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Review

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Review

Choosing an Effective PCR-Based Approach for Diet Analysis of Insect Herbivores: A Systematic Review

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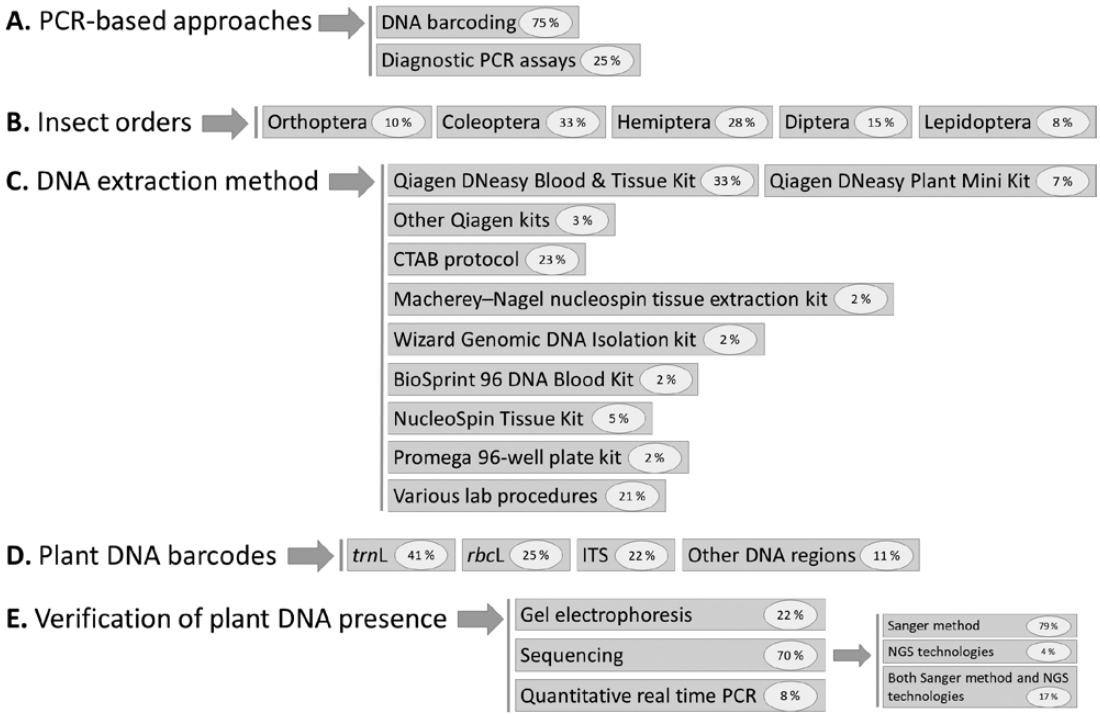
Abstract

Identification of ingested plant species using polymerase chain reaction (PCR)-based methods is an increasingly useful yet challenging approach to accurately determine the diet composition of insect herbivores and thus their trophic interactions. A typical process of detection of DNA of ingested plants involves the choice of a DNA extraction method, a genomic target region, and/or the best approach for an accurate plant species identification. The wide range of available techniques makes the choice of the most appropriate method for an accurately and timely identification of ingested plants from insect guts difficult. In our study, we reviewed the commonly used PCR-based approaches in studies published from 1977 to 2019, to provide researchers with the information on the tools which have been shown to be effective for obtaining and identifying ingested plants. Our results showed that among five insect orders used in the retrieved studies Coleoptera and Hemiptera were prevalent (33 and 28% of all the records, respectively). In 79% of the studies a DNA barcoding approach was employed. In a substantial number of studies Qiagen DNA extraction kits and CTAB protocol were used (43 and 23%, respectively). Of all records, 65% used a single locus as a targeted plant DNA fragment; *trn*L, *rbc*L, and *ITS* regions were the most frequently used loci. Sequencing was the dominant type of among DNA verification approaches (70% of all records). This review provides important information on the availability of successfully used PCR-based approaches to identify ingested plant DNA in insect guts, and suggests potential directions for future studies on plant–insect trophic interactions.

Graphical abstract



Keywords: DNA barcoding, gut content analysis, insect plant usage, plant–insect interactions

Various approaches are used to investigate insect diet and feeding preferences, including but not limited to direct observations and controlled feeding experiments (Siemann and Rogers 2003, Jogesh et al. 2008, Branson and Sword 2009, Fielding and Conn 2011, Fan et al., 2013), microscopical identification of ingested plants and pollen (Joern 1979, Joern 1983, Silberbauer et al. 2004), stable isotope methods (see review by Hood-Nowotny and Knols 2007), and biochemical approaches (Stephens et al. 2008). Our review here focuses on molecular gut content analysis of insect herbivores, and here specifically on polymerase chain reaction (PCR)-based approaches, primarily DNA barcoding, to identify ingested plant species in insect guts.

DNA barcoding approaches have been identified to be highly useful to rapidly and accurately identify the diet composition of insect herbivores (Miller et al. 2006; Jurado-Rivera et al. 2009; Valentini et al. 2009; Bafeel et al. 2012; Wallinger et al. 2012, 2013; Garcia-Robledo et al. 2013; Traugott et al. 2013; Avanesyan 2014; Huang et al. 2017; De la Cadena et al. 2017; Zhu et al. 2019; Avanesyan and Lamp 2020). These techniques allow us to identify ingested food plants, determine the feeding behavior of insect herbivores, and draw conclusions on the role of insects in trophic interactions. DNA barcoding approaches have many advantages over classical approaches by providing a better species resolution, as well as detection of plants which can be missed during field surveys (Valentini et al. 2009, Hereward and Walter 2012, Kitson et al. 2013, De la Cadena et al. 2017). In particular, the use of high-throughput DNA sequencing can decipher complex multitrophic interactions of generalist insect species (Taberlet et al. 2012, Firlej et al. 2013, Mollot et al. 2014, Sow et al. 2020). Knowledge of diet composition is especially critical for understanding feeding preferences of invasive agricultural or forest pests, as well as detecting and predicting novel plant–insect associations (Garcia-Robledo et al. 2013, González-Chang et al. 2016, Avanesyan and Lamp 2020). This information also has important implications for biological control of weeds by determining the field host range of a potential biocontrol agent and ultimately reducing the use of herbicides (Frei et al. 2019, Ollivier et al. 2020).

