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DNA barcoding and elucidation of cryptic aphid species (Hemiptera: Aphididae) in India

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Abstract

Rapid, precise and timely identification of invasive pest insects such as aphids is important and a challenge worldwide due to their complex life cycles, parthenogenetic reproduction, sex and colour morphs. In this respect, DNA barcoding employing a 658 bp fragment of 5′ region of the mitochondrial cytochrome oxidase I (CO-I) gene is an effective tool in addressing the above. In the present study, we employed CO-I for discriminating 142 individuals representing 32 species of aphids from India. Sequence analyses revealed that the intraspecific and interspecific distances ranged from zero to 3.8% and 2.31 to 18.9%, respectively. In addition, the study also showed for the first time the prevalence of three cryptic species, namely *Brevicoryne brassicae* (Linnaeus), *Hyperomyzus carduellinus* (Theobald) and *Brachycaudus helichrysi* (Kaltenbach) from India. Our work has clearly demonstrated that DNA barcoding is an efficient and accurate method for identification of aphid species (including cryptic species), an approach that potentially could play an important role in formulating viable pest management strategies, more especially biocontrol.

Keywords: aphid, biocontol, CO-I, cryptic species, cytochrome oxidase, DNA barcoding, invasives

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Introduction

Among the many challenges in sustaining crop productivity and nutritional security, direct and indirect damages by insect pests is of paramount importance. Pests such as aphids and thrips pose the dual problem of direct physical damage to crop plants as well as vectoring of many plant pathogenic viruses (Blackman & Eastop, 2000; Mound, 2005). Management of plant pathogens vectored by insect pests is all the more complex because of the factors influencing the epidemiology of these diseases. Among the many plant viruses transmitted by insects, aphid-transmitted viruses are the most numerous and predominant worldwide (Blackman & Eastop, 2000). Aphids (Hemiptera: Aphididae), with a recorded diversity of about 5000 species, are small, soft-bodied insects with sucking mouth parts that feed mainly on phloem and are considered as economically-important, often invasive pests throughout the world (van Emden & Harrington, 2007; Foottit *et al.*, 2008). In light of this, a quick, accurate and timely

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identification of aphids is important for their management. However, the evolutionary tendency towards the loss of taxonomically useful characters and phenotypic plasticity due to host and environmental factors make their identification difficult (Foottit *et al.*, 2008). In addition, the presence of unusual morphological forms of species on different host plants under various climatic conditions, complex life cycles, colour polymorphisms and a cyclically parthenogenetic mode of reproduction in the majority of species, often involving an alternation of hosts between a winter primary host and spring-summer secondary host/s (Dixon, 1998), add to the difficulty of precise identification. Furthermore, morphological examination of aphids to species is usually restricted to certain life stages or asexual forms, since there are generally no reliable keys for the identification of the immature stages (Henderson *et al.*, 1976) or for that matter, the sexual morphs themselves, and which may prove difficult for a non-expert to use.

Considering all these factors, it is even so necessary to detect invasive quarantine pest species introduced into particular countries along with agricultural or horticultural products at the port of entry, where speed and accuracy of identification are paramount (Glover *et al.*, 2010). In this regard, Hebert *et al.* (2003*a*, *b*) proposed the concept of DNA barcoding as a rapid and precise way of species discrimination of a broad range of biological specimens using a selected 658 bp fragment of the 5′ end of the mitochondrial cytochrome oxidase-1 (CO-I) gene. DNA barcoding can be employed as a useful approach for molecular identification of species in their various life stages and forms (Foottit *et al.*, 2010), host-associated genetic differences (Brunner *et al.*, 2004), discrimination of cryptic species (Smith *et al.*, 2006), as well as biotypes (Eastop, 1973; Shufran *et al.*, 2000). Potentially, DNA barcoding could be easily incorporated into pest management programmes involving pest complexes – such as in the case of the apple aphid, *Aphis pomi* (De Geer) and small raspberry aphid, *Aphis spiraecola* Patch, where both selection and timing of the management practices can be affected by the insect's polymorphism and overwintering host adaptation (Lowery *et al.*, 2006; Footit *et al.*, 2010).

The purpose of the present study was to discriminate 142 individual aphids representing 32 species collected on various host plants in South India using CO-I barcoding and to record the presence of cryptic species and host-associated genetic forms among these taxa, if any.

Materials and methods

Taxon sampling

Taxon assignments were performed according to the Current World Catalogue of Aphids (Remaudiere & Remaudiere, 1997). Specimens were collected in 95% ethanol during 2008–2012 and kept at −80°C until DNA testing. Prior to molecular work, aphid species were identified morphologically by Dr. Sunil Joshi of the National Bureau of Agriculturally Important Insects (NBAII), Bangalore, India. The complete data set, including 142 individual specimens representing 32 species of aphids, is listed in table 1. In order to understand and document intraspecific variations in the barcoding region of each species (Meyer & Paulay, 2005), we analysed all the sequences for aphids available from NCBI-GenBank. Specimen details and sequences are available in BOLD (www.barcodingoflife.org, 'Barcoding of aphids in Karnataka', project) and also in NCBI-GenBank.

DNA extraction and Polymerase Chain Reaction (PCR)

Total genomic DNA was extracted from individual aphids using a non-destructive method (Rowley *et al.*, 2007), while at the same time voucher specimens were mounted on glass slides and deposited with the National Pusa Collection (NPC), Indian Agricultural Research Institute (IARI) Delhi. Depending on the concentration, the DNA samples were diluted with sterile distilled water in order to obtain a working solution of 20–25 ng μl^{-1} purified DNA. A portion of the total DNA was preserved in glycerol (10%) at −80°C for future reference. Standard protocols were followed for PCR, cloning, sequencing of the CO-I region, and sequence alignment (Toda & Komazaki, 2002; Hajibabaei *et al.*, 2006).

