Video Article

Laboratory Protocol for Genetic Gut Content Analyses of Aquatic Macroinvertebrates Using Group-specific rDNA Primers

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Abstract

Analyzing food webs is essential for a better understanding of ecosystems. For example, food web interactions can undergo severe changes caused by the invasion of non-indigenous species. However, an exact identification of field predator-prey interactions is difficult in many cases. These analyses are often based on a visual evaluation of gut content or the analysis of stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$). Such methods require comprehensive knowledge about, respectively, morphologic diversity or isotopic signature from individual prey organisms, leading to obstacles in the exact identification of prey organisms. Visual gut content analyses especially underestimate soft bodied prey organisms, because maceration, ingestion and digestion of prey organisms make identification of specific species difficult. Hence, polymerase chain reaction (PCR) based strategies, for example the use of group-specific primer sets, provide a powerful tool for the investigation of food web interactions. Here, we describe detailed protocols to investigate the gut contents of macroinvertebrate consumers from the field using group-specific primer sets for nuclear ribosomal deoxyribonucleic acid (rDNA). DNA can be extracted either from whole specimens (in the case of small taxa) or out of gut contents of specimens collected in the field. Presence and functional efficiency of the DNA templates need to be confirmed directly from the tested individual using universal primer sets targeting the respective subunit of DNA. We also demonstrate that consumed prey can be determined further down to species level via PCR with unmodified group-specific primers combined with subsequent single strand conformation polymorphism (SSCP) analyses using polyacrylamide gels. Furthermore, we show that the use of different fluorescent dyes as labels enables parallel screening for DNA fragments of different prey groups from multiple gut content samples via automated fragment analysis.

Video Link

The video component of this article can be found at https://www.jove.com/video/56132/

Introduction

Predator-prey interactions, which constitute the majority of trophic interactions and food web dynamics, are key aspects to characterize the fluxes of matter and energy throughout food webs within and between ecosystems, which is one of the major goals of ecology[1]. The determination of the source and flow of carbon and nutrients is furthering the understanding of ecological connectivity between ecosystems[2]. However, ecosystems, such as rivers and their catchments, are not only linked by fluxes of organic matter and nutrients but also by the movements of organisms[3]. Thus, habitat alterations interrupting the flow of resources that link those systems can strongly alter the food webs of both ecosystems, not only directly but also indirectly by changing the respective composition of predator-prey communities. For instance, changes of food webs have been shown to be linked to the movements of single predator species (*e.g.*, rainbow trout)[4]. Such changes potentially threaten biodiversity and the functioning of aquatic ecosystems. Therefore, analyzing predator-prey interactions in the field is essential to determine the impact of human-induced environmental changes, such as water management practices, on the native biodiversity of aquatic ecosystems.

Since tracking trophic linkages is difficult in complex systems, several approaches have been established that enable the assessment of feeding interactions in the field[5]. Traditionally, investigations of feeding interactions in the field are based on visual identification of prey remains in dissected guts and require an extensive knowledge about morphologic prey diversity. Visual gut content analyses have provided insights in resource use of several groups of consumers (*e.g.,* seasonal variation in diet of lobster[6] and fish[7,8] or feeding preferences of copepods). However, the physical process of digestion makes visual gut content analyses difficult and usually misses soft bodied prey-organisms[9]. For species feeding by liquid ingestion or for some invertebrate consumers that intensively comminute their food before ingestion, like amphipods, visual identification of prey species in gut contents is impossible[10]. Due to these limitations, molecular analysis provides a promising alternative.

Molecular analyses have now become a common tool allowing rapid and precise prey detection in gut contents. The range of such techniques is diverse: strategies based on monoclonal antibodies or polymerase chain reactions (PCR) are often used, because of their high specificity and sensitivity[11]. The development of new monoclonal antibodies is time- and cost-intensive, therefore, the application of other molecular techniques

is more useful when antibodies do not already exist[11]. Another common approach is the amplification of regions of deoxyribonucleic acid (DNA), like ribosomal ribonucleic acid (RNA) genes present in most species, using universal primer sets[12,13]. When using this technique, it is often not possible to identify the whole range of prey organisms within mixed sources of DNA[14]. An effective approach to avoid such a drawback is to use group-specific primer sets for genetic gut content analyses. Designed to amplify only DNA regions of particular target groups and exclude all other species[15,16], group-specific primers enable identification of prey organisms on the taxonomic level of the specified groups without time- and cost-intensive secondary analyses. However, like all gut content analysis, such analyses provide only a snapshot of feeding behavior. Therefore, combining molecular gut content analyses with analyses of time-integrating natural tracers (*e.g.,* stable isotopes) is considered beneficial[1,2].

Here, we describe a detailed method for PCR-based investigations of predator-prey interactions using group-specific primer sets for nuclear ribosomal DNA (rDNA) regions to be combined with stable isotope analyses of the same specimen. We describe the detection of the DNA of single prey groups via agarose gel electrophoresis. Additionally, we present an opportunity for further downstream analyses of PCR products of such group-specific primers applicable whenever a higher taxonomic resolution than the primers' specificity is required. Because single stranded DNA (ssDNA) fragments form tertiary conformations that are determined by their primary sequence[17], small variations in fragments amplified by such group-specific primers lead to conformational changes. Such changes can be detected by single strand conformation polymorphism (SSCP) analyses with polyacrylamide gels[17,18], enabling a more precise identification of prey organisms (down to the species level).

While agarose gel electrophoresis is a common and inexpensive tool to visualize DNA fragments and determine their approximate length[19], the resolution depends on the amount of DNA and the staining dye used[20]. Usually, the visualization is straight forward when working with pure DNA samples, but potentially low amounts of prey DNA in the gut contents of consumers can complicate the scoring of agarose gel electrophoresis results. Still, this detection method is feasible to screen a low number of consumer specimens from the field for one or a few prey groups, but complication in the scoring makes the screening of a high number of samples for multiple prey taxa extremely time intensive and thus impracticable. A more sensitive detection method is the automated analysis of fragments via capillary electrophoresis, which additionally allows the determination of the exact length of fragments[21]. Several microsatellite based studies have shown that by using different fluorescent dyes as labels, it is possible to detect and determine different fragments of comparable length by automated fragment analysis[22,23,24]. Therefore, we also present a detailed protocol for parallel detection of DNA from multiple prey groups using PCR with labeled group-specific primer sets and detection via automated fragment analysis with an automated sequencer. Additionally, we present results from a case study demonstrating that the detection of prey DNA via automated fragment analysis is an approach which also enables a relative quantification of ingested prey.

