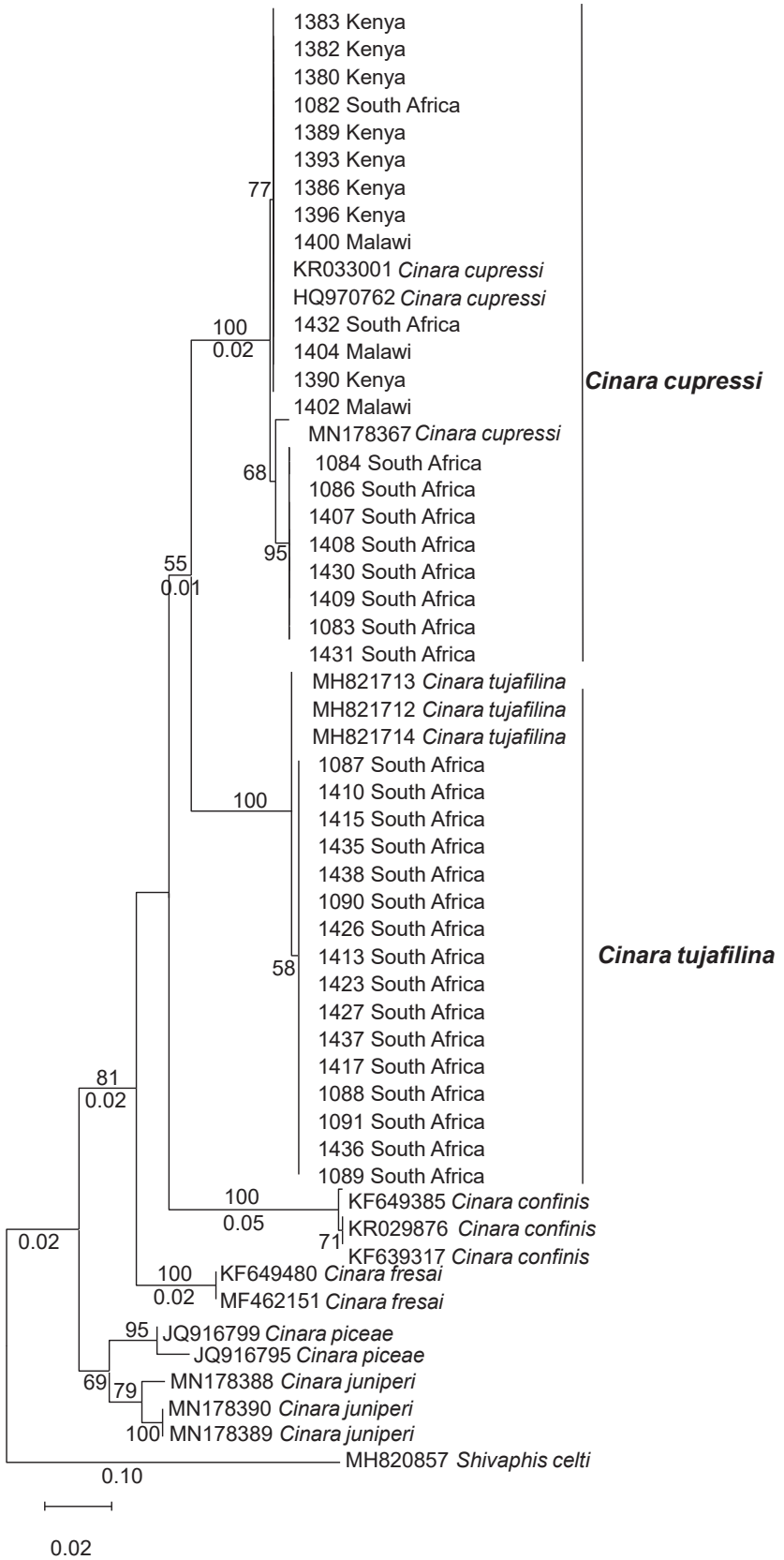

Figure 2: A phylogenetic tree based on maximum likelihood analysis of 526 bp sequences of the barcoding region of the COI gene for aphid specimens collected from Kenya, Malawi and South Africa. A sequence of the Asian Wooly Hackberry Aphid (*Shivaphis celti*) was used as an outgroup. The numbers above and below the branches indicate bootstrap value and branch length, respectively

Kenya, and in Ethiopia *C. cupressi* caused over US$10 million worth of damage between 2003 and 2005 (Orondo and Day 1994; FAO 2011). Negative impacts have also been reported for indigenous *Juniperus procera* in Kenya and *W. whytei* in Malawi (Chilima 1991; Ciesla 1991). According to the IUCN Environmental Impact Classification for Alien Taxa (EICAT), the impact of *C. cupressi* is classified as Moderate (MO; causing a decline in the population of a taxon) (Hawkins et al. 2015). Unlike the situation in other parts of Africa, very little is known regarding the ecology and impact of *C. cupressi* in South Africa. However, the damage by *C. cupressi* to *Widdringtonia* spp. observed in Kirstenbosch NBG (with individual trees exhibiting dieback and death) presents evidence for Moderate impact.

A risk analysis of *C. cupressi* s.l. for South Africa supports the category 1b NEM:BA A&IS listing, based on its high risk and challenges to management (SANBI, unpublished; Appendix S1). Further studies should be undertaken to better understand the threat posed by *C. cupressi*, particularly to South African *Widdringtonia.* There is also an urgent need for management options to be explored and implemented.

C. tujafilina is a cosmopolitan species, with its presence previously confirmed in east and southern Africa, including in Kenya, Malawi and South Africa (Millar 1990; Millar 1994; Schabel 2006). This aphid is considered a pest of minor importance in some states of the USA and parts of South America (El Mutjar et al. 2009; Mech et al. 2019), but little is known regarding its importance in Africa. An interesting outcome of this study was that *C. tujafilina* was not found amongst the specimens from Kenya and Malawi included for comparative purposes. But our samples from those countries were very limited in number and future sampling and DNA-base identifications could show that it is present there.

C. cupressi and possibly *C. tujafilina* could pose a threat to *Widdringtonia* spp. in South Africa, considering the economic and environmental impact of the *C. cupressi* invasion elsewhere in the world. This is particularly relevant for *W. wallichii,* a species confined to a small area in the Cederberg mountains and currently listed as Critically Endangered (Farjon et al. 2013). While not endemic to South Africa, *W. whytei* has a similarly restricted range, occurring only on Mount Mulanje in Malawi. This species is also listed as Critically Endangered, with *C. cupressi* recognised as a threat to its survival (Chanyenga et al. 2019).

Conclusions

Identification of the two closely related aphid species and their host species in the current study forms an important foundation in the development of sustainable and effective management strategies. It also illustrates the value of monitoring pests at sentinel sites such as botanical gardens for the detection of closely related pest species and novel host-pest associations as highlighted by Wondafrash et al. (2021).

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