PCR was performed in a thermal cycler (ABI-Applied Biosystems, Veriti, USA) using the following cycling parameters; an initial denaturation step at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, an annealing step at 47°C for 45 s, an extension step at 72°C for 45 s and a final extension step at 72°C for 20 min using the universal CO-I primers: LCO-1490; 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and HCO-2198; 5′- TAA ACT TCA GGG TGA CCA AAA AAT CA-3′ (Hebert *et al.*, 2003*a*, *b*). The total reaction volume of 25 µl contained ∼20 picomoles of each primer, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5 units of Taq DNA polymerase (Fermentas Life Sciences, UK). The amplified products were resolved on 1.0% agarose gel, stained with ethidium bromide (10 µg ml^{-1}) and visualized in a gel documentation system (UVP).

Sequencing and sequence analysis

The amplified products were eluted using a gel extraction kit (Nucleospin® Extract II, Macherey Nagel, Germany) according to the manufacturer's protocol, whilst the eluted products were ligated into general purpose cloning vector, InsT/A clone (Fermentas Life Sciences, UK), again according to the manufacturer's protocol. Blue–white selection was carried out and plasmids were isolated using GenJET™ plasmid MiniPrep kit (Fermentas Life Sciences, UK), according to the manufacturer's protocol from the overnight culture of positive clones cultured in LB broth. Sequencing was performed in triplicates of the above clones in an automated sequencer (ABI prism® 3730 XL DNA Analyzer; Applied Biosystems, USA) using M13 universal primers, both in the forward and reverse directions. A homology search was done using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/) and sequence alignment was performed using BioEdit version 7.0.9.0 (Hall, 1999). All the sequences generated were deposited in NCBI-GenBank (Supplementary material 1) and also accessible in BOLD.

CO-I sequences were aligned using the Clustal W program in BioEdit.7.0. The sequences were further analysed using MEGA.5.0 (Kumar *et al.*, 1993) to obtain conspecific and congeneric distances, while Neighbour-Joining (NJ) trees were constructed using the Kimura-2-parameter (K2P) distance model (Kimura, 1980; Saitou & Nei, 1987).

Results

Data analysis

The CO-I from all the 32 aphid species (table 1) were successfully sequenced, further analyses revealing that

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Table 1. Analysed samples of Aphid species with description of the sampling locations, GenBank accession numbers, name, date and voucher specimen details.