Protocol

1. Collect Macroinvertebrates from the Field and Prepare the Samples for Genetic Gut Content Analyses.

1. Collect macroinvertebrates at each site, sort out individuals of consumer species of interest into single tubes, and directly freeze them in liquid nitrogen or dry ice to prevent gut clearance and degradation of DNA.
   NOTE: Avoid storing samples in alcohol, because some macroinvertebrates tend to regurgitate before the alcohol kills them.
2. **Only use the gastro-intestinal tract of mid-sized and big (approximately >3 mm) macroinvertebrate species for genetic gut content analyses so the remaining matter of the specimen can be used for other procedures (*e.g.,* stable isotope analysis). Note that this is not applicable when investigating very small taxa like water mites. Therefore, steps 1.2.1 and 1.2.2 can be skipped.**
   1. Slightly defrost an individual and carefully dissect its gastro-intestinal tract under a stereomicroscope using fine stainless steel forceps. Make sure not to puncture the gastro-intestinal tract to avoid loss of matter.
   2. Clean forceps using decontamination solution to remove DNA and RNA contaminations after the preparation of each gastro-intestinal tract. Therefore, incubate forceps for 10 min in the decontamination solution. Afterwards, rinse them with sterile water to remove residual traces and wipe them with a lint-free paper towel.
   3. Transfer the dissected gastrointestinal tract to a 2.0 mL safe-lock reaction tube containing 440 µL (450 µL possible for use of repetitive pipette) salt extraction buffer [SEB; 0.4 M sodium chloride (NaCl), 10 mM Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) pH 8.0, and 2 mM ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) pH 8.0]. Store prepared samples at −20 °C immediately until the extraction of DNA. Ensure that DNA will be extracted within a couple of days.

3. **Use a modified high-salt extraction protocol[25] to extract DNA.**
   1. Take samples out of the freezer. Use forceps to add one 5 mm stainless steel bead to each sample tube, and leave frozen samples on the bench at room temperature (RT) to thaw. Use a bead mill to homogenize samples for 1 min at 15 Hz.
   2. Spin down samples with a short run of a centrifuge. Use microliter pipettes to add 90 µL (100 µL possible for use of repetitive pipette) 10% sodium dodecyl sulfate solution (SDS) and 5 µL of 10 mg/mL proteinase K.
   3. Vortex samples thoroughly and incubate mixtures for 1 h at 60 °C with constant shaking at 400 rpm on a thermomixer. Additionally, thoroughly vortex samples every 10 min to further promote lysis.
   4. Spin down samples with a short run of a centrifuge, add 350 µL of 5 M NaCl with a microliter pipette and vortex thoroughly for approximately 0.5 - 1 min. Centrifuge samples for 40 min at 16,200 x g at 5 °C.
      NOTE: If multiple sets in a row have to be done, the temperature setting of the centrifuge needs to be slightly raised (not higher than 10 °C) because SDS tends to flocculate if the temperature is too low.
   5. Use microliter pipettes to transfer approximately 600 µL supernatant into a new 1.5 mL reaction tube. Be careful not to remove anything from the pellet.
   6. Tip stainless steel beads out of the reaction tubes into a stainless steel tea strainer, clean them with running water and incubate them in a Petri dish with the decontamination solution for 10 min. Thoroughly rinse off the decontamination solution with sterile water and store cleaned beads in ethanol (>99%, p.a. grade) or dry until reuse.
      NOTE: Cleaning of beads does not need to be done in this step directly, but rather can be done whenever there is waiting time in the following steps.

7. Add 600 µL of ice-cold isopropanol (p.a. grade) to the supernatant with a microliter pipette and carefully invert the tube a couple of times. Store samples overnight at -20 °C.
8. Take samples out of the freezer and centrifuge them for 20 min at 16,200 × g and room temperature. Carefully discard the supernatant and dry the tube carefully with lint-free tissue paper. Note that if the surrounding temperature is high (more than 25 °C), centrifuge at 5 °C to avoid the pellets slipping while discarding the supernatant.
9. Wash pellets containing DNA with 70% ethanol. To do this, add 200 µL of ice-cold ethanol (70%, p.a. grade) using a microliter pipette and centrifuge samples for 10 min at 16,200 x g and room temperature (or 5 °C, if needed). Carefully discard the supernatant and dry the tube carefully with lint-free tissue paper. Use a 10 µL pipette adjusted to approximately 8 µL to carefully pipette off the remaining supernatant. Make sure not to disturb the pellet.
10. Dry the pellet for approximately 5-20 min at 60 °C or at room temperature at the bench until all the ethanol is evaporated.
11. Dissolve pellet in 50-100 µL nuclease-free (steam sterilized and DEPC treated) water (ddH_2O), wait for at least 1 h (though better results will be obtained overnight at 4 °C).
12. Measure the amount of DNA contained in each sample with a micro-volume spectrophotometer, according to the manufacturer's instructions. Make a working dilution of each sample containing approximately 2-10 ng DNA/µL. Keep the stock solution in the freezer. Keep the working solution in the fridge and ensure that further sample processing is done shortly.

