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Cryptic diversity and habitat partitioning in an economically important aphid species complex

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ABSTRACT

Cardamom Bushy Dwarf Virus (CBDV) is an aphid-borne nanovirus which infects large cardamom, *Amomum subulatum* (Zingiberaceae family), in the Himalayan foothills of Northeast India, Nepal and Bhutan. Two aphid species have been reported to transmit CBDV, including *Pentalonia nigronervosa* and *Micromyzus kalimpongensis* (also described as *Pentalonia kalimpongensis*). However, *P. nigronervosa* was recently split into two species which exhibit different host plant affiliations. Whilst *P. nigronervosa* primarily feeds on banana plants, *Pentalonia caladii* (previously considered a 'form' of *P. nigronervosa*) typically feeds on plants belonging to the Araceae, Heliconiaceae and Zingiberaceae families. This raises the possibility that CBDV vectors that were originally described as *P. nigronervosa* correspond to *P. caladii*. Accurate identification of vector species is important for understanding disease dynamics and for implementing management strategies. However, closely related species can be difficult to distinguish based on morphological characteristics. In this study, we used molecular markers (two mitochondrial loci and one nuclear locus) and Bayesian phylogenetic analyses to identify aphid specimens collected from 148 CBDV infected plants at a range of locations and elevations throughout Sikkim and the Darjeeling district of West Bengal (Northeast India). Our results revealed the presence of a diversity of lineages, comprising up to six distinct species in at least two related genera. These included the three species mentioned above, an unidentified *Pentalonia* species and two lineages belonging to an unknown genus. Surprisingly, *P. caladii* was only detected on a single infected plant, indicating that this species may not play an important role in CBDV transmission dynamics. Distinct elevation distributions were observed for the different species, demonstrating that the community composition of aphids which feed on large cardamom plants changes across an elevation gradient. This has implications for understanding how competent vector species could influence spatial and temporal transmission patterns of CBDV.

1. Introduction

Many vector-borne pathogens are transmitted by multiple vector species which overlap in geographic distributions and host ranges. Characterising the diversity and composition of vector communities is important for making predictions about future disease dynamics because vector species can influence pathogen evolutionary trajectories and transmission patterns in different ways (Power and Flecker, 2008; Gómez-Díaz et al., 2010; Lord, 2010). For example, variation among vector species in host affiliations can lead to host specific divergence among pathogen strains (Van Putten et al., 2007), and differential preferences for host phenological stages or climatic conditions can influence the temporal and spatial dynamics of disease outbreaks (Almeida et al., 2005). Accurate identification of vector species is also important for disease management as different species may vary in their susceptibility to control strategies.

Large cardamom, *Amomum subulatum* (Zingiberaceae family), is an important cash crop which is cultivated in the Himalayan foothills of Northeast India, Nepal and Bhutan. Productivity has dramatically declined in recent decades due to a high incidence of fungal and viral pathogens (Bhattarai et al., 2013). One of the key problems is an aphid-borne nanovirus known as *Cardamom Bushy Dwarf Virus* (CBDV; Nanoviridae, Babuvirus). CBDV is the causal agent of foorkey disease, which is characterised by excessive sprouting of dwarf tillers, reduced yield and mortality (Mandal et al., 2004). Two aphid species within the Macrosiphini tribe (Aphidinae subfamily) have been reported to transmit CBDV. These include the banana aphid, *Pentalonia nigronervosa* (Varma and Capoor, 1964), and a species which was originally designated as *Micromyzus kalimpongensis* (Basu and Ganguly, 1968), but has since

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been described as a member of the *Pentalonia* genus (Blackman and Eastop, 2008). Both species are small (adults are 1–2 mm in length) and dark (reddish-brown to black), and alate individuals have characteristic dark wing venation (Blackman and Eastop, 2008).

P. nigronervosa was previously considered to have two forms, 'typica' and 'caladii'. However, these were recently described as separate species (*P. nigronervosa* and *P. caladii*) based on molecular and morphological analyses and differences in host plant affiliations (Foottit et al., 2010). Although both can potentially feed on the same host plants (Bhadra and Agarwala, 2010), *P. nigronervosa* primarily feeds on banana and abaca plants (Musaceae family), whilst *P. caladii* appears to have greater affinity for plants within the Araceae, Heliconiaceae and Zingiberaceae families (Foottit et al., 2010; Bhadra and Agarwala, 2010, 2012; Rebijith et al., 2013; Duay et al., 2014). *P. nigronervosa* and *P. caladii* are competent vectors of *Banana bunchy top virus* (BBTV; Nanoviridae, Babuvirus) (Hu et al., 1996; Watanabe et al., 2013), which infects banana plants and is closely related to CBDV (Mandal et al., 2004). However, the differences in host plant affiliations suggest that *P. nigronervosa* may be a more relevant vector of BBTV than *P. caladii* and that *P. caladii* may be a more important vector of CBDV than *P. nigronervosa*. Although *P. caladii* has not explicitly been reported as a vector of CBDV, this may simply reflect that it was only recently recognised as a distinct species and is difficult to distinguish from *P. nigronervosa* based on morphology alone (Foottit et al., 2010).