Sl no.	Location	Locality	Accession Number	Name of Aphid species	Host plant	Date of collection	Specimen voucher
1	Karnataka	Hebbal	HQ112196	*Astegopteryx bambusae*	*Bambusa tulda*	November-2010	ORP-2010 – 61
2		Bangalore	JX051408	*A. bambusae*	*B. tulda*	January-2012	KBRIIHR-172
3		Hessaraghatta	JX051385	*A. bambusae*	*B. tulda*	January-2012	KBRIIHR-149
4		Jigani	JX051384	*A. bambusae*	*B. tulda*	January-2012	KBRIIHR-148
5		Bannarghatta	JX051383	*A. bambusae*	*B. tulda*	January-2012	KBRIIHR-147
6		Cubbon park	JX051382	*A. bambusae*	*B. tulda*	January-2012	KBRIIHR-146
7	Karnataka	Kolar	JX051386	*Brevicoryne brassicae*	*Raphanus sativus*	January-2012	KBRIIHR-150
8		Hessaraghatta	JX051387	*B. brassicae*	*R. sativus*	January-2012	KBRIIHR-151
9		IIHR	JX051403	*B. brassicae*	*R. sativus*	January-2012	KBRIIHR-167
10		Doddaballapur	HQ112183	*B. brassicae*	*R. sativus*	November-2010	ORP-2010 – 48
11	Karnataka	Mandya	JX051388	*Melanaphis sacchari*	*Sorghum bicolor*	January-2012	KBRIIHR-152
12		Maddur	JX051389	*M. sacchari*	*S. bicolor*	January-2012	KBRIIHR-153
13		Bijapur	JX051390	*M. sacchari*	*S. bicolor*	January-2012	KBRIIHR-154
14		Maddur	JX051402	*M. sacchari*	*S. bicolor*	January-2012	KBRIIHR-166
15		Devihosur	HQ112185	*M. sacchari*	*S. bicolor*	November-2010	ORP-2010 – 50
16	Karnataka	Mandya	JX051391	*Aphis nerii*	*Calotropis* spp.	January-2012	KBRIIHR-155
17		Maddur	JX051392	*A. nerii*	*Calotropis* spp.	January-2012	KBRIIHR-156
18		Hessaraghatta	JX051393	*A. nerii*	*Calotropis* spp.	January-2012	KBRIIHR-157
19		Kolar	HQ112187	*A. nerii*	*Calotropis* spp.	November-2010	ORP-2010 – 52
20	Karnataka	Maddur	JX051394	*Rhopalosiphum padi*	*S. bicolor*	January-2012	KBRIIHR-158
21		Hessaraghatta	JX051395	*R. padi*	*S. bicolor*	January-2012	KBRIIHR-159
22		Hessaraghatta	JX051427	*R. padi*	*Zea mays*	January-2012	KBRIIHR-191
23	Karnataka	Hessaraghatta	JX051396	*Rhopalosiphum maidis*	*Z. mays*	January-2012	KBRIIHR-160
24		Maddur	JX051397	*R. maidis*	*Z. mays*	January-2012	KBRIIHR-161
25		Hebbal	HQ112195	*R. maidis*	*Z. mays*	November-2010	ORP-2010 – 60
26	Karnataka	Mandya	JX051398	*Ceratovacuna lanigera*	*Sacharum officinarum*	January-2012	KBRIIHR-162
27		Maddur	JX051399	*C. lanigera*	*S. officinarum*	January-2012	KBRIIHR-163
28		Hebbal	HQ112193	*C. lanigera*	*S. officinarum*	November-2010	ORP-2010 – 58
29	Karnataka	IIHR	JX051401	*Aphis craccivora*	*Vigna unguiculata*	January-2012	KBRIIHR-165
30		Hessaraghatta	HQ112189	*A. craccivora*	*V. unguiculata*	November-2010	ORP-2010 – 54
31		IIHR	HM237330	*A. craccivora*	*V. unguiculata*	July-2008	NIL
32	Karnataka	Bangalore	JX051404	*Aphis spiraecola*	*Aralia* spp.	January-2012	KBRIIHR-168
33		IIHR	JX051405	*A. spiraecola*	Ornamentals	January-2012	KBRIIHR-169
34		Girinagar	JX051406	*A. spiraecola*	Ornamentals	January-2012	KBRIIHR-170
35		Kolar	JX051407	*A. spiraecola*	Ornamentals	January-2012	KBRIIHR-171
36		Hebbal	HQ112181	*A. spiraecola*	*Aralia* spp.	November-2010	ORP-2010 – 46
37	Karnataka	Bangalore	JX051409	*Hysteroneura setariae*	*Eleusine coracana*	January-2012	KBRIIHR-173
38		Mandya	JX051431	*H. setariae*	*E. coracana*	January-2012	KBRIIHR-195
39		Chikballapur	JX051432	*H. setariae*	*E. coracana*	January-2012	KBRIIHR-196
40		Hessaraghatta	JX051433	*H. setariae*	*E. coracana*	January-2012	KBRIIHR-197
41		Hebbal	HQ112194	*H. setariae*	*E. coracana*	November-2010	ORP-2010 – 59
42	Karnataka	Bangalore	JX051411	*Toxoptera odinae*	*Anacardium occidentale*	January-2012	KBRIIHR-175
43		IIHR	JX051423	*T. odinae*	*Hibiscus* spp.	January-2012	KBRIIHR-187
44		Hessaraghatta	JX051424	*T. odinae*	*Hibiscus* spp.	January-2012	KBRIIHR-188
45		Mandya	JX051425	*T. odinae*	*Hibiscus* spp.	January-2012	KBRIIHR-189
46		Hebbal	HQ112186	*T. odinae*	*A. occidentale*	November-2010	ORP-2010 – 51
47	Karnataka	Mandya	JX051412	*Aphis fabae*	*Solanum nigrum*	January-2012	KBRIIHR-176
48		Doddaballapur	HQ112182	*A. fabae*	*S. nigrum*	November-2010	ORP-2010 – 47
49	Karnataka	Bangalore	JX051413	*Cinara tujafilina*	*Thuja chilensis*	January-2012	KBRIIHR-177
50		Bangalore	HQ443318	*C. tujafilina*	Unknown	January-2012	NIL
51	Karnataka	IIHR	JN160720	*Pentalonia nigronervosa*	*Musa acuminata*	March-2011	ORP-PN-13
52		Hebbal	HQ112184	*P. nigronervosa*	*M. acuminata*	November-2008	ORP-2010 – 49
53	Kerala	Payyoli	JN160724	*P. nigronervosa*	*M. acuminata*	March-2011	ORP-PN-17
54		CPCRI	JN160722	*P. nigronervosa*	*M. acuminata*	March-2011	ORP-PN-15
55		Idukki	JN160718	*P. nigronervosa*	*Coloccassia* spp.	March-2011	ORP-PN-11
56		Wayanadu	JN160716	*P. nigronervosa*	*Coloccassia* spp.	March-2011	ORP-PN-09
57		IISR	JN160714	*P. nigronervosa*	*Elettaria cardamomum*	March-2011	ORP-PN-07
58		Wayanadu	JN160712	*P. nigronervosa*	*E. cardamomum*	March-2011	ORP-PN-05
59		Wayanadu	JN160710	*P. nigronervosa*	*E. cardamomum*	March-2011	ORP-PN-03
60	Kerala	Wayanadu	JN160708	*P. nigronervosa*	*E. cardamomum*	March-2011	ORP-PN-01
61		Koyilandy	JN160725	*P. nigronervosa*	*Musa acuminata*	March-2011	ORP-PN-18
62		Ulliyeri	JN160723	*P. nigronervosa*	*M. acuminata*	March-2011	ORP-PN-16
63		Balussery	JN160721	*P. nigronervosa*	*M. acuminata*	March-2011	ORP-PN-14
64		Wayanadu	JN160719	*P. nigronervosa*	*M. acuminata*	March-2011	ORP-PN-12

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Table 1. (*Cont.*)