Typically, a DNA barcoding process involves the following steps: 1) plant DNA extraction, 2) amplification of the targeted plant DNA fragment, 3) validation of the presence of the amplified plant DNA fragment, 4) sequencing of the plant DNA fragment, and 5) plant species identification via comparison with reference databases. At each step of the DNA barcoding process, an investigator makes a choice about what tool, kit, targeted gene, or sequencing method to use. Previous studies demonstrated successful plant DNA detection from insect guts using various Qiagen DNA extraction kits (Pumarino et al. 2011, Hereward and Walter 2012, Cooper et al. 2016, Avanesyan and Lamp 2020) and CTAB protocols (Staudacher et al. 2011, Cooper et al. 2016). Several plant DNA loci, such as *trn*L, *rbc*L, *mat*K, and *ITS* (*ITS-1* and *ITS-2*) have been successfully utilized for amplification of ingested plant DNA (Matheson et al. 2008, Hereward and Walter 2012, Garcia-Robledo et al. 2013, Wang et al. 2019, Avanesyan et al. 2021). Both, sequencing a single DNA template using the Sanger approach (hereafter 'DNA barcoding') and metabarcoding using next-generation sequencing (NGS) technologies (hereafter 'metabarcoding') have been used in various studies to identify ingested plant species, explore source of honey and plant community composition, as well as infer various species interactions (e.g., Hawkins et al. 2015, Pornon et al. 2016, Deiner et al. 2017, Matesanz et al. 2019, Baksay et al. 2020; see also reviews by González-Chang et al. 2016, Dormontt et al. 2018, Adamowicz et al. 2019, Kennedy et al. 2020).

In addition to traditional DNA barcoding, diagnostic PCR assays involving the detection of products via amplification with plant-specific primers have been shown to be effective for both

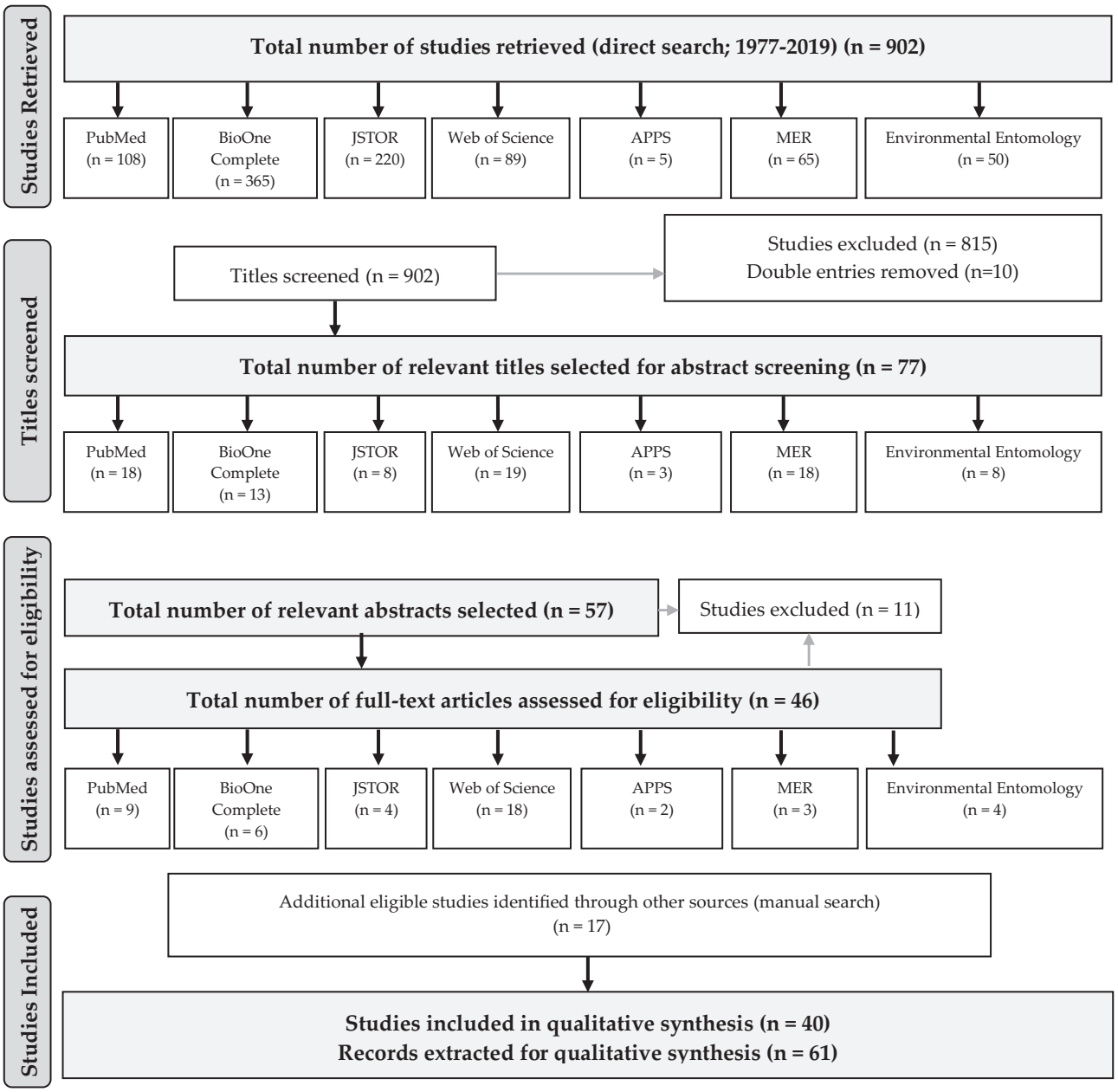


Fig. 1. PRISMA flowchart: literature search and data extraction (modified from Moher et al. 2009).

aboveground insect herbivores (e.g., Pumariño et al. 2011, Wang 2018) and belowground insect herbivores (e.g., Staudacher et al. 2011, Wallinger et al. 2012). Such PCR assays are especially advantageous for confirming the diet of insect herbivores with a limited number of host plants (Wang et al. 2018).

The wide range of available techniques makes the choice of the most appropriate method for an accurately and timely identification of ingested plants from insect guts difficult. To address this, we reviewed PCR-based approaches that were commonly used in dietary studies to identify the most promising tools for identifying ingested plant species. This information will help researchers to make well-founded choices and design experimental protocols applicable to their specific research question and study species.

Specifically, we designed our study to help researchers in 1) gathering preliminary information necessary for successful detection of host plant DNA from an insect of interest, and 2) reducing troubleshooting time. In this review, we summarize available information based on a systematic literature search (following Moher et al. 2009), using the keywords which researchers might typically use, and restricting the summary to studies with a successful identification of plants ingested by insects.