In phytophagous insects, differences in host plant specialisations may allow different races and/or closely related species to co-occur in sympatry. However, when host plant ranges overlap, alternative modes of niche partitioning can promote coexistence in the same community assemblages (Abbot and Withgott, 2004). Large cardamom is cultivated in a heterogeneous mountain landscape across an elevation gradient ranging from approximately 500 m to 2000 m. Habitat partitioning across this elevation gradient may be a mechanism by which closely related aphid species with overlapping host ranges avoid interspecific competition for resources. If the species are competent vectors of CBDV, this scenario could have implications for CBDV transmission as differences among vector species in preferences for microclimatic conditions can influence pathogen persistence (Glass, 2005) and future disease dynamics in the face of climate change (Sternberg and Thomas, 2014).

Aphids often exhibit high levels of phenotypic plasticity and closely related species can be difficult for non-specialists to distinguish using conventional taxonomic approaches based on morphological characteristics (Foottit et al., 2008). Molecular markers, particularly mitochondrial *cytochrome c oxidase subunit 1* (COI) gene sequences, have proven useful for species level identification in aphids and can reveal the existence of morphologically cryptic species (Foottit et al., 2008, 2009; Wang and Qiao, 2009; Rebijith et al., 2013). In this study, we used COI and additional mitochondrial and nuclear markers to identify aphid specimens collected at a range of locations and elevations throughout Sikkim and the Darjeeling district of West Bengal (Northeast India) from CBDV infected large cardamom plants expressing symptoms of foorkey disease. We examined the species composition of the aphid community to determine which of the reported (*P. nigronervosa* and *M. kalimpongensis*) and putative (*P. caladii*) CBDV vector species were present and assessed if the species exhibit different elevation distributions.

2. Methods and materials

2.1. Sample collection

Aphids were collected during 2011–2013 from 148 CBDV infected plants at a range of locations and elevations throughout Sikkim and the Darjeeling district of West Bengal, Northeast India (Fig. 1; Supplementary Table 1). The infection status of the plants was visually assessed based on the expression of foorkey disease symptoms. CBDV DNA had also been extracted from a subset of the plants (approximately 10%) during previous studies (Savory and Ramakrishnan, 2014; Savory et al., 2014). Aphid specimens from each infected plant were collected using a paintbrush and were preserved in 95% ethanol until DNA extraction. Sampling locations and elevations were recorded using a Garmin eTrex GPS.

2.2. DNA extraction and PCR amplification of mitochondrial and nuclear markers

Genomic DNA was extracted from individual adults (1 adult per infected plant) using a DNeasy Blood and Tissue Kit (Qiagen). In a few cases, no adult specimens were available so DNA was extracted from multiple nymphs (i.e. 2–3 nymphs per infected plant). For the majority of samples, we obtained DNA sequences corresponding to three loci (for a small number of samples sequences for only two loci were obtained; Supplementary Table 1). These included a 658 bp fragment of the COI gene, which has been widely used as a 'DNA barcode' for identifying aphid species and other animal species (Hebert et al., 2003; Foottit et al., 2008, 2009), as well as partial mitochondrial *cytochrome b* (cyt b; 437 bp) and *nuclear elongation factor 1α* (EF1α; 747–752 bp) sequences. Primers are listed in Supplementary Table 2. PCRs were performed in 10 μl reaction volumes, containing 3.4 μl H_2O, 5 μl Multiplex PCR Master Mix (Qiagen), 0.3 μl of each primer (10 pmol/μl) and 1 μl DNA template. PCR protocols for COI and EF1α involved initial denaturation for 15 min at 95 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C (COI) or 55 °C (EF1α) and extension for 1 min at 72 °C, and then a final extension period of 10 min at 72 °C. The PCR protocol for cyt b involved initial denaturation for 15 min at 95 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at 52 °C and extension for 1 min at 72 °C, and then a final extension period of 10 min at 72 °C. PCR products were purified using Exonuclease (New England Biolabs) combined with Shrimp Alkaline Phosphatase (USB) as specified in the product manuals. Sequencing was performed with the primers listed in Supplementary Table 2 using a 3130 × l Genetic Analyzer from Applied Biosystems (Life Technologies).

2.3. Sequence alignment and annotation

Sequences were aligned using the MUSCLE algorithm implemented in MEGA version 5.05 (Tamura et al., 2011). Open reading frame positions were determined following alignment and comparison with annotated sequences available on Genbank for conspecifics and/or other aphid species within the Macrosiphini tribe. DNA sequences were then translated into amino acid sequences to check for the presence of frame shift mutations or premature stop codons, which can indicate amplification of transposed sequences and/or pseudogenes. Heterozygous positions in the nuclear EF1α sequences were coded according to the International Union of Pure and Applied Chemistry (IUPAC) ambiguity code.

2.4. Specimen identification

We attempted to assign species identities to the unknown specimens by using Basic Local Alignment Search Tool (BLAST) analyses and phylogenetic analyses to compare the haplotypes observed in this study with publicly available aphid sequences. The utility of this approach is clearly constrained by the taxonomic coverage of existing reference libraries. Representative sequences were avail-