Sl no.	Location	Locality	Accession Number	Name of Aphid species	Host plant	Date of collection	Specimen voucher
65		Idukki	JN160717	*P. nigronervosa*	*Coloccassia* spp.	March-2011	ORP-PN-10
66		Appangala	JN160715	*P. nigronervosa*	*E. cardamomum*	March-2011	ORP-PN-08
67		Idukki	JN160713	*P. nigronervosa*	*E. cardamomum*	March-2011	ORP-PN-06
68		Wayanadu	JN160711	*P. nigronervosa*	*E. cardamomum*	March-2011	ORP-PN-04
69		Wayanadu	JN160709	*P. nigronervosa*	*E. cardamomum*	March-2011	ORP-PN-02
70	Karnataka	Bangalore	JX051415	*Hyperomyzus carduellinus*	*Sonchus* spp.	January-2012	KBRIIHR-179
71		IIHR	JX051437	*H. carduellinus*	Ornamentals	January-2012	KBRIIHR-201
72		Bangalore	HQ443319	*H. carduellinus*	Unknown	July-2010	NIL
73	Karnataka	Bangalore	JX051416	*Macrosiphoniella sanborni*	*Chrysanthemum* spp.	January-2012	KBRIIHR-180
74		Bangalore	HQ443315	*M. sanborni*	Unknown	July-2010	NIL
75	Karnataka	Bangalore	JX051417	*Schoutedenia emblica*	*Phyllanthus* spp.	January-2012	KBRIIHR-181
76		Bangalore	HQ443313	*S. emblica*	Unknown	July-2010	NIL
77	Karnataka	Bangalore	JX051418	*Toxoptera citricida*	*Citrus* spp.	July-2010	KBRIIHR-182
78		Bangalore	HQ443316	*T. citricida*	*Citrus spp*	July-2008	NIL
79	Maharashtra	Nagpur	JX051419	*T. citricida*	*Citrus* spp.	December-2010	KBRIIHR-183
80		NRCC	JX051420	*T. citricida*	*Citrus* spp.	December-2010	KBRIIHR-184
81		Pipla	JX051421	*T. citricida*	*Citrus* spp.	December-2010	KBRIIHR-185
82	Karnataka	Bangalore	JX051422	*Greenidea artocarpi*	*Artocarpus* spp.	July-2008	KBRIIHR-186
83		Bangalore	HQ443317	*G. artocarpi*	Unknown	July-2008	NIL
84		Hessaraghatta	JX051426	*Cerataphis lataniae*	*Areca catechu*	July-2008	KBRIIHR-190
85		Bangalore	HQ632647	*C. lataniae*	*Chrysalidocarpus* spp.	July-2008	IIHR-BT-25
86	Karnataka	IIHR	JX051434	*Uroleucon sonchi*	Ornamentals	July-2008	KBRIIHR-198
87		Chikballapur	JX051435	*U. sonchi*	Ornamentals	July-2008	KBRIIHR-199
88		Hebbal	HQ632649	*U. sonchi*	*Sonchus arvensis*	July-2008	IIHR-BT-19
89		IIHR	HQ632653	*Greenidea psidii*	*Psidium guajava*	July-2008	IIHR-BT-23
90		Bangalore	HQ632654	*Toxoptera aurantii*	*Artocarpus heterophyllus*	July-2008	IIHR-BT-24
91		Bangalore	HQ632652	*Brachycaudus helichrysi*	*Chromolaena* spp.	July-2008	IIHR-BT-22
92		Bangalore	HQ632650	*Hyadaphis coriandri*	*Foeniculum vulgare*	July-2008	IIHR-BT-20
93		Bangalore	HQ632648	*Aphis punicae*	*Punica granatum*	July-2008	IIHR-BT-18
94	Karnataka	Bangalore	HQ632646	*Paoliella nirmalae*	*Terminalia arjuna*	July-2008	IIHR-BT-17
95		Bangalore	JX051400	*Acyrthosiphon pisum*	*Pisum sativum*	January-2012	KBRIIHR-164
96	Tamilnadu	Irudupallam	HQ112191	*A. pisum*	*P. sativum*	November-2010	ORP-2010 – 56
97	Karnataka	Bangalore	JX051410	*Macrosiphum rosae*	*Rosa chinensis*	January-2012	KBRIIHR-174
98	Tamilnadu	Ooty	HQ112192	*M. rosae*	*R. chinensis*	November-2010	ORP-2010 – 57
99	Karnataka	Hessaraghatta	JX051428	*Aphis gossypii*	*Hibiscus* spp.	October-2011	KBRIIHR-192
100		Chikballapur	JX051429	*A. gossypii*	Ornamentals	October-2011	KBRIIHR-193
101		Hessaraghatta	JX051430	*A. gossypii*	*Chrysanthemum* spp.	October-2011	KBRIIHR-194
102		Shimoga	JQ067101	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-07
103		Bangalore	JQ067099	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-05
104		Kolar	JQ690329	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-37
105		Bangalore	JQ067100	*A. gossypii*	*Citrullus lanatus*	October-2011	KBRIIHR-06
106		Hessaraghatta	HQ112188	*A. gossypii*	*Gossypium* spp.	November-2010	ORP-2010-53
107		Hessaraghatta	HM237329	*A. gossypii*	*C. lanatus*	July-2010	NIL
108	Rajasthan	Kauroli	JQ690335	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-42
109		Jaipur	JQ690333	*A. gossypii*	*Solanum melongena*	October-2011	KBRIIHR-40
110		Dausa	JQ690336	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-43
111		Jaipur	JQ690334	*A. gossypii*	*Cucurbita maxima*	October-2011	KBRIIHR-41
112		Jaipur	JQ690332	*A. gossypii*	*Luffa* spp.	October-2011	KBRIIHR-39
113	Gujarat	Gujarat	JQ067108	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-14
114	Tamilnadu	TNAU	JQ690330	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-38
115	Maharashtra	Akola	JQ690331	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-38
116		Pune	JQ067107	*A. gossypii*	*Gossypium* spp.	October-2011	KBRIIHR-13
117		Nagpur2	JQ067098	*A. gossypii*	*Citrus sinensis*	December-2010	KBRIIHR-04
118		NRCC2	JQ067096	*A. gossypii*	*C. sinensis*	December-2010	KBRIIHR-02
119		NRCC1	JQ067095	*A. gossypii*	*C. sinensis*	December-2010	KBRIIHR-01
120		Nagpur	JQ067097	*A. gossypii*	*C. sinensis*	December-2010	KBRIIHR-03
121	Kerala	Balussery	JQ067105	*A. gossypii*	*C. lanatus*	October-2011	KBRIIHR-11
122		Balussery	JQ067106	*A. gossypii*	*Hibiscus* spp.	October-2011	KBRIIHR-12
123		CPCRI	JQ067104	*A. gossypii*	*C. lanatus*	October-2011	KBRIIHR-10
124		IISR	JQ067102	*A. gossypii*	*C. lanatus*	October-2011	KBRIIHR-08
125	Karnataka	Shimoga	JQ808460	*Myzus persicae*	*Solanum melongena*	October-2011	KBRIIHR-107
126		Bangalore	JQ808458	*M. persicae*	*C. lanatus*	October-2011	KBRIIHR-105
127		IIHR	JQ808456	*M. persicae*	*S. melongena*	October-2011	KBRIIHR-103
128		Kolar	JQ808467	*M. persicae*	*Abelmoschus esculentus*	October-2011	KBRIIHR-114
129		IIHR	JQ808461	*M. persicae*	*S. melongena*	October-2011	KBRIIHR-108
130		Bangalore	JQ808459	*M. persicae*	*C. lanatus*	October-2011	KBRIIHR-106