In our systematic review, we were specifically interested in the following questions:

(1) *What insect groups were used for detecting ingested plant species using a PCR-based approach?* Previous studies have demonstrated successful detection of ingested plants from guts of multiple insect species, including but not limited to the orders Coleoptera and Orthoptera (Kishimoto-Yamada et al. 2013, Staudacher et al. 2013, Avanesyan 2014, Avanesyan and Culley 2015), as well as Hemiptera (Cooper et al. 2016, 2019; Avanesyan and Lamp 2020; Avanesyan et al. 2021). In this review, we explored the range of insect groups utilized.

(2) *Which DNA extraction methods have been used for detecting plant DNA from insect guts*? In our study, we reviewed the DNA extraction methods which have been successfully used for plant DNA isolation from insect guts. We also addressed whether researchers tend to use a whole-body extract or isolated insect body parts for DNA extraction. For the purpose of this review, we also included studies which used insect regurgitates.

(3) *Which DNA regions have been shown to allow for successful identification of plant species?* The choice of plant DNA regions certainly depends on multiple factors, including but not limited to a researcher's previous experience, available laboratory tools, study species, etc. Hollingsworth et al. (2011) reviewed the most commonly used plant DNA barcodes from intact plants and discussed the discriminatory power for each targeted gene. However, the choice of plant DNA barcodes becomes more difficult when a researcher deals with ingested plants (i.e., degraded DNA). The balance between resistance of a targeted DNA fragment to degradation and a good species resolution is important but not always possible. In this review, we specifically focused on which plant DNA region(s) the authors found to be effective and whether a single locus or multiple loci are commonly used in such studies.

(4) *How is the presence of the targeted plant DNA fragment verified?* It is critical to confirm that the targeted DNA fragment was amplified. A researcher can confirm the presence of a specific amplified DNA fragment by its length via gel electrophoresis or alternatively by performing BLAST comparisons against sequences in public databases such as NCBI GenBank or the Barcode of Life DataSystems (BOLD). Depending on a research question and previous work on the study species, the researcher might choose to follow another routine to validate the obtaining the targeted DNA fragment. We summarize the validation methods the authors used and discuss their applications.

Based on the results from previous studies and our own experience of choosing a specific approach, we expected that the retrieved studies would show a wide range of DNA extraction methods and targeted plant DNA loci. We also expected a DNA barcoding to be applied when the study species is a specialist insect; whereas metabarcoding could be the most often choice when the study species is a polyphagous insect. We also suggest potential applications of using a PCR-based approach for diet analysis of agricultural insect pests.

Materials and Methods

Literature Search and Inclusion Criteria

For this study, we conducted literature search following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al. 2009) (Fig. 1). First, we searched four databases (Web of Science, JSTOR, PubMed, and BioOne Complete) for relevant studies on molecular gut content analysis of insect herbivores published between 1 January 1977 and 30 September 2019. The year 1977 was chosen as the year of the development of Sanger's 'chain-termination' technique (Sanger et al. 1977, Heather and Chain 2016). We searched for relevant studies using the following keywords: 'molecular' AND 'gut' AND 'content' AND 'analysis'; the search was then refined by keyword 'insects'.

In addition to these four databases which we used as the primary sources to search for relevant studies, we screened three journals (Environmental Entomology, Molecular Ecology Resources, and Applications in Plant Sciences) for the same time period using 'insect molecular gut content analysis' as a search query. By adding these three journals and using a slightly different combination of the keywords we intended to retrieve the studies which might have been missed during the primary search in the databases. Due to the focus of this review, only experimental research articles published in English were included in the screening. Using these search terms, we retrieved 902 articles in total; of those, 10 double entries were removed, and 77 relevant titles containing terms such as 'feeding', 'host plants', 'plant DNA', 'ingested plants', 'PCR', 'DNA barcoding', 'insect herbivores', or their combination were chosen for abstract screening. Based on abstract screening, 46 relevant articles were selected for further screening of full texts.

To include a study in our analysis, we used the following inclusion criteria:

(1) the study had to report successful detection of plant DNA from insect guts only (for the purpose of this review, the studies which

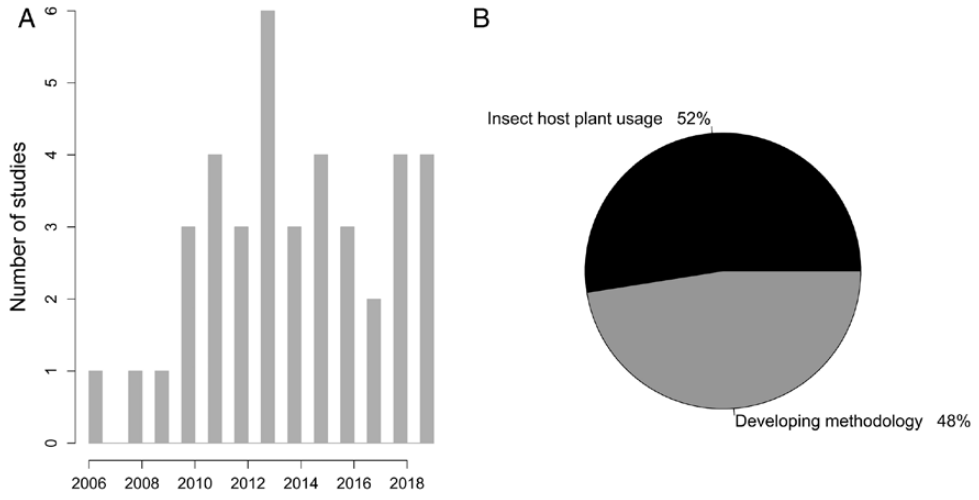


Fig. 2. Number of studies relevant to using a DNA barcoding approach in insect gut content analysis published during 1977–2019: all retrieved studies (A), and percentage of studies that reported different research objectives (B).