2. Verify Functional Efficiency of DNA-extracts for Subsequent Detection of Recently Ingested Prey.

1. **To ensure the presence and functional efficiency of templates, test each DNA extract (steps 1.1 to 1.3.11) using universal primer sets suitable for the respective food source (*e.g.,* invertebrates[16,26]) that are targeting the same nuclear subunit as the group-specific primer, used for subsequent detection of prey[25,27]. The following steps refer to the use of universal primer sets NSF1419/20 and NSR1642/16[16] and LSU D1,D2 fw1 and LSU D1,D2 rev2 or LSU D1,D2 rev4[26]. To prove that the PCR reaction has been successful and that no contamination did occur, use a positive control containing DNA that had already been amplified successfully and one control sample containing ddH_2O instead of DNA within each PCR run.**
    1. To prepare primer working solutions that contain a final concentration 10 µM of the primer, pipette the appropriate amount of the respective primer stock solution and ddH_2O (depending on the concentration of the stock solution) into a fresh 1.5 mL reaction tube using micropipettes.
    2. Add primers, deoxynucleotide mix (dNTPs), reaction buffer, Taq DNA polymerase and ddH_2O in a 1.5 or 2.0 mL (depending on the number of samples to be processed) reaction tube, then vortex this mixture. Detailed volumes for the standard reaction mixture for a 10 µL PCR reaction are given in **Table 1** (for details of chemicals used, see **Table of Materials**).
    3. Use microliter pipettes to transfer each 1 µL of DNA extract into 9 µL of the standard reaction mixture within a PCR tube (or a well of a PCR plate). Close the reaction tube or the reaction wells of the plate (with cap stripes).
    4. Program the thermocycler: The standard protocol for the NSF1419/20 and NSR1642/16[16] primer set is: 94 °C for 4 min (denaturation), followed by 30 cycles of 94 °C for 30 s, 50 °C (annealing temperature) for 30 s, 72 °C for 90 s, and final extension at 72 °C for 10 min. For the LSU primer sets[26], use the following: 94 °C for 4 min, followed by 45 cycles of 94 °C for 20 s, 52.5 °C for 20 s, 72 °C for 90 s, and a final extension at 72 °C for 8 min.
    5. Place PCR tubes or plates into the thermocycler, close the lid of the cycler, and start the respective program.
    6. Prepare a 1.5% agarose gel using a buffer solution containing Tris base, boric acid and EDTA (TBE buffer), and agarose. For preparation of a 1.5% agarose gel, using a gel casting tray with overall dimensions of 10 cm × 7.5 cm × 3 cm, weigh 0.45 g agarose into a conical flask, use a measuring cylinder to gauge 30 mL of 1x TBE buffer (89 mM Tris, pH 7.6, 89 mM boric acid and 2 mM EDTA, pH 8.0) and pour it into the conical flask. Heat the mixture using a microwave. Stop the microwave whenever there are large air bubbles and carefully swirl the flask until the solution has only one phase.
    7. To cool down solution to approximately 60 °C, swirl the flask in a water bath. Swiftly pour the solution into a gel casting tray, remove air bubbles, and insert combs. Wait approximately 20 min until gel is hardened.
    8. Fill electrophoresis unit with 0.5x TBE and insert gel casting tray containing the agarose gel. Make sure that the gel slots are free of air bubbles.
    9. Spin down the PCR products using the short run option of a mini (plate) centrifuge. Use a microliter pipette to mix each 3 µL of PCR product with 1 µL of 6x loading dye (40% sucrose and 0.25% bromophenol blue) and load it into the gel slots of the agarose gel. Pipette 1.5 µL of a 100 bp DNA ladder into one slot of each line with a microliter pipette as size standard. Replace the lid of the electrophoresis unit correctly and connect leads to a power supply. Run the gel at 100 V/cm for 35 min.
    10. To prepare a staining bath, pour 1,000 mL of 1x TBE into a square-shaped plastic box impermeable to light. Add 50 µL of ethidium bromide with a microliter pipette, close the lid of the plastic box and shake it to ensure equal distribution of the ethidium bromide.
    11. Remove the gel from the electrophoresis unit and place it into the staining bath for approximately 10 min. Then, take the gel out of the staining bath and place it onto a gel documentation system and visualize it according to the manual.
    12. Analyze only samples that showed positive results (fragments of adequate size visual in agarose gel) in terms of presence and functional efficiency of templates for subsequent detection of recently ingested prey.

PCR-Mix	Concentration working solution	Mix for 10 µl PCR reaction	Final concentration in PCR
Primer FW	10 µM	0.5 µl	0.5 µM
Primer RW	10 µM	0.5 µl	0.5 µM
Reaction buffer (20 mM Tris-HCl, pH 8.55, 16 mM (NH4)2SO4 and 2 mM MgCl2 final concentrations)	1 µM	1 µl	1 µM
dNTP	10 mM	0.25 µl	0.25 mM
Taq polymerase	5 U	0.1 µl	0.05 U
ddH_20		6.65 µl	
Total amount reaction mixture		9 µl	
DNA		1 µl	

Table 1: Detailed protocol for the preparation of the standard reaction mixture for a 10 µL PCR reaction.

3. Detect Recently Ingested Prey/Food Items with Group-Specific Primer Sets Using Gel Electrophoresis.

1. **Conduct PCR using the working solution of DNA extracts (prepared according to steps 1.1 to 1.3.11) and the group-specific primer set (unlabeled, *i.e.,* without modification) appropriate for the prey group targeted like described in steps 2.1.1 to 2.1.3 (for variations in the reaction mixture for the respective primer set, see Table 2). Run PCR using the standard protocol given in step 2.1.4 (for annealing temperatures for the respective primer set, see Table 2).**
    1. Use one control with pure DNA from a specimen belonging to the respective target group (positive control) and one negative control with pure DNA from the consumer species in each PCR run.

2. Check the success of the PCR reaction using agarose gel electrophoresis described in steps 2.1.6 to 2.1.11. The PCR reaction was successful if a fragment of appropriate length (see **Table 2**) is visible for the positive control but not for the negative control. If one or both of these criteria are unfulfilled, repeat PCR.
3. Use agarose gel electrophoresis, as described in steps 2.1.6 to 2.1.11, to detect whether the prey group targeted had been consumed by the different consumer specimens tested (via judgment of whether the fragment of appropriate length is visible or not). Make sure not to miss fragments with low visual resolution.
4. Store PCR products at 4 °C, if further analyses will be done within the next 24 h, or at -20 °C if analyses are to be performed later.

4. Subsequently Determine Consumed Prey Further Downstream Using SSCP Analysis via Polyacrylamide Gel.

1. **Prepare a 9% acrylamide gel for SSCP electrophoresis. Prepare the working solutions in a laboratory hood.**
    1. To prepare a 200 mL acrylamide working solution, fill a measuring cylinder with 20 mL of 10x TBE (109 g of Tris base, 55 g of boric acid and 40 mL of 0.50 M EDTA, pH 8.0 dissolved in 1000 mL of ddH_2O) and another one with 45 mL of 40% (29:1) acrylamide mix. Pour TBE and acrylamide mix into a graduated flask (a graduated glass bottle with a 200 mL mark is also appropriate here) and add ddH_2O up to the 200 mL mark. Keep working solution in the fridge (4 °C) until needed. Do not use working solution older than one week.
    2. Weigh 0.1 g of ammonium persulfate (APS; use a balance with at least 0.01 g accuracy) into a 1 mL volumetric flask and dissolve it by adding ddH_2O up to the graduation mark to prepare a 10% APS stock solution. Transfer aliquots (approximately 350 µL) into fresh reaction tubes.
    NOTE: One aliquot is needed for each polyacrylamide gel. Store the remaining aliquots at -20 °C and only defrost aliquots shortly before they are needed.
    3. Assemble a gel cassette compromised of two glass plates, one blank and one notched glass plate (each 20 cm x 20 cm), with spacers (1.5 mm thick) between them running up the sides of the glass. Use each three fold-back clips (32 mm) on the right and left side to fixate gel cassette (**Figure 1**).
    4. Mix 5 g of agarose and 250 mL of 1x TBE buffer in a conical flask to prepare solution for a 2% agarose gel. Heat and afterwards cool down mixture as described in steps 2.1.6 and 2.1.7. Swiftly pour the solution into a square-shaped plastic box (21 cm x 6.5 cm x 5 cm) and place the lower end of the gel cassette (step 4.1.3) into the agarose solution. Adjust the fold-back clips at the lower end of the gel cassette to a height so that they sit on the rim of the square-shaped plastic box (**Figure 1**). Wait approximately 20-30 min until the agarose plug has completely hardened.
    5. Fill a 100 mL measuring cylinder with the acrylamide working solution (step 4.1.1) until the 60 mL mark and pour solution into a beaker. Use a micropipette to add 300 µL of one aliquot of the APS stock solution (step 4.1.2) and to subsequently add 37.5 µL of tetramethylethylenediamine (TEMED). Swiftly, pour the solution between the glass plates of the gel cassette. Alternatively, inject the solution between the glass plates with a 100 mL disposable syringe with a 1,000 µL pipette tip adjusted to the injection head of the syringe.
    6. Carefully insert a 1.5 mm thick standard comb at the upper end between the glass plates. The comb must be surrounded by the polyacrylamide solution. Wait approximately 1 h until the polyacrylamide gel is polymerized. Avoid ventilation during polymerization as far as possible because it increases the time needed for the gel to polymerize.