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Table 1. (*Cont.*)

Sl no.	Location	Locality	Accession Number	Name of Aphid species	Host plant	Date of collection	Specimen voucher
131		IIHR	JQ808457	*M. persicae*	*A. esculentus*	October-2011	KBRIIHR-104
132		Malleswaram	HQ112190	*M. persicae*	*Duranta erecta*	November-2010	ORP-2010 – 55
133		Chikballapur	JX051436	*M. persicae*	*Amaranthus* spp.	January-2012	KBRIIHR-200
134		Hessaraghatta	HM237331	*M. persicae*	*S. melongena*	July-2010	NIL
135	Maharashtra	Nagpur	JQ808455	*M. persicae*	*A. esculentus*	October-2011	KBRIIHR-102
136		Nagpur	JQ808454	*M. persicae*	*S. melongena*	October-2011	KBRIIHR-101
137		Akola	JQ808466	*M. persicae*	*A. esculentus*	October-2011	KBRIIHR-113
138	Kerala	Balussery	JQ808462	*M. persicae*	*S. melongena*	October-2011	KBRIIHR-109
139		CPCRI	JQ808468	*M. persicae*	*S. melongena*	October-2011	KBRIIHR-115
140	Rajasthan	Jaipur	JQ808464	*M. persicae*	*S. melongena*	October-2011	KBRIIHR-111
141	Tamilnadu	TNAU	JQ808465	*M. persicae*	*S. melongena*	October-2011	KBRIIHR-112
142	Gujarat	Gujarat	JQ808463	*M. persicae*	*A. esculentus*	October-2011	KBRIIHR-110

Table 2. Maximum composite likelihood estimate of the pattern of nucleotide substitution from 142 individuals of 32 species of aphids.

	A	T	C	G
A	–	*5.3*	*1.85*	6.89
T	*4.49*	–	11.34	*1.32*
C	*4.49*	32.42	–	*1.32*
G	23.43	*5.3*	*1.85*	–

308 characters were variable and 270 characters were parsimony informative from the 658 bp regions investigated. No pseudogenes were amplified as indicated by the absence of stop codons within the sequences and the base composition was similar with no indels (Rebijith *et al*., 2012). Reliability of the clustering pattern in the trees was determined using the bootstrap test with 1000 replications employing MEGA 5.0 (Tamura *et al*., 2011) (table 2). Nucleotide frequencies were 34.7% (A), 40.9% (T), 10.2% (C) and 14.3% (G). The base composition of the CO-I gene fragment was found to be biased towards Adenine and Thymine, which together constituted 75.5% of the total as expected from earlier studies on aphids (Wang *et al*., 2011). The overall transition (ti)/transversion (tv) bias of nucleotide sequence was $R = 2.2$.

NJ analysis

The CO-I data set yielded one NJ tree representing the 32 species of aphids studied, which formed distinct clusters (fig. 1). The intraspecific COI sequence divergences ranged from 0 to 3.8% (table 3), whereas interspecific divergences ranged from 2.3 to 18.9%, with a mean of 11.6% (Supplementary material 1). Intrageneric distances ranged from 2.0 to 6.3% (table 4) and intergeneric divergences from 5.0 to 18.2% with a mean of 11.6% (Supplementary material 2). Thus, a discrete barcoding gap between the intra and interspecific distances (Hebert *et al*., 2004) was observed in the current study, except for the cotton–melon aphid, *Aphis gossypii* Glover and the pomegranate aphid, *Aphis punicae* Passerini (fig. 1). The study also revealed that aphids within the genus *Toxoptera* (citrus aphids) are polyphyletic as inferred from the NJ tree.