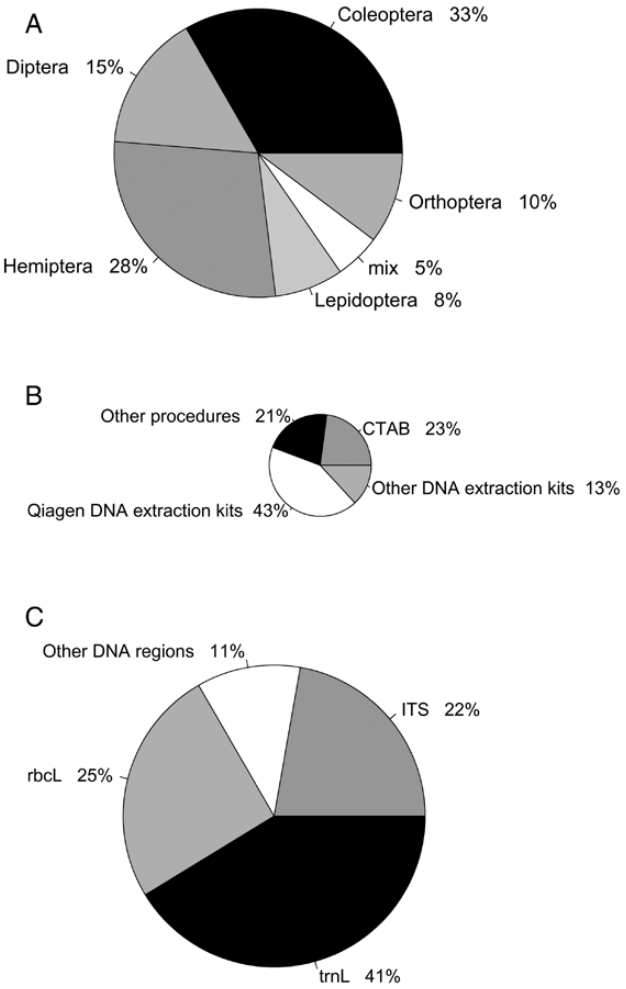


Fig. 3. Percentage of studies that used various insect orders (A), various DNA extraction methods (B), and various plant DNA barcoding regions (C). (A) Mix orders include the following: Lepidoptera and Hemiptera (Pumarino et al. 2011); Orthoptera, Coleoptera, Hemiptera, and Lepidoptera (Matheson et al. 2008). (B) Various kits include: Macherey–Nagel nucleospin tissue extraction kit (McClenaghan 2015), Wizard Genomic DNA Isolation kit (Lovejoy and Johnson 2014), Promega 96-well plate kit (Navarro et al. 2010), BioSprint 96 DNA Blood Kit (Wallinger et al. 2015), and NucleoSpin Tissue Kit (Kajtoch 2014, Kajtoch and Mazur 2015). (C) Other DNA regions include: *mat*K (Kajtoch 2014), short and long RAPD fragments amplified by primers OPA3, OPA4, OPB10, OPD3, pUC/M13F, BOXAIR, pehA#6, and pehA#3 (Latif et al. 2012); a 169-bp region of plastid DNA amplified by strawberry-specific primers (Diepenbrock et al. 2018); multiplex PCR assays (Staudacher et al. 2013).

used plant DNA detection from insect feces or focused on DNA barcoding of honey and pollen were not included);

(2) the study had to report the plant DNA region used for PCR amplification;

(3) the study had to indicate an approach used to confirm successful plant DNA detection (i.e., method to validate the amplification of the targeted DNA region).

We expected that the keywords we used for the literature search might yield both the studies which employed a diagnostic PCR using specific primers and the studies that used a traditional DNA barcoding approach. We aimed to keep our literature search as close as possible to a typical literature search a researcher might use. Subsequently, all such studies were included in the analysis if they satisfied the inclusion criteria described above.

The authors' rationale for conducting a study and study objectives were not considered: the articles which focused on developing a method or using a method to decipher insect feeding preferences were included in the analysis. As a result, a total of 23 articles which satisfied all the inclusion criteria were selected for the analysis. Additionally, reference lists of these 23 articles were screened and nine eligible studies (i.e., secondary references) were added. Finally, five eligible studies were manually retrieved through ResearchGate, and three eligible unpublished studies from our own laboratory trials (two studies are now published) were added. As a result, 40 articles which satisfied all the inclusion criteria were used in our data analysis (Fig. 1; Supp Table S1 [online only]).

Data Extraction

To investigate and summarize available information on the approaches which were used for detecting host plants ingested by insects, from each study we retrieved separate records which included: 1) one plant DNA region, applied to 2) one insect species, and 3) one validation method used for this plant DNA region and insect species. As in some studies several plant regions were used for more than one insect species, we retrieved a total of 61 records from 40 articles (Fig. 1; Supp Table S2 [online only]). Additionally, the following information was extracted from each article: the purpose of the study (developing a method or investigating insect feeding preferences), insect order, number of plant DNA loci used, validation method (an approach used to confirm successful amplification of the targeted DNA region), and sequencing approach, if used (a DNA barcoding or metabarcoding) (Supp Table S1 [online only]). Additionally, we also extracted the following data: DNA extraction method, body parts used, and kind of plant DNA locus used (Supp Table S2 [online only]).

Data Analysis

All data on insect orders, DNA extraction method, PCR amplification, and validation methods were synthesized using proportions, and the prevalence of studies reporting of a certain approach or tool was determined using a binomial test. Kruskal–Wallis test was used to determine the difference between the number of studies published at different time periods. The differences among validation methods, targeted genes, and extraction methods in application to different insect feeding guilds were determined using Pearson's chi-squared test and *MASS* package in R. All the data analysis was conducted in R, v.3.4.3 (R Core Team 2014).

Results

Focus of the Studies and Insect Orders

Identified 40 articles, which met the inclusion criteria, were published between 2006 and 2019; number of publications ranged from 1 to 5 per year (Fig. 2A). The number of studies published between 2011 and 2019 were significantly prevalent ($\chi^2 = 6.124$, df = 1, $P = 0.01334$). Studies aiming to develop a method and explore insect host plant usage were almost equally presented (Fig. 2B); 75% of the studies utilized a DNA barcoding approach and 25% employed diagnostic PCR assays (Supp Table S2 [online only]).