NOTE: It is possible to store casted gels within sealed plastic bags with a wet tissue in the fridge up to 3 days.

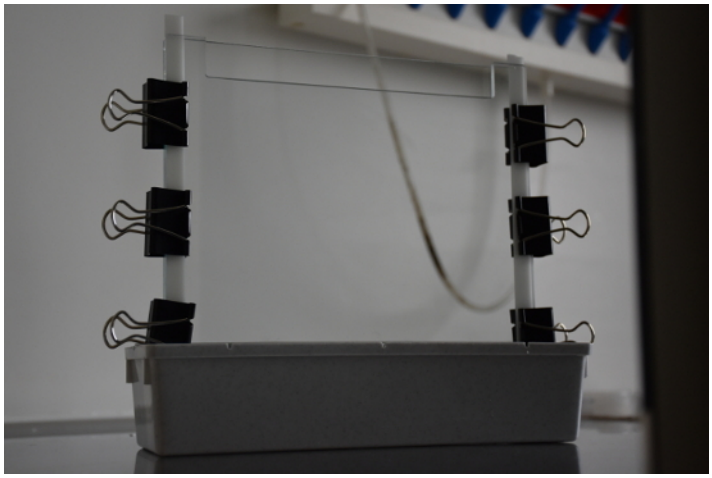

Figure 1: Picture illustrating the construction to pour polyacrylamide gels. Please click here to view a larger version of this figure.

2. Place the gel cassette, containing the polyacrylamide gel prepared, into a vertical electrophoresis unit. Fill the electrophoresis unit with the appropriate amount (depending on the type of electrophoresis unit) of 0.5x TBE and cool the unit down to 10 °C using a refrigerated circulator.
3. For DNA denaturation, preheat a thermal-block/-cycler to 96 °C. Prepare denaturation buffer with 95% formamide (99.5% p.a. grade) and 5% aqueous solution containing 0.25% bromophenol blue and 40% (w/v) sucrose.
4. Take the PCR products out of the fridge or the freezer and defrost them. Spin down the PCR products using the short run option of a mini (plate) centrifuge. Then, mix each 4 µL of a PCR product with 4 µL of denaturation buffer. Heat mixtures for 5 min at 96 °C immediately followed by a cooling step in iced water for 10 min.
   NOTE: Samples should be loaded onto the polyacrylamide gel (step 4.5) immediately after this.
5. Remove comb from the polyacrylamide gel (step 4.1.6) and use a micropipette to completely load each sample into a gel slot. Run electrophoresis at 8 W per gel while constantly cooling the electrophoresis unit to 10 °C. Running time needed depends on the size of fragment to be separated. For fragment sizes of approximately 210 bp, a running time of about 3.5 h is considered appropriate.
6. To prepare a staining bath, pour 1,000 mL of 1x TBE into a square-shaped plastic box impermeable to light and add 30 µL of staining dye (suitable to stain single stranded DNA) with a microliter pipette. Close the lid of the plastic box and shake it to ensure equal distribution of the staining dye.
7. Remove the gel cassette from the electrophoresis chamber and carefully separate the notched glass plate from the gel. Slide the polyacrylamide gel from the blank glass plate into the staining bath. Place the staining bath onto a shaking platform and stain gel for 20 to 30 min with constant shaking.
8. Take the gel carefully out of the staining bath and place it onto the UV table of a documentation system.
   NOTE: Due to the instability of the gel it is advisable to handle the prior step with two people. One person should hold the staining bath next to the UV table and another one should reach under the gel with both hands, take it out, and slide it onto the UV table.
9. Close the lid or door of the documentation system and take a photograph according to the manufacturer's instruction manual.

5. Detect Multiple Recently Ingested Prey Groups in Parallel Using Automated Fragment Analyses.

1. **Analyze the gut content DNA extracts using the 16 group-specific primer sets given in Table 2, with one primer of a set labelled due to modification with a staining dye (see Table 2 column titled Modification), and use the parallel detection methods of an automated sequencer.**
   1. Use the working solution of DNA extracts (prepared according to steps 1.1 to 1.3.11) and conduct separate PCRs for each primer set like described in steps 2.1.1 to 2.1.5 (variations in the mixture are given in **Table 2**). Use at least one control with pure DNA from a specimen belonging to the respective target group (positive control) and one negative control with pure DNA from the consumer species in each PCR run.
   2. Run PCRs in the thermocycler using the standard PCR protocol given in step 2.1.4, adjusted to the respective annealing temperature (and number of cycles) for each primer set given in **Table 2**.
      NOTE: Use the same DNA allocation of reaction wells for PCR with each of the 16 different primer sets to simplify subsequent pooling of PCR products for batched automated fragment analyses.
   3. Store PCR products at -20 °C until all PCRs belonging to one batch for the automated fragment analyses (see **Table 2**) are finished. Keep PCR products in the dark and do not store them longer than 1 week prior to fragment analyses.