With regard to the vectoring potential of the aphids studied, we analysed all the available sequences for both *A. gossypii* and the peach-potato aphid, *Myzus persicae* (Sulzer), which revealed that these species are apparently individual cosmopolitan, polyphagous species without any obvious cryptic species or biotypes. However, the NJ tree of 46 samples of the banana aphid, *Pentalonia nigronervosa* revealed that samples collected from banana formed a first clade (belonging to *P. nigronervosa* Coquerel *sensu stricto*) and samples collected from cardamom, alpinia, colocasia and ginger formed a second clade (belonging to *Pentalonia caladii* van der Goot) as described by Foottit *et al*. (2010). For the first time, we were also able to record the existence of cryptic species within the three species, *Brevicoryne brassicae* (Linnaeus), *Hyperomyzus carduellinus* (Theobald) and *Brachycaudus helichrysi* (Kalt.) from India, based on the mean intraspecific variations of CO-I within a group (10X rule) (Hebert *et al*., 2004). These findings were further supported by the NJ bootstrap values of 99, 98 and 100 for *B. brassicae*, *H. carduellinus* and *B. helichrysi*, respectively (fig. 1) and by the calculated intra and interspecific distances for Group 1 & 2 of these three species (fig. 2).

Discussion

Rapid and timely identification of invasive insects such as aphids is important and challenging worldwide, as these particular pests outnumber all other insects in terms of both number and diversity (Footit *et al*., 2008). In this regard, while classical taxonomy has its own strengths, molecular identification employing CO-I barcoding has the added advantage of not being limited by polymorphism, sexual form (asexual/sexual) and life stages of the target species (Asokan *et al*., 2011). All the aphid species employed in the present study were differentiated clearly on the basis of DNA barcodes, which proved to be a valuable tool for the identification of these serious insect pests, an approach complementing classical taxonomy.

1 *DNA barcoding and current taxonomy of aphids*

Morphological identification of aphids poses a serious problem due to the smaller size, polymorphism, insufficient discerning morphological characters, and the complex association with multiple hosts (Miller & Foottit, 2009; Lee *et al*., 2010). Because of this, DNA barcoding employing the CO-I gene sequence (Hebert *et al*., 2004) has become an alternative and effective tool for species identification (Foottit *et al*., 2008; Glover *et al*., 2010; Lee *et al*., 2010). In addition, CO-I may be suitably employed to elucidate the prevalence of biotypes (Shufran *et al*., 2000) and for the discovery of new species

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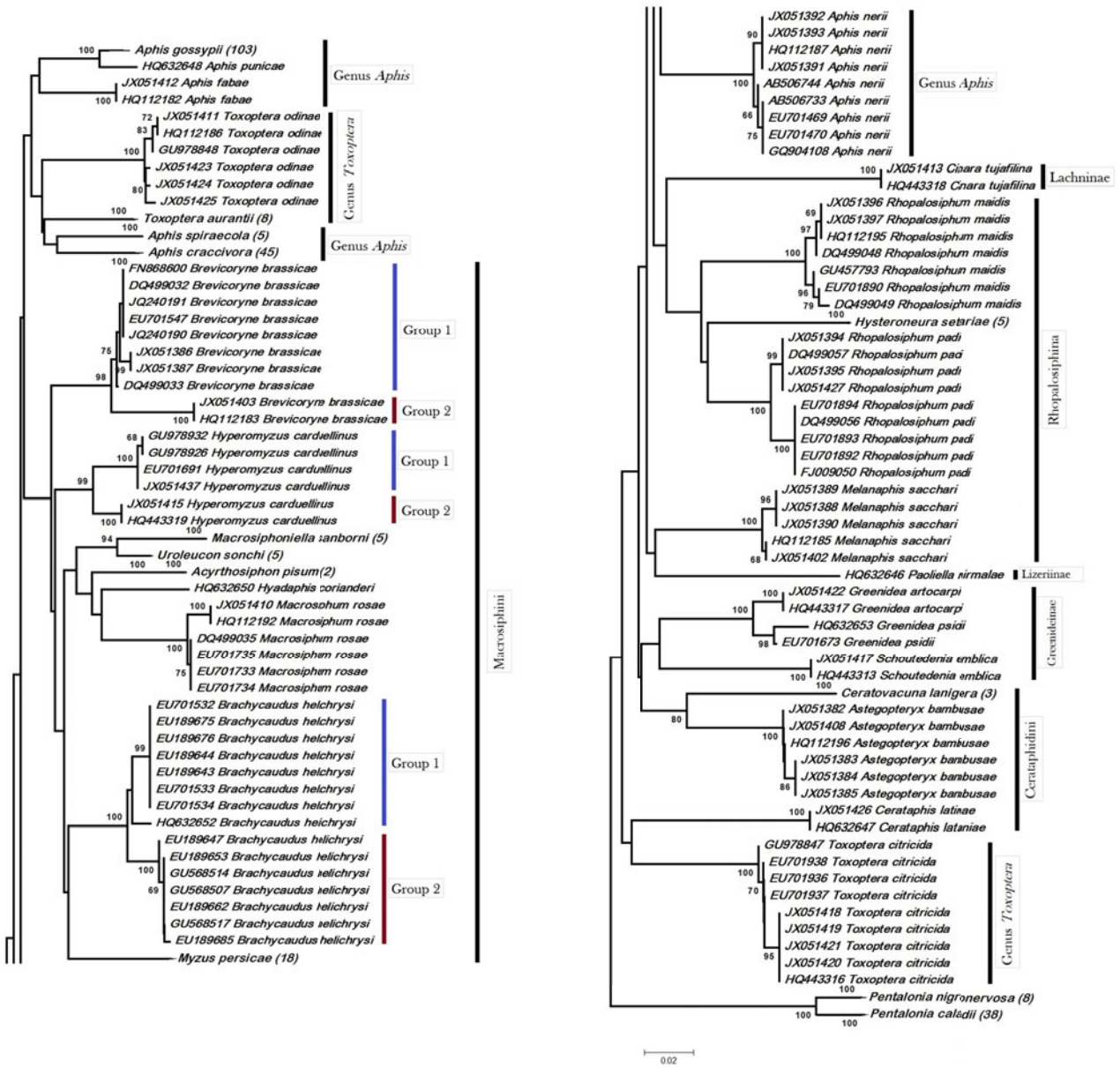