Five insect orders were primarily used in the studies: Orthoptera, Coleoptera, Hemiptera, Diptera, and Lepidoptera; Coleoptera and Hemiptera were significantly prevalent (61% of all records; binomial test: $P = 0.0003$; Fig. 3A). Two studies used a mix of insect species from these five orders: various species from Lepidoptera and Hemiptera (Pumarino et al. 2011); and various species from

Table 1. Primer sequences (5′–3′) reported for detecting *trn*L, *rbc*l, and *ITS* regions in Coleoptera and Hemiptera

Author, year	Insect order	Primer sequences (5′–3′)	Amplicon sizes (bp)
Avanesyan and Lamp (2020)	Hemiptera	rbcLaF: ATGTCACCACAAACAGAGACTAAAGC rbcLaR: GTAAAATCAAGTCCACCRCG	530
Cooper et al. (2019)	Hemiptera	B49873-e: GGTTCAAGTCCCTCTATCCC A50272-F: ATTTGAACTGGTGACACGAG B49317-c: CGAAATCGGTAGACGCTACG A49855-d: GGGGATAGAGGGACTTGAAC ITS2F: ATGCGATACTTGGTGTGAAT ITS3R: GACGCTTCTCCAGACTACAAT	400–600
Cooper et al. (2016)	Hemiptera	trnL575F: CGACCCCCTTTCCTTAGCG trnL755R: TCGGGAATCATTCAACTAGGGA	180
García-Robledo et al. (2013)	Coleoptera	ITS4_Rev: GCATATCAATAAGCGGAGGA ITS3_Rev: ATTGTAGTCTGGAGAAGCGTC ITS2-2_For: ATGCGATACTTGGTGTGAAT	360
		rbcL_F: ATGTCACCACAAACAGAGACTAAAGC rbcL_Rev: GTAAAATCAAGTCCACCRCG rbcL_230_Rev: TTACCAGYCTTGATCGTTACAAAGG rbcL_260_For: CTTTGTAACGATCAAGRCTGGTAAG	N/A
		psbA: GTTATGCATGAACGTAATGCTC trnH: CGCGCATGGTGGATTCACAATCC	N/A
Hereward and Walter (2012)	Hemiptera	trnL e B49873: GGTTCAAGTCCCTCTATCCC trnF f A50272: ATTTGAACTGGTGACACGAG	161–567
Hereward et al. (2013)	Hemiptera	c A49325: CGAAATCGGTAGACGCTACG d B49863: GGGGATAGAGGGACTTGAAC	400, 600
Jurado-Rivera et al. (2009)	Coleoptera	c A49325: CGAAATCGGTAGACGCTACG d B49863: GGGGATAGAGGGACTTGAAC	313–581
Kajtoch (2014)	Coleoptera	1F: ATGTCACCACAAACAGAAAC 636F: GCGTTGGAGAGATCGTTTCT rbcL-724R: TCGCATGTACCTGCAGTAGC A49325: CGAAATCGGTAGACGCTACG B49863: GGGGATAGAGGGACTTGAAC	400
Kajtoch and Mazur (2015)	Coleoptera	A49325: CGAAATCGGTAGACGCTACG B49863: GGGGATAGAGGGACTTGAAC A49425: GGGCAATCCTGAGCCAA B49466: CCATTGAGTCTCTGCACCTATC	343–574
Kitson et al. (2013)	Coleoptera	A49325: CGAAATCGGTAGACGCTACG M13(-21) B49863: TGTAAAACGACGGCCAGTGGGGATA GAGGGACTTGAAC H-rev: GATAGGTGCAGAGACTCAATG	255–409
Kishimoto-Yamada et al. (2013)	Coleoptera	rbcLa forward: ATGTCACCACAAACAGAGACTAAAGC rbcLa reverse: GTAAAATCAAGTCCACCRCG	547–553
Lovejoy and Johnson (2014)		trnL-c: CGAAATCGGTAGACGCTACG trnL-d: GGGGATAGAGGGACTTGAAC	300–600
Matheson et al. (2008)	Coleoptera Hemiptera	rbcL19: AGATTCCGCAGCCACTGCAGCCCCTGCTTC rbcLZ1: ATGTCACCACAAACAGAGACTAAAGCAAGT	157
Navarro et al. (2010)	Coleoptera	c A49325: CGAAATCGGTAGACGCTACG d B49863: GGGGATAGAGGGACTTGAAC	379–609
Wang et al. (2017)	Hemiptera	*trn*L-*trn*F Cotton: Forward: GTTGAAGAAAGAATCGAATAGAATAG Reverse: ATAGACAGCAAACGGGCTTT	236
		Mungbean: Forward: ATGTCAATACCGACAACAATGAA Reverse: AAATCCAAATTCCAATTTAGTTG	199
Wang et al. (2019)	Hemiptera	psbA: GTTATGCATGAACGTAATGCTC trnH: CGCGCATGGTGGATTCACAATCC	430
		rbcL_F: ATGTCACCACAAACAGAGACTAAAGC *rbcL*_Rev: GTAAAATCAAGTCCACCRCG	599
		ITS1-F-rc*: GACTCTCGGCAACGGATATC ITS4: TCCTCCGCTTATTGATATGC	410

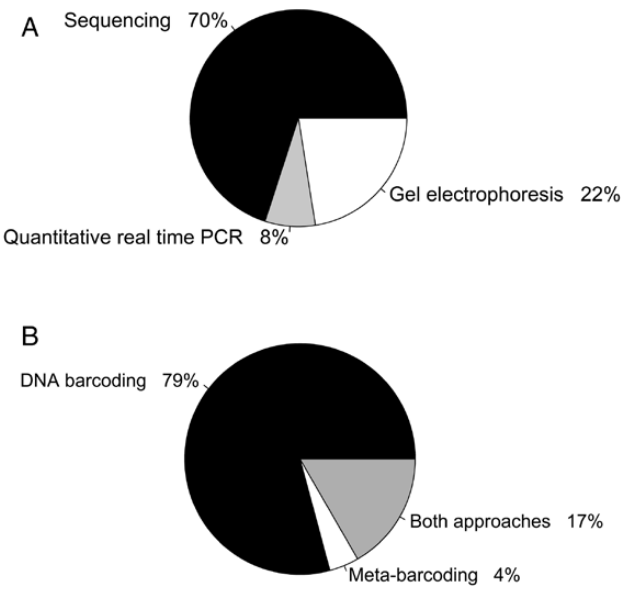


Fig. 4. DNA verification methods used in retrieved relevant studies: percentage of studies used various DNA verification methods (A), and percentage of studies used a DNA barcoding, metabarcoding, and both approaches (B).

Orthoptera, Coleoptera, Hemiptera, and Lepidoptera (Matheson et al. 2008).