	Target taxon	Primer names	Primer sequences 5'-3'	Modification	Annealing temperature [°C]	Product size [bp]
Batch A	Ephemeroptera	Epa28Sf	CGGGGCGTCTCGATCTA	Cy5	56	129-135
		Epa28Sr	GCTACGGACCTCCATCAGGG	none		
	Caenis + *Ephoron*	Caep28Sf	CCAAGGAGTCTAACACGTGT	Cy5	60	182-185
		Caep28Sr	TGCTCTTGGTGCACCTCAC	none		
	Bithynia	Bit18Sf	ACAGCTCCGACCCCTTCAAC	IRD700	60	203
		Bit18Sr	TTCCCCGCTACCCGTTGCC	none		
	Mysidae	Mys28Sf	TCATTGGGTCTACTAAACCC	Cy5	60	240
		Mys28Sr	ATCGATTTGCACGTCAGA	none		
	Ecnomus + *Psychomyia*	Ecpsy18Sf	TCATTTGCCTGAATATTGTGC	IRD700	55	298
		Ecpsy18Sr	ACCGAATCAAAATAAAAAA	none		
	Rhyacophila	Rhya18Sf	CTCAAAGCGGGCTAAGTT	Cy5	60	314
		Rhya18Sr	CCACCGGGTTAAAATAATG	none		
	Dreissena *; ****	Dreis18Sf	GTTAGCCCAGACCAACGCG	IRD700	62	339-346
		Dreis18Sr	TCCTCGCTCAAGGATCTAAA	none		
	Heptageniidae	Hpt28Sf	TAGAAGGTGCCAGGCCTGTG	IRD700	55	550-562
		Hpt28Sr	GACAAGTCCGCACAACGACA	none		
Batch B	*Asellus*	Ase28Sf	AACGGACGAGACGGGGATTT	IRD700	60	135
		Ase28Sr	CCTAGGGGTTTAGCCAGCC	none		
	Jaera	Jae28Sf	AACTATCCGTCCCGTCTTGA	Cy5	60	179
		Jae28Sr2	CCAACATATACGCTCTGAG	none		
	Ephemera	Eph28Sf	TAACGCTCGAACGAGGGG	Cy5	58	254-256
		Eph28Sr	CCCGCGAAGAACAACCGTCG	none		
	Corophium	Coro18Sf	AGTTCCCTCTTGCGCTTGTC	IRD700	60	245
		Coro18Sr	AAGCCATCGTTCGCGTGTA	none		
	Gammarus *; **; ***	Gam18Sf	GGGAATCGGTCAAACGGAGT	Cy5	67	312
		Gam18Sr	ACAGAATTTCACCTCTCGCCT	none		
	Simulium	Sim28Sf	GAGCTTACATATTTAATG	IRD700	48	336
		Sim28Sr	GATACCCAGGGATGGAGAAA	none		
	Chironomidae	Chiro18Sf	GAACTAGTTAACTATGTT	Cy5	50	355-360
		Chiro18Sr	TATTCCATGCAAAAATATTCA	none		
	Corbicula	Corbicul28Sf	TCGGATCCGCAAGGACCG	IRD700	55	375
		Corbicul28Sr	TTCGTACGGACCCCATGAC	none		

The product size is the fragment length for species belonging to the respective target group found by automated fragment analyses in our lab. Deviations from the standard PCR protocol: *, 25 cycles; **, 0.025 mM dNTPs; ***, 1× reaction buffer S (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$ final concentrations;); ****, 4.5 mM $MgCl_2$ final concentration.

Table 2: Details of the 2 batches of 15 group-specific primer pairs established by Koester *et al.* (2013) and the newly designed Jae28S primer pair amplifying regions of the 18S or 28S rDNA from 16 target groups of aquatic macroinvertebrates. f, forward primers; r, reverse primers; 18S, primer targets the 18S rDNA subunit; 28S, primer targets the 28S rDNA subunit. "Hydrobiologia, Is *Dikerogammarus villosus* (Crustacea, Gammaridae) a 'killer shrimp' in the River Rhine system?, 768, 2016, Table S1, Supplementary material page 2, Koester Meike, Bayer Bastian, Gergs René, (© Springer International Publishing Switzerland 2015)" With permission of Springer. Please click here to view a larger version of this table.

2. **To detect the amplified fragments of all 16 primer sets conduct automated fragment analyses in the 2 batches given in Table 2. Use an internal size standard (DNA Size Standard Kit - 400 [Batch B] and - 600 [Batch A], respectively) to determine fragment length. Prepare the sequencing plate and automatic sequencer as described in the following steps. Note that this procedure might have to be adjusted if using another platform than that given in the Materials Table.**
    1. To run each batch-sample, prepare a mixture containing Sample Loading Solution (SLS) and the respective size standard. Pipette 21.6 µL of SLS and 0.4 µL of DNA Size Standard Kit - 600 per sample of Batch A and, respectively, 21.7 µL of SLS and 0.3 µL of DNA Size Standard Kit - 400 per sample of Batch B.
    2. Take the PCR products of the 8 PCRs belonging to the respective batch out of the freezer and defrost them. Ensure that they are still kept in the dark.
    3. Use a microliter pipette to load the number of wells of a sequencing plate needed (number of samples to be analyzed) with each 22 µL of the respective mixture described in step 5.2.1. Spin down the PCR products of each of the 8 PCRs using the short run option of a mini (plate) centrifuge. Transfer each 1 µL per PCR product of each of the 8 PCRs into the respective wells of the sequencing plate with a microliter pipette.
    4. Carefully spin down this mixture within the sequencing plate with a short run of a mini plate centrifuge and overlay each of the wells used with one drop of mineral oil. Fill respective reaction wells of a buffer plate with 250-300 µL of DNA separation buffer.
    5. Use the software for the capillary sequencer to create a Sample Setup according to the manufacturer's instructions. Assign the methods to control sample sets and determine the sequence of methods to be used to process data (see **Table 3** for running conditions adequate for use with the size standard given in the **Materials Table**). Note that it is crucial that the allocation of samples within the Sample Setup matches the sample allocation in the sequencing plate (including positive and negative controls, see step 5.1.1) and that the method adequate for fragment analyses with the respective size standard is chosen.
    6. Fill a wetting tray with deionized water and insert the sequencing plate, buffer plate and wetting tray into the capillary sequencer. Insert a gel cartridge containing a sufficient amount of fresh DNA separation gel which is needed for the sample run. Run the sequencing plate using the prepared Sample Setup.

	Denature		Seperate		Capillary	Inject	
	Temperature	Time	Voltage	Time		Voltage	Time
size standard 600	90 °C	120 s	4.8 kV	60 min	50 °C Wait for Temp: YES	2.0 kV	30 s
size standard 400	90 °C	120 s	6 kV	45 min	50 °C Wait for Temp: YES	2.0 kV	30 s

Table 3: Specific conditions for fragment analyses upon the automated sequencer using the size standards given in the Materials Table.

3. **To analyze the results export raw data and upload those data to a fragment analysis program. Choose standard dye color orders of the respective manufacturer to set dye color channels.**
    1. Create a Panel outlining the expected range of fragment length amplified by the single group-specific primer sets within a batch according to the user manual of the program.
    2. To process the data, select the respective Panel, appropriate size standard, standard color, and type of analysis and run procedure. Choose the electropherogram option to display fluorescent signal intensities from capillary electrophoresis instruments as a single line trace for each dye color. Markers/primer sets will be displayed indicating the respective fragment range and the exact length of the detected fragment associated with the center of each called peak will be highlighted (in most programs by coloration or name assigned) automatically.
    3. For each sample, calculate how closely the sample's internal size standard matches the selected size standard to reassure the success of the size calling. Most fragment analyses programs have an option to automatically calculate and visualize this match. If the size calling failed, repeat fragment analyses for those samples.
    4. Visually recheck the electropherogram of each sample and confirm/unconfirm each peak called for each marker/primer set separately. Manually insert uncalled peaks that fit the criteria of a marker/primer set or delete wrong ones according to the user manual of the program used. Apply the base pair size or bin name in the 'Allele Label'. Calculate and display the peak specifications in the 'Peak Table'.