Fig. 1. NJ tree with bootstrap support (1000 replicates) showing clusters of species for COX-1 sequences. Distinct clades for 32 species of aphids can be seen in the figure, in which three species viz. *B. brassicae*, *H. carduellinus* and *B. helichrysi* showing two distinct groups with >90% bootstrap support. The numbers indicated in brackets represents the individuals analysed in the corresponding species.

within the Aphididae (Foottit, 1997). Recently, Foottit *et al.* (2010) examined *P. nigronervosa* using integrated taxonomic approaches and designated *P. nigronervosa* form *typica* as *P. nigronervosa* (infesting banana) and *P. nigronervosa* form *caladii* as *P. caladii* (infesting plants belonging to the families Zingiberaceae (ginger) and Araceae (Arums). Our present study based on CO-I supports this classification. In yet another study, the genus *Toxoptera* raised by Koch in 1856, comprising six species namely *Toxoptera aurantii* (Boyer de Fonscolombe), *Toxoptera celtis* Shinji, *Toxoptera citricida* (Kirkaldy), *Toxoptera odinae* (van der Goot), *Toxoptera victoriae* Martin and *Toxoptera chaetosiphon* Qiao, Wang & Zhang, has a lot of morphological similarity with the genus *Aphis*, except for the presence or absence of stridulatory apparatus. In this respect, our study showed that *T. aurantii* and *T. odinae* form a clade with members of the genus *Aphis*, supporting recent COI barcoding and phylogenetic studies by Foottit *et al.* (2008), Lee *et al.* (2010) and Kim *et al.* (2011). Recently, Blackman *et al.* (2011) studied the sexual morphs and colour variants of *T. odinae* and placed it again in the genus *Aphis*. Similarly based on the present molecular studies, we propose that *T. aurantii* also be placed in the genus *Aphis*.

Our studies showed the possible existence of cryptic species in three aphid species, namely, *B. brassicae*, *H. carduellinus* and *B. helichrysi*. Two biotypes – NZ-1 and 2 – of *B. brassicae* were previously reported by Lammerink

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Table 3. The intraspecific genetic divergences of 22 species that have two or more sequences of aphids with minimum, maximum and average values.

Sl. no.	Species	No. of individuals	Min.	Max.	Average
01	*A. craccivora*	45	0.00	1.27	0.40
02	*A. gossypii*	105	0.00	2.49	0.55
03	*M. persicae*	18	0.00	0.76	0.24
04	*A. fabae*	02	0.00	0.00	0.00
05	*A. nerii*	09	0.00	0.77	0.43
06	*A. spiraecola*	05	0.00	0.00	0.00
07	*A. bambusae*	06	0.00	0.61	0.37
08	*B. helichrysi*	15	0.00	2.54	1.33
09	*B. brassicae*	10	0.00	3.83	1.50
10	*H. carduellinus*	06	0.00	3.06	1.72
11	*H. setariae*	05	0.00	0.46	0.27
12	*M. sanborni*	05	0.00	0.00	0.00
13	*M. rosae*	06	0.00	1.54	0.73
14	*M. sacchari*	05	0.00	0.61	0.37
15	*R. maidis*	07	0.00	1.70	0.88
16	*R. padi*	09	0.00	1.55	0.78
17	*T. aurantii*	08	0.00	0.61	0.15
18	*T. citricida*	09	0.00	0.64	0.30
19	*T. odinae*	06	0.00	1.38	0.84
20	*U. sonchi*	05	0.00	0.82	0.33
21	*P. nigronervosa*	07	0.00	0.00	0.00
22	*P. caladii*	38	0.00	0.78	0.13

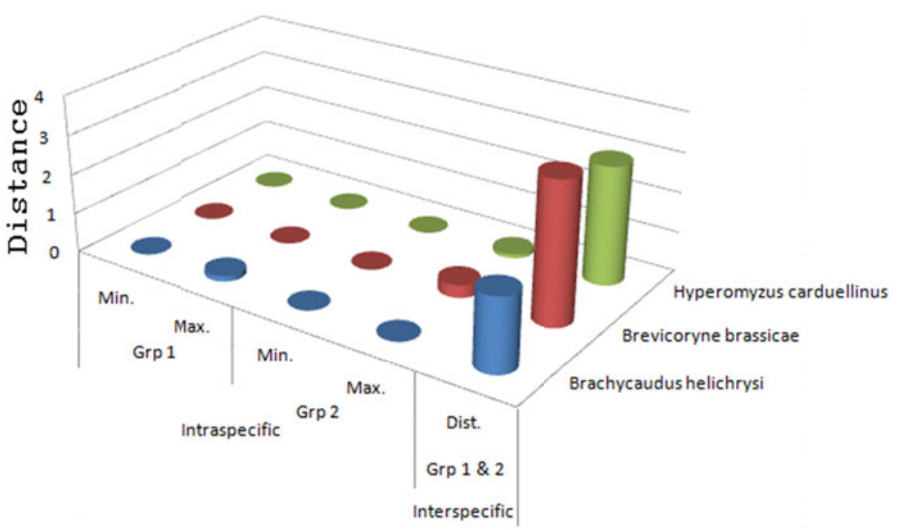
(1968) based on field experiments. This contention was supported by our molecular data, even though clades corresponding to host plants were unclear. Our observations of the existence of sibling species of *B. helichrysi* have been well supported by the recent studies of Madjdzadeh *et al.* (2009) and Piffaretti *et al.* (2012) employing morphometrics and molecular methods, respectively.