Choice of DNA Extraction Method

A substantial number of the retrieved records (54%) used various DNA extraction kits, primarily from Qiagen (43% of all the records) (Fig. 3B). Other studies employed DNA extraction kits such as (as reported by the authors) Macherey–Nagel nucleospin tissue extraction kit (McClenaghan 2015), Wizard Genomic DNA Isolation kit (Lovejoy and Johnson 2014), Promega 96-well plate kit (Navarro et al. 2010), BioSprint 96 DNA Blood Kit (Wallinger et al. 2015), and NucleoSpin Tissue Kit (Kajtoch 2014, Kajtoch and Mazur 2015). The rest of the retrieved records used a CTAB-based protocol or developed other extraction procedures (23 and 21%, respectively) (Fig. 3B). Other procedures were described as the DNA extractions using the reagents available in a laboratory (Matheson et al. 2008; Junnila et al. 2010, 2011; Latif et al. 2012; García-Robledo et al. 2013; Hereward et al. 2013, Huang et al. 2017). Regarding DNA extraction, the number of studies with insect whole-body extraction was significantly prevalent (64% of all records; binomial test: $P = 0.006$). Other options included: isolated guts, various body parts (e.g., thorax), and regurgitates.

Choice of Plant DNA Barcode

On average, one to three plant DNA loci were used in the reviewed studies; the number of those which used a single locus was significantly prevalent (65% of all records; binomial test: $P = 0.006$). The plant DNA regions which were used more often included: *trn*L (41% of the studies), *rbc*L (25%), and *ITS* (22%) (Fig. 3C). Primer sequences used to detect various portions of these DNA regions in gut contents of Coleoptera and Hemiptera are presented in Table 1. Other DNA regions were: *mat*K (Kajtoch 2014); the plastid gene *ycf*1 (Diepenbrock et al. 2018); and specific primers located in the *trn*T–*trn*F region (Staudacher et al. 2013), as well as short and long RAPD fragments amplified by primers OPA3, OPA4, OPB10, OPD3, pUC/M13F, BOXAIR, pehA#6, and pehA#3 (Latif et al. 2012).

Verification of Plant DNA Presence

The prevalent number of studies used sequencing to validate the amplification of the targeted plant DNA fragment (70% of all records; binomial test: $P < 0.0001$) (Fig. 4A). The rest of the studies employed a diagnostic PCR (30%); of these, most studies conducted gel electrophoresis as a final step of the experimental procedures (Fig. 4A). DNA barcoding was significantly prevalent (79% of all records; binomial test: $P < 0.0001$); 17% of studies used both a DNA barcoding using Sanger method and metabarcoding using various NGS technologies (Fig. 4B). Interestingly, validation methods significantly differed among the studies which amplified different plant DNA loci ($\chi^2 = 24.673$, df = 9, $P = 0.003$): most of the studies which used *trn*L, *rbc*L, and *ITS* genes (96, 93, and 63%, respectively) used sequencing as a validation method, while 80% of studies which used other plant DNA regions used gel electrophoresis as a validation method.

Discussion

In this study, we explored PCR-based approaches that researchers commonly used for diet analysis of insect herbivores. We were specifically interested in retrieving records of successful detection of ingested plant DNA from insect guts published between 1977 and 2019. Our main goal was to review the chosen approaches and to provide researchers with a brief summary of the available information on the most often used tools, kits, and targeted plant genes for detecting ingested plants from insect guts, which ultimately helps the researchers make an informed choice for the most suitable tool for their specific research question.

Retrieved Studies, Studies' Focus, and Insect Orders

Since only studies that reported successful plant DNA detection from insect guts were included in our data analysis, such a relatively small number of studies published during 1977–2019 may be associated with challenges accompanying the process of detection of ingested plant DNA, and unsuccessful attempts were not published.

The choice of the study species and incorporating DNA barcoding tools certainly depends on a researcher's goals and the research focus. Kajtoch and Mazur (2015), for example, indicated that they chose the weevil, *Centricnemus leucogrammus* (Germar, 1824) (Coleoptera: Curculionidae) for their study as a well-explored species in terms of ecology and which feeding preferences was intensively explored using molecular biology tools. The authors advanced this knowledge in their study by exploring feeding activity of *C. leucogrammus* under harsh environmental conditions using DNA barcoding tools (Kajtoch and Mazur 2015). The prevalence of study species of the orders Coleoptera and Hemiptera, many of which are important agricultural pests and/or potential biocontrol agents were somewhat expected. From the plant DNA perspective, the leaf-grazing species of the order Coleoptera, as well as their larvae, are potentially a convenient study system due to less destroyed DNA of ingested plants compared to that in the guts of fluid-feeding insects (Cooper et al. 2016, Avanesyan and Lamp 2020, Gonella et al. 2020). As for Hemiptera, many species of this order are economically important agricultural pests (Sethusa et al. 2014), some are vector species (Cooper et al. 2016, 2019; Cavalieri et al. 2019), and therefore, they represent valuable study species. Among the retrieved studies, Wang et al. (2018), for example, focused on the bug, *Apolygus lucorum* (Meyer-Dür) (Hemiptera: Miridae), a highly mobile pest of cotton and other crops; Lovejoy and Johnson (2014) used the

invasive kudzu bug, *Megacopta cribraria* (Fabricius) (Hemiptera: Plataspidae), an important pest of various legumes. Despite the substantial number of studies on Hemiptera, several authors emphasized the challenges associated with detection of plant DNA from piercing–sucking insects; among which a potential lack of plant tissue in insect guts and limited knowledge on stylet penetration through the plant tissue (Cooper et al. 2016, 2019; Avanesyan and Lamp 2020; Avanesyan et al. 2021). We did not find, however, that the insect species which were easier to handle in terms of molecular gut content analysis were often chosen as study species. Future studies might focus on insect species which were less often used in the gut content analysis, such as lepidopterans, and develop DNA barcoding protocols applicable to these species.

Choice of DNA Extraction Method

A wide variety of extraction methods was used in the retrieved studies: studies which used 'ready-to-go' extraction kits and studies which developed the extraction procedures were almost equally presented. Such a variety of extraction methods can be explained by at least three factors: 1) investigating unique study species, 2) specific research goals (whether it is developing a method or deciphering diet composition), and 3) needs and funds of a research team.

Both, plant DNA extraction kit (DNeasy Plant Mini Kit) and insect DNA extraction kit (DNeasy Blood & Tissue Kit) worked well for the purpose of the studies. The plant DNA extraction kit apparently worked best with the leaf-grazing insect species of a relatively large size, such as grasshoppers (Avanesyan 2014, Avanesyan and Culley 2015). In grasshopper gut contents the ingested plant DNA can be preserved and detected for up to 22 h postconsumption (Avanesyan 2014). Additionally, the DNA extraction method was not associated with the insect orders which were used in the studies; which is valuable information for researchers who overcome challenges working with fluid-feeding insects.