Representative Results

We successfully used steps described in this protocol for diet analyses within different studies. In this section, we present examples describing the applicability of the different sections of the protocol.

As an example, to demonstrate the effectiveness of group-specific primer sets established by the authors[28] and the potential of further downstream analyses of PCR products by SSCP analyses, a feeding experiment was conducted. Individuals of *Dikerogammarus villosus* as predators and *Caenis* spp. larvae as prey were captured in the River Rhine and backwaters near Karlsruhe (Germany). In the laboratory, predator and prey taxa were kept separately in filtered stream water at 20 °C and were starved for 36 hours. For the experiment, amphipod specimens were placed in beakers with filtered stream water (100 mL) individually, fed with two to three *Caenis* larvae for 30 min, and subsequently frozen in liquid nitrogen. The gastrointestinal tract of each *D. villosus* was dissected and DNA was extracted as described in Protocol 1. DNA extracts were tested with the Caep28S primer set suitable to detect *Caenis* spp. using the appropriate PCR protocol[28] as described in Protocol 3. To verify differences between amplified fragments of different *Caenis* species, PCR was conducted with the gut content samples and pure DNA samples of three *Caenis* reference species. Agarose gel electrophoresis led to single bands of double stranded DNA, which cannot be distinguished between the different *Caenis* species with this type of electrophoresis (**Figure 2A**). In contrast, by SSCP gel electrophoresis, as described in Protocol 4, it was possible to identify prey organisms to species level by comparing the gut content samples with the reference samples (**Figure 2B**). The SSCP fragment pattern of the three reference taxa *Caenis beskidensis* (**Figure 2B**, lane 1), *Caenis horaria* (**Figure 2B**, lane 2), and *Caenis luctuosa* (**Figure 2B**, lane 3) consisted of four bands with differentiable patterns. Two gut content samples (**Figure 2B**, lane 4 and 5) had a fragment pattern equal to that of *C. luctuosa* (**Figure 2B**, lane 3). The fragment pattern of the third gut content sample (**Figure 2B**, lane 6) was identical to that of *C. horaria* (**Figure 2B**, lane 2). Sequence analyses of the two gut content samples identified as *C. luctuosa* and *C. horaria* (**Figure 2B**, lane 4 and 6) and the respective reference species verified this result (see electronical supplement alignment fasta file).

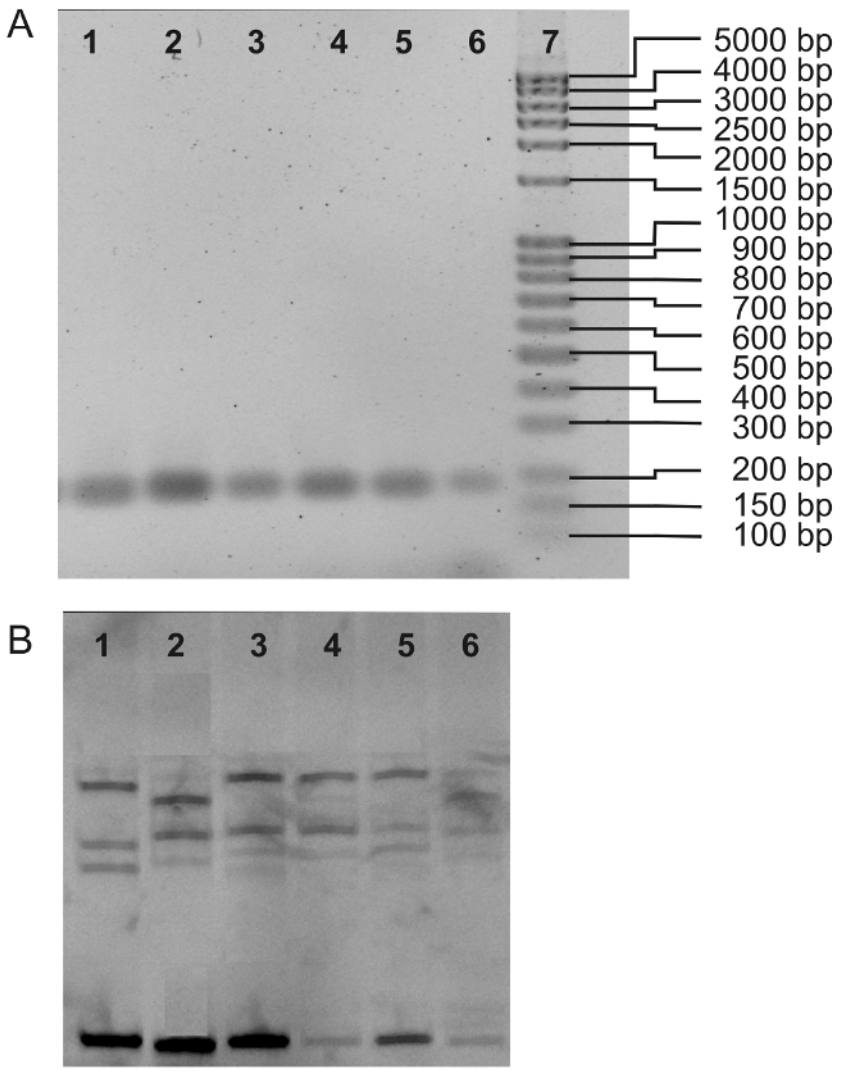

Figure 2: Original image of (**A**) agarose gel electrophoresis and (**B**) SSCP analysis of PCR fragments of gut content and reference samples amplified with the Caep28S primer set. Lanes 1-3 show the reference samples of the species *Caenis beskidensis* (1), *Caenis horaria* (2) and *Caenis luctuosa* (3). In lanes 4-6, the fragment pattern of different gut content samples of the amphipod *Dikerogammarus villosus* are presented. Lane 7 shows the fragment pattern of a 100 bp DNA ladder. Please click here to view a larger version of this figure.