Table 4. The intrageneric divergences of 5 genus that have two or more sequences of aphids with mean distance values.

Sl. no.	Genus	No. of individuals	Distance (%)
1	*Aphis*	166	4.93
2	*Rhopalosiphum*	16	4.32
3	*Toxoptera*	23	6.26
4	*Greenidea*	3	2.29
5	*Pentalonia*	45	1.97

2. *Host-associated genetic differentiation*

Host association in aphids is likely to influence reproductive isolation when migration occurs from one host to other. This could be due to pre-mating or post-mating selection against migrants and hybrid progeny (Liou & Price, 1994; Brunner *et al.*, 2004). Even though some aphid species may, at a population level, appear to be polyphagous over large spatial scales, they tend to be monophagous at the colony level due to the availability of suitable host at this much smaller spatial scale (Eastop, 1979). This might have cascading effects on evolution of biotypes and cryptic species favouring host adaptation (Wang & Qiao, 2009), which is evident in the greenbug aphid, *Schizaphis graminum* (Rondani) (Shufran *et al.*, 2000). However in our study, none of the species showed host-associated genetic differences as previously reported by Wang *et al.* (2011) in the cowpea aphid, *Aphis craccivora*, although Foottit *et al.* (2010) did show that *P. nigronervosa*,



	Intraspecific				Interspecific
	Grp 1		Grp 2		Grp 1 & 2
	Min.	Max.	Min.	Max.	Dist.
Brachycaudus helichrysi	0	0.14	0	0.01	1.86
Brevicoryne brassicae	0	0	0	0.32	3.56
Hyperomyzus carduellinus	0	0	0	0.1	2.98

Fig. 2. The range of intra and interspecific distances of group 1 and 2 of three newly identified cryptic species of aphids viz. *B. brassicae*, *H. carduellinus* and *B. helichrysi* according to Hebert's barcoding gap of 10X intraspecific to interspecific distances.

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which feeds only on banana and *P. caladii* that infests several hosts, including cardamom, ginger and alpinia, are host specific and our study indicates the same too.

3 *DNA barcoding implications in pest management*

In recent decades, aphids continue to pose a major threat to agriculture, horticulture and forestry, including *Bt*-transgenic plants (e.g., Faria *et al.*, 2007), more especially due to the evolution of pesticide resistance in some pest species infesting crops treated with conventional pesticides, including organophosphates, carbamates and pyrethroids (Foster *et al.*, 2007). Although many aphid species are damaging in their own right due to the physical injury they inflict on plants, their potential as disease vectors transmitting pathogenic plant viruses of one sort or another has field level implications. In plant disease management, it is advisable to control the vectors (e.g., aphids) rather than the viruses. However, it is difficult to control aphids using insecticides due to their parthenogenetic mode of reproduction (i.e., high rate of reproduction), life cycles (including alternation from crop to non-crop and hence chemically untreated plants), and apparent polyphagy in some species (e.g., *M. persicae*); yet, many farmers still use the chemical approaches as their primary control measure, which may well ultimately lead to the development of resistance, as has indeed occurred in many species of aphids (Devonshire, 1989; Foster *et al.*, 2007).

Insect pest management approaches require a clear understanding on the pest species in question in terms of their particular biology, ecology and population structure/genetics. In this respect, the identification of *P. nigronervosa*, which infests banana transmitting Banana Bunchy Top virus (BBTV) demands quick control measures using insecticides in order to limit the spread of BBTV, whereas in the case of *P. caladii*, the aphid can probably be managed by employing biological agents such as ladybird beetles (Coleoptera: Coccinellidae) and hoverfly larvae (Diptera: Syrphidae) which can reduce pesticide usage and hence slow – and may be even prevent – the developement of insecticide resistance, as well as reducing the polluting impact of these poisons in the environment.

Among the known 376 species of *Liriomyza* flies (Diptera: Agromyzidae), four are difficult to diagnose morphologically and are significant pests globally (EPPO, 2005). Biological differences in susceptibility to pesticides and fecundity (Gao *et al.*, 2012) led to the displacement of *L. sativae* (Blanchard) by *L. trifolii* (Burgess) in China and vice versa in Japan (Gao *et al.*, 2011). However, use of DNA barcoding was able to readily discriminate among these four polymorphic *Liriomyza* species (Scheffer *et al.*, 2006) and has proved highly useful in pest management programmes involving biocontrol. In a nutshell, DNA barcoding can play an important role in pest management when polymorphic pest species have potential impact on the agroeconomy (i.e., direct feeding/vectoring diseases), phenology and susceptibility to specific management practices.

Conclusion

In this study, we generated CO-I barcoding sequences for 142 individual specimens representing 32 aphid species from India. We trust that our work will serve as a rapid, precise, independent identification approach for the discrimination of aphid species of different life stages and colour morphs, both for the species presently studied, and in the future, for other pest species of agricultural, horticultural and forestry interest and importance. This will in turn help in further elucidation of the epidemiology of viruses, their management and serve as a potentially valuable tool in quarantine at the port of entry. Moreover, as our study has revealed, the prevalence of three cryptic aphid species, namely, *B. brassicae, H. carduellinus* and *B. helichrysi*, shows that further studies on the evolution of these particular species (and doubtless others too) are required before we can collectively be sure that we are looking at individual species (*sensu stricto*) rather than complexes of cryptic species (*sensu lato*), perhaps of differing disease vectoring capability. Here, as we show, DNA barcoding is proving an effective tool that can be employed for species identification, elucidation of cryptic species, biotypes and also in the discovery of new species.

The supplementary materials for this article can be found at http://www.journals.cambridge.org/ber

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