The prevalence of studies which used an insect whole-body extract to amplify ingested plant DNA can be explained by convenience of DNA extraction process as this limits the time needed for sample preparation for extraction (Jurado-Rivera et al. 2009). Also, using a whole-body extract may be an easier option when researchers work with relatively small insects, such as the spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (Briem et al. 2018), or potato psyllid, *Bactericera cockerelli* (Sulc) (Hemiptera: Triozidae) (Cooper et al. 2016, 2019). Some of the studies pointed out, however, that using a whole-body extract may increase risk of contamination of insect body surface with host plant DNA (e.g., Briem et al. 2018). Even though this issue can be addressed by emerging an insect in a bleach solution (Cooper et al. 2016; Avanesyan and Lamp 2020), future studies might further explore this issue and determine whether the amount of plant DNA on insect surface differs between leaf-grazing insects and fluid-feeders, to increase yield of targeted DNA.

Choice of Plant DNA Region

PCR has been shown to be the most common molecular method for determining diet composition and evaluating trophic interactions in the agricultural settings (González-Chang et al. 2016). Dormontt et al. in their review of DNA barcoding applications (2018) indicated several loci which are routinely used in DNA barcoding of plant species, such as *rbc*L, *mat*K, *ITS*, and *psb*A–*trn*His. Similar to the previous studies (e.g., Matheson et al. 2008; Kress et al. 2009; Kajtoch and Mazur 2015), we found that *trn*L and *rbc*L are the two most often used plant DNA barcodes.

The *trn*L region was first suggested by Taberlet et al. (1991), and since then was widely used for various ecological studies focusing on determining insect associations with their host plants (Jurado-Rivera et al. 2009, Navarro et al. 2010, Staudacher et al. 2011, Avanesyan 2014, Avanesyan and Culley 2015, Wallinger et al. 2015, Avanesyan et al. 2021). A substantial portion of the retrieved studies used the universal primers proposed by Taberlet et al. (1991) (Table 1). It has also been demonstrated in the previous studies that the *trn*L intron is relatively tolerant to degradation and it can provide a higher species resolution than *rbc*L gene (Kress et al. 2009, Sundari and Papuangan 2019). In our previous study on the spotted lanternfly, however, we were not able to successfully amplify a *trn*L region, while *rbc*L gene provided a good amplification and species resolution (Avanesyan and Lamp 2020). To improve taxonomic resolution of a specimen, multiple loci can be combined (Kress et al. 2009, Li et al. 2017, Zhu et al. 2019). Keeping in mind differences in species resolution provided by *trn*L and *rbc*L genes (Kress et al. 2009, Sundari and Papuangan 2019, Avanesyan and Lamp 2020), a researcher may want to make choice to better address the goal of the study—whether it is plant species identification, or confirmation of plant consumption.

Additionally, when the ingested plant DNA is a DNA source, the DNA is more likely degraded, and amplification of long DNA regions can be challenging (Dormontt et al. 2018). This can pose limitation for utilizing metabarcoding as some sequencing platform can have shorter sequence read length than some of the long DNA barcoding loci; as a result, only partial sequence can be obtained (Dormontt et al. 2018). Some authors suggested using short but informative loci if metabarcoding approach is chosen by a researcher (Coissac et al. 2012, Dormontt et al. 2018).

Verification and Identification of Plant DNA Presence

The most important step toward successful identification of plants is a validation of the presence of the targeted DNA template in the PCR product: this step not only provides the results but ultimately demonstrates the effectiveness of the utilized PCR-based approach. Obtaining a DNA sequence is critical for plant species identification, and many sequencing procedures become more accessible for the researchers (Stein et al. 2014). The observed prevalence of the studies which used a DNA barcoding using Sanger method (79%) can be explained by high utilization of specialist insects as study species, as well as using direct feeding assays. Sanger sequencing was the first method used for DNA barcoding of insect host plants (Kajtoch and Mazur 2015); it has also been a relatively cheap method with regard to sample submission (especially when submitting a large number of individual samples) compared to metabarcoding (Wallinger et al. 2012). Despite its effectiveness demonstrated in the retrieved studies, Sanger method is limited to the identification of only one species only within a single reaction (Kajtoch and Mazur 2015). So, while a DNA barcoding is effective for work with specialist insects, it is impossible to apply it for polyphagous insect species which require sequencing multiple amplicons (Kajtoch and Mazur 2015, Avanesyan and Lamp 2020). This limitation can be partially overcome if a researcher, for example, design the primers which amplify multiple amplicons of different length within the same reaction (Wallinger et al. 2012). However, metabarcoding is certainly highly efficient to address this issue; it has also been advantageous for identification of various plant food sources under natural conditions (e.g., Briem et al. 2018, Pitteloud et al. 2020, Zhang et al. 2021). Also, pollen DNA metabarcoding, which was outside of the focus of