Furthermore, a laboratory experiment was conducted in which water mites belonging to the species *Hygrobates fluviatilis* were fed on chironomid larvae to not only determine if DNA of chironomid individuals consumed can be detected in the water mites, but also to test if the amount of DNA detected depends on the elapsed time since consumption of the prey[29]. After starving the mites for approximately one week, each mite was offered one chironomid larva as food. Each 3-4 water mite individuals were fixed in ethanol (absolute) for genetic analysis using the Chiro18S primer set specific to the dipteran family Chironomidae[28] at 1, 2, 3, 7, 9, 24, 32 and 50 h after being fed[29]. As a degree of consumption by the water mite individuals, the respective prey individuals were classified into the following five feeding categories: 1 = completely sucked out, integument completely emptied (>90%), 2 = almost completely sucked out (>75-90%), 3 = semi sucked out (>50-75%), 4 = only partially sucked out (>5-50%), 5 = no visible change in the prey's body structure (≤5%)[29]. DNA was extracted from the water mites as described in Protocol 1 (without steps 1.2.1 and 1.2.2) and the presence of 18S rDNA in the extract and the functional efficiency of the template were verified as described in Protocol 2. Detection of prey DNA was performed according to Protocol 4 using only the Chiro18S primer set with the forward primer labeled with a fluorescent dye (see **Table 2**) and the DNA Size Standard Kit - 400. To allow comparisons between samples analysed within different runs of an automated sequencer, the ratio between sample peak height and the peak height of the nearest internal standard (*i.e.*, 360 bp in this case) of each sample were calculated and used as a proxy for the amount of DNA detected. Results showed that chironomid DNA was detectable in virtually all individuals of *Hygrobates fluviatilis* fed on chironomid larvae[29]. From the shortest interval (1 h after feeding) to the longest period after feeding (50 h) the relative amount of detected prey DNA was significantly reduced (**Figure 3A**)[29]. Furthermore, the relation between time after feeding and the prey classification as a proxy for the ingested amount of prey DNA could be confirmed (**Figure 3B**)[29]. The reduction of prey DNA detected with increasing time after feeding was most likely completely reflected by the degradation of DNA and digestion of the prey, because egestion of prey DNA is highly implausible due to the lack of an anus in water mites[29]. These results confirm the suitability of this approach for the quantification of ingested prey.

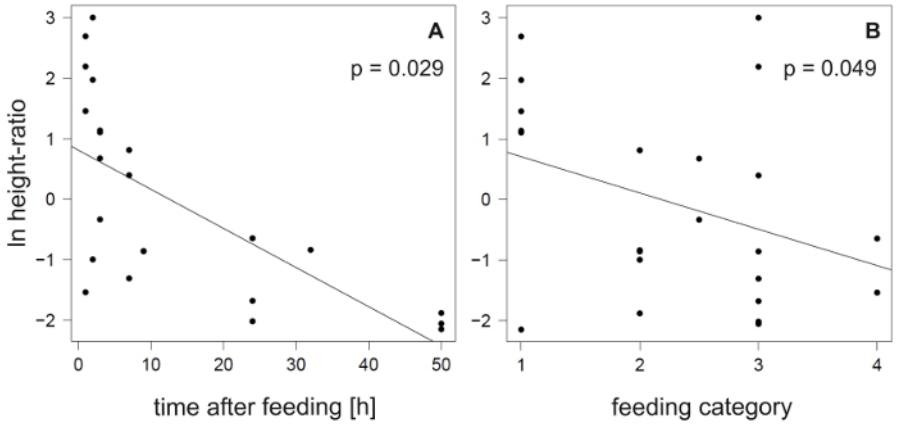

Figure 3: Relationships between the national ln height-ratio (sample-peak heights/standard$_{360}$ peak height) and (**A**) time after feeding of mites and (**B**) feeding category (n = 23). "Experimental and Applied Acarology, First detection of prey DNA in *Hygrobates fluviatilis* (Hydrachnidia, Acari): a new approach for determining predator-prey relationships in water mites, 67, 376, P. Martin, M. Koester, L. Schynawa, R. Gergs, (© Springer International Publishing Switzerland 2015)" With permission of Springer. Please click here to view a larger version of this figure.

To investigate the importance of predation by the invasive amphipod *D. villosus* in the field, intensive stable isotope analyses of $\delta^{13}C$ and $\delta^{15}N$ of *D. villosus* and potential food resources were furthered by molecular detection of recently ingested prey within the gut contents of the exact same individuals. In total, 206 *D. villosus* individuals from different sites were collected and the gastrointestinal tract of each individual was dissected and DNA was extracted according to Protocol 1. The presence and functional efficiency of the templates used was assured as described in Protocol 2. Recent predation by the *D. villosus* individuals on multiple macroinvertebrate prey taxa was tested as described in Protocol 4. Overall, only 16% of the *D. villosus* gut contents tested positive for DNA belonging to any of the 16 targeted macroinvertebrate prey taxa, and therefore supported the results of the stable isotope analyses that indicated a low predacious feeding behavior of the invasive amphipod in the field[27]. While, within the genetic analyses, DNA of 12 tested prey taxa was found in at least 1 gut content sample, DNA of the 4 other prey taxa was never detected (**Figure 4**)[27].

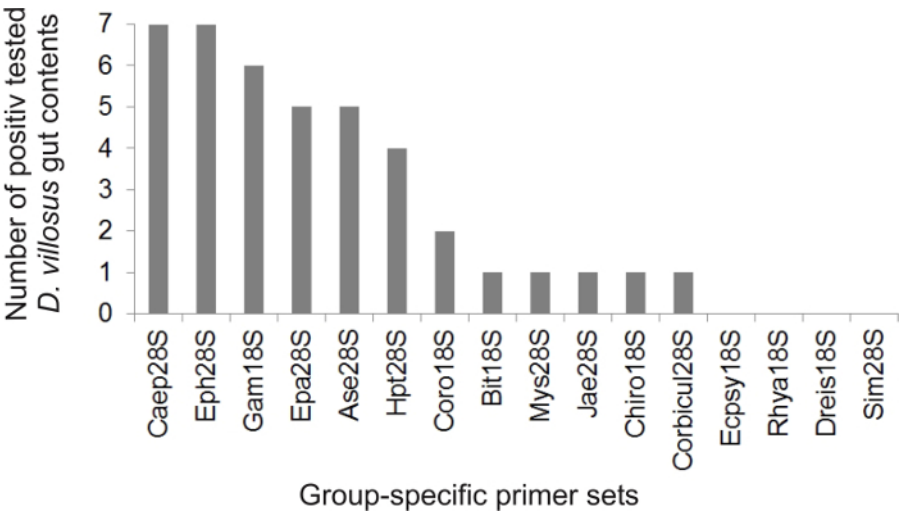

Figure 4: Histogram illustrating the respective number of *D. villosus* individuals from all ten sites whose gut contents tested positive for macroinvertebrate prey DNA. PCR was carried out with 16 group-specific primer sets as indicated. "Hydrobiologia, Is *Dikerogammarus villosus* (Crustacea, Gammaridae) a 'killer shrimp' in the River Rhine system?, 768, 2016, 310, Koester Meike, Bayer Bastian, Gergs René, (© Springer International Publishing Switzerland 2015)" With permission of Springer. Please click here to view a larger version of this figure.