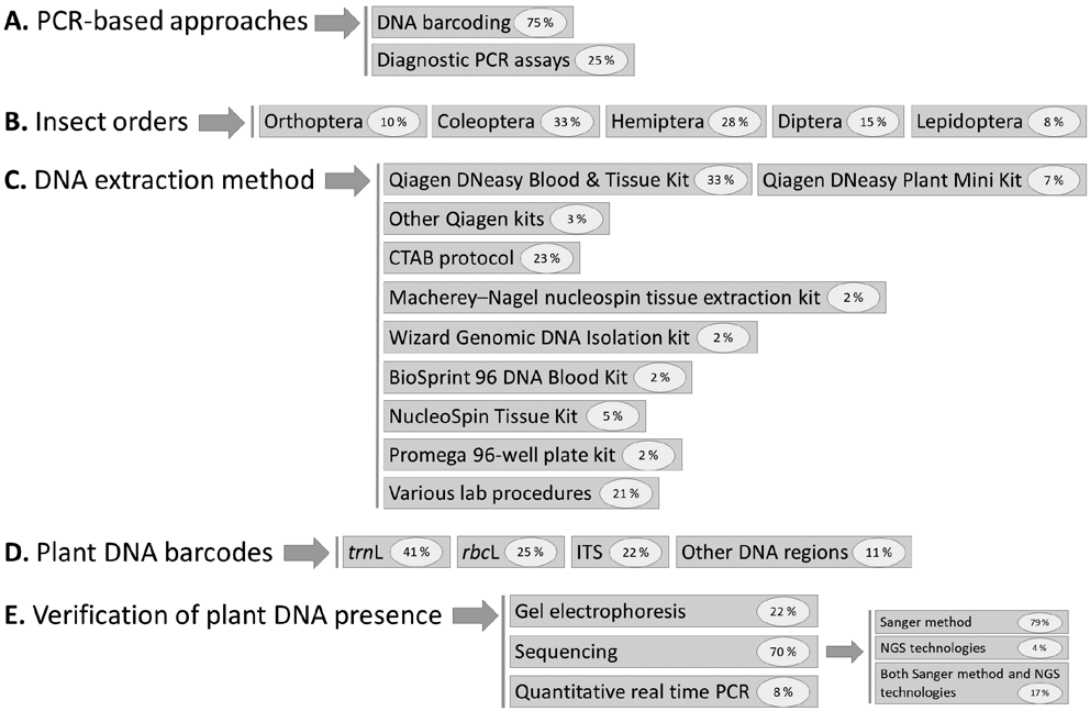


Fig. 5. Summary of the results of the systematic review of the retrieved studies on detection of ingested plants from insect guts using PCR-based approaches. The presented results include: overall PCR-based approaches (A), insect orders (B), DNA extraction methods (C), plant DNA barcodes (D), and approaches the studies used to verify the obtaining of the targeted plant DNA fragment (E). The results demonstrated the prevalence of a DNA barcoding (A), using insects of Coleoptera and Hemiptera orders (B), utilizing 'ready-to-go' DNA extraction kits (C), amplifying plant DNA loci such as *trn*L, *rbc*L, and *ITS* (D), and performing sequencing, particularly Sanger sequencing, as a validation method (E).

the present manuscript, is a promising research direction (please see a review by Evans and Kitson 2020), which can be applied even to historic insect specimens (e.g., Gous et al. 2021).

Our review also indicates that metabarcoding approach was used for polyphagous insect herbivores and here primarily for the analysis of field-collected individuals (McClenaghan et al. 2015). In this context, the targeted genes *trn*L, *rbc*L, and *ITS* were most frequently used as target loci since there are numerous sequences available in public databases such as NCBI GenBank. Looking at the expected development in this area, it is to be expected that metabarcoding will gain even more importance for the investigation of the feeding behavior of polyphagous insects at a high resolution in the future. Using this approach, a researcher cannot only identify the range of consumed plants, but also the identity of the insects in parallel.

Potential Applications of PCR-Based Approaches for Diet Analysis of Agricultural Insect Pests

Insect herbivores, both generalists and specialists, represent an important part of agricultural food webs, often damaging crop plants and causing substantial economic problems; the estimated annual costs associated with only non-native crop arthropod pests are $13.9 billion (Pimentel et al. 2005). González-Chang et al. (2016), in their review of molecular techniques used to study trophic interactions in agricultural systems, emphasized the importance of using molecular tools for analysis of food webs, discovering rare or cryptic interactions, as well as understanding the effect of landscape complexity on species interactions. Below we briefly indicate a few specific applications of PCR-based approaches for detecting ingested plants from insect guts that researchers could utilize in the agricultural setting, along with several examples from previous studies.

First, PCR-based approaches are extremely valuable for the analysis of diet composition of generalist species (Sow et al. 2020). In diet analysis, metabarcoding of ingested plant species becomes especially advantageous (McClenaghan et al. 2015) as it is highly effective for the analysis of complex food webs (González-Chang et al. 2016) and for investigating feeding activity of highly polyphagous pests (Avanesyan and Lamp 2020, Pitteloud et al. 2020, Zhang et al. 2021). Thus, Pitteloud et al. (2020) have successfully utilized a DNA metabarcoding method to investigate changes in plant–Orthoptera interactions across several elevational gradients.

Exploring pest migration between crop plants is another valuable application of plant DNA detection from insect guts. Wang et al. (2017, 2018, 2019) in series of experiments with the polyphagous mirid bug *A. lucorum* (Heteroptera: Miridae) successfully demonstrated utilization of DNA barcoding of plants ingested by this insect pest for determining the consumption of novel food plants and migration of *A. lucorum* between cotton (*Gossypium hirsutum* L.) and mungbean (*Vigna radiata* (L.) Wilczek).

The safety of biological control and a better understanding of interactions between weeds and potential biocontrol agents is yet another venue for utilizing PCR-based approaches. Biocontrol agents directly or indirectly could consume nontarget species (Willis and Memmott 2005). Accurate detection and confirmation of the ingested food plants using PCR-based methods is a valuable tool to 'track' trophic interactions in the agricultural environment and to identify a promising biocontrol candidate (Ollivier et al. 2020). Frei et al. (2019) in their study with carabid beetles found that weed seeds were a substantial part of carabid diet and demonstrated a potential of carabid beetles for biocontrol of weeds.

Finally, using DNA barcoding approaches can be very helpful for exploring host-finding process by agricultural pests. It becomes

especially important if insects (such as, for example, aphids and whiteflies) can transmit plant pathogens. In aphids, such virus transmission can occur during series of steps in the process of insect search of host plants and identifying feeding sites (Irwin et al. 2007, Fereres and Moreno 2009). During this process, aphid stylets can frequently penetrate plant cells in peripheral plant tissues following various sensory cues, tasting various plants even outside of the crop field, and then perform sap ingestion from the acceptable food plants (Irwin et al. 2000, Powell et al. 2006, Irwin et al. 2007; Fereres and Moreno 2009). Such stylet probing and feeding behavior after the insect arrival on the host plant is common among many hemipteran pests (Backus 1988, Backus and Hunter 1989, Backus et al. 2005). Tasting various plants during the host search can result in ingestion of small amount of DNA from the plants which are not the insect's primary host plants. Future studies might focus on further exploring host-finding process in insect vectors and identifying such 'nonhost' ingested DNA.

Conclusions

A PCR-based analysis of ingested plants is an exciting and challenging approach used to confirm direct trophic interactions between insect species and specific food plants. Our study demonstrated a significant increase in the number of studies successfully used this approach during last decade. We found that using 'ready-to-go' DNA extraction kits, amplifying plant DNA loci such as *trn*L, *rbc*L, and *ITS*, and utilized sequencing as a validation method are commonly used approaches which researchers used (Fig. 5). This review provides important information on availability of successfully used PCR-based approaches to detect ingested plant DNA from insect guts, as well as potential directions for future studies on plant–insect interactions. In conclusion, we designed this review systematic review to help researchers conduct effective PCR-based assays for their study species; and we also want to encourage researchers to further develop and publish PCR-based approaches especially for insect pests of high economic importance.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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