Supplemental File. Sequence_alignement_feeding_trial.fasta Please click here to download this file.

Discussion

Here, we present an easy and cost-efficient method for PCR-based determination of predator-prey interactions from field samples via group-specific primers for rDNA regions, which can be combined with other non-molecular analyses of the same specimens. However, there are several critical steps within the protocol. Firstly, assuring the specificity of the primers used is essential. Secondly, it is crucial to avoid contamination and loss of gut content material during the preparation of the gastro-intestinal tract and the subsequent extraction of DNA. While cross-sample contamination can lead to false positive detection of prey DNA, loss of gut content material might lead to missing rarely consumed prey species. Verification of the presence of rDNA belonging to the respective subunit and the functional efficiency of templates used for the analyses (section 2 of the protocol) is essential for gathering representative information about the prey consumed. This is in particular crucial, if no DNA of prey groups the consumer is supposed to feed on can be detected[25,27]. Furthermore, when preparing the PCRs (steps 3.1, 5.1.1

and 5.1.2) it is crucial that the mixture and protocol used exactly fit the criteria in which the respective primer was specified to a certain group of taxa. Otherwise, either amplification of DNA within the PCR reaction might fail completely, or the DNA of taxa not targeted might be amplified as well. Therefore, it is obligatory to use positive and negative controls within each PCR run to assure that the PCR reaction worked and no contamination occurred. Additionally, it is necessary that the array of the samples for each PCR run is documented exactly to enable the correct assignment of prey consumed to the respective consumer specimen. For this task, particular caution needs to be taken while pooling PCR products for parallel detection of multiple prey groups via automated fragment analyses (step 5.2.3).

For the detection of amplified fragments using agarose gel electrophoresis (steps 3.2 and 3.3) agarose gels should be as thin as possible and care has to be taken during the visual inspection of the image to avoid missing fragments with low visual resolution. For results and an appropriate conclusion about prey consumption by a consumer, it is crucial that even rarely consumed prey taxa are being recognized. Thick agarose gels diminish the visibility of potentially low amounts of prey DNA and thus reduce the probability to miss rarely consumed prey and introduce uncertainties in the conclusions drawn from the analysis. Furthermore, from a health perspective, using a less- or non-toxic alternative than ethidium bromide for staining the gels might be advisable. The protocol for subsequent further downstream analysis of amplified fragments via SSCP analyses using polyacrylamide gels includes some steps that should be performed with particular caution. It is important that gel cassette assembled is leak-proof (step 4.1.3) and the polyacrylamide solution is given enough time to polymerize completely (step 4.1.6). Opening the cassette too early will lead to leaking liquid acrylamide solution and, despite the fact that the preparation has to be started from the beginning, extensive cleaning up needs to be done as well. Furthermore, transferring the polyacrylamide gel into the staining bath (step 4.7) and from there onto the UV table (step 4.8) needs to be done very carefully due to the instability of such gels. Otherwise, the gel might tear and fragments could be disrupted, which leads to the necessity to repeat the procedure.

One major requirement for successfully processing data received from automated fragment analyses is that the sample's internal size standard closely matches the size standard pattern given by the manufacturer (step 5.3.3) to enable determination of the appropriate fragment length. This is especially crucial because otherwise, different fragments, labelled with the same fluorescent staining dye, might be assigned to the wrong prey taxa and therefore lead to a complete misjudgment of predator-prey interactions. Furthermore, electropherograms often show background noise. To establish confidence in the interpretation, it is essential that the peaks of the internal size standard are significantly higher than the background noise of the sample. Consequently, peaks for each marker/primer should only be counted if they are significantly higher than the background noise.

This protocol can be modified to detect several groups of prey taxa of interest within various consumers of interest. Admittedly, group-specific primers may not already be available for every potential prey taxon of interest. Nevertheless, group-specific primers are available not only for several freshwater macroinvertebrate taxa[28], but also for a broad range of other taxa (*e.g.,* several marine macroinvertebrate taxa[16,30] and common taxa of flying insects[31]). Thereby, the selection of group-specific primer sets suitable to amplify DNA of all the prey species of a given taxon, but unsuccessful in the amplification of DNA from species not belonging to this taxon, is essential[32]. Many group-specific primers that are now available have been specified for a given range of taxa (*e.g.*, 130 taxa of freshwater macroinvertebrates common in the River Rhine system[28]). Therefore, to avoid false-negative or false-positive results, the specificity of such primers should be rechecked for the range of taxa common in the ecosystem of interest prior to their application for target taxa and consumers not included in the range of taxa for which the primers had been established. Furthermore, if those molecular analyses are not to be combined with other analyses of the exact same individual, dissection of the gastrointestinal tract might be skipped.

Nevertheless, dissecting the gastrointestinal tract of larger specimens also prevents failure of DNA extraction because of too much material, and reduces the presence of consumer-DNA in the sample extract. However, studies have shown that using specific blocking primers, which attach to the DNA of the consumer and thereby block it, for the PCR reaction can effectively enhance the amplification of rare sequences in mixed samples[33]. Therefore, dissection of the gastrointestinal tract can be avoided, even when dealing with larger consumer specimens, by the use of specific blocking primers. For parallel detection of amplicons derived from multiple group-specific primer sets via automated fragment analyses, care should be taken in the choice of staining dye as label. Thereby, primer sets amplifying fragments of approximately the same length should be labelled with clearly distinguishable fluorescent dyes. Moreover, there are a range of capillary electrophoresis systems available from several manufacturers. Some of these systems supposedly allow the determination of multiple fragments even via unspecific dyes. The protocol presented here could easily be adjusted to detect amplified fragments with any of those systems. Apart from that, to reduce the time needed for the analyses, it would be possible to optimize single multiplex-PCR assays to amplify DNA of prey groups of one fragment analysis batch simultaneously (for instance, simultaneous amplification of 12 prey organisms of beetles[34] or up to six flying insect taxa[31]).

A potential disadvantage of gut content analyses in general, and therefore also the analyses described in this protocol, is the low temporal resolution. With such methods, it is only possible to identify recently ingested organisms, which might lead to uncertainties in the importance of single prey organisms[2]. However, we demonstrated that genetic analysis according to this protocol can easily be combined with analyses of stable isotopes ($\delta^{15}N$ and $\delta^{13}C$) of the exact same consumer specimens[25,27]. As a time-integrating natural tracer of predator-prey interactions, stable isotope analyses are frequently used to characterise food web structures[1], but potential overlapping isotopic values from different prey species often impede an exact definition of predator-prey interactions[2]. Therefore, using this protocol for genetic analyses in combination with stable isotope analyses to overcome the disadvantages of each method can further our understanding of food webs and their relationship to nutrient or energy fluxes.

Disclosures

The authors have nothing to disclose.

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