

Supplementary File 1: PNA Clamp Development

The PNA clamp (PNA Bio, Thousand Oaks, CA) was developed to reduce amplification of *Apis mellifera*, to be used in conjunction with the COI metabarcoding primers FwhF2 (5'-GGDACWGGWTGAACWGTWTAYCCHCC-3') and FwhR2n (5'-GTRATWGCHCCDGCTARWACWGG-3') (Vamos et al. 2017). These metabarcoding primers were chosen as they have been shown to recover >95% of species in mock arthropod communities, show minimal variation in species recovery based on changes to annealing temperature, and amplify a short ~200 bp region ideal for degraded eDNA samples (Elbrecht et al. 2019). The PNA clamp was designed for high specificity to *A. mellifera* and multiple nucleotide mismatches to relevant bee pests including *Varroa* spp. and Asian honeybee species.

Confirmation of reduced A. mellifera amplification

Tissue samples of *A. mellifera*, *A. cerana*, *A. florea*, *V. destructor*, and *V. jacobsoni* were extracted using a chelex DNA extraction method and quantified using a Nanodrop 8000 Spectrophotometer (Thermo Scientific). Samples were then diluted to 2 ng/μL. All samples were initially amplified via a gradient quantitative PCR (qPCR) for the COI markers only to confirm appropriate cycling conditions. qPCR reactions were carried out in 15 μL volumes using 7.5 μL of SensiFAST SYBR No-ROX mix (Bioline), 0.6 μL each of forward and reverse primer (10 uM), 4.3 μL of ultrapure H₂O, and 2 μL of DNA. Samples were amplified using a CFX96 Touch Real-Time PCR Detection System (BioRad) following an initial denaturation at 95°C for 5 minutes, followed by 45 cycles of 95°C for 30 seconds, annealing step (45-50°C) for 30 seconds, and an extension of 72°C for 50 seconds. Following the identification of a suitable annealing temperature the PNA clamp was tested at final reaction concentrations of 1, 2, 3, 4, and 5 uM to identify optimal performance of the clamp. qPCR reactions and protocols were followed as described above, with the following modifications: amount of H₂O adjusted to account for the addition of the PNA clamp, 50°C annealing temperature, and the number of qPCR cycles reduced to 40. qPCRs were run in triplicate to ensure the results produced were consistent with DNA negative controls run throughout, and samples amplified without any PCR blockers in each reaction as an additional control.

The PNA clamp showed a reduction in amplification of *A. mellifera* by six cycles, while only impacting *V. destructor* amplification by one cycle (Fig.1). No other non-target species tested showed any reduced amplification when the PNA clamp was included in the PCR reaction. The effectiveness of the PNA clamp did not considerably differ across any of the concentrations tested; we elected to use a 2uM concentration for metabarcoding samples for this study.

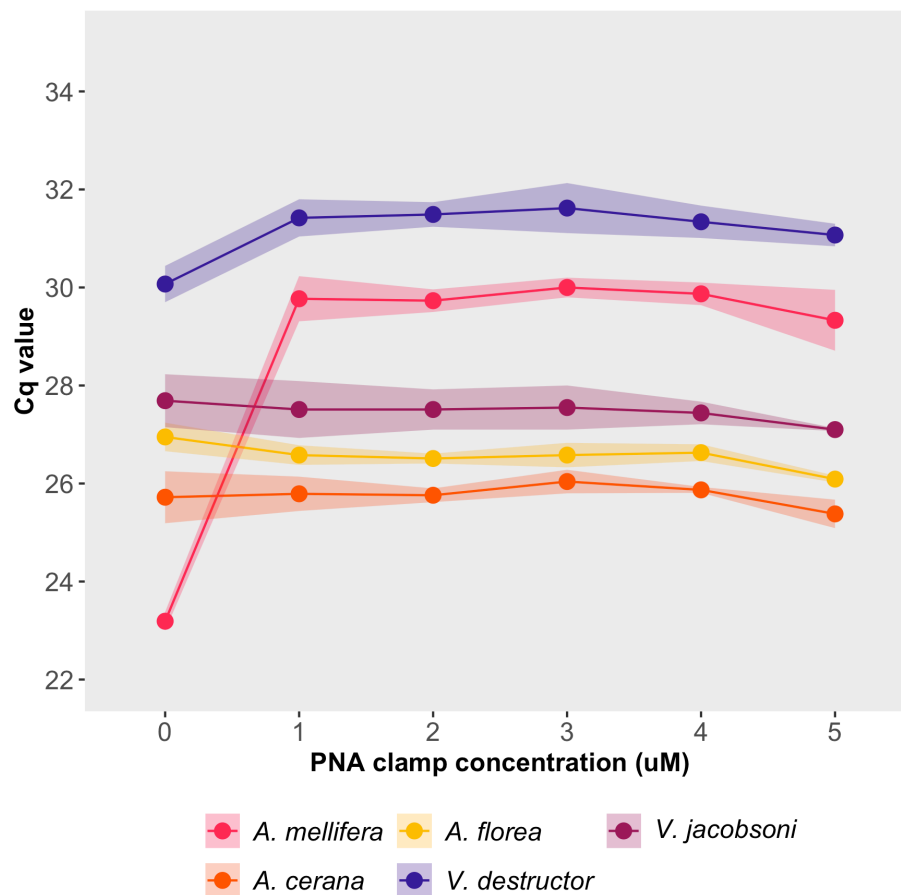


Fig. 1 Mean Cq results across three replicate runs, tested on three honeybee species (*A. mellifera*, *A. cerana*, and *A. florea*) and two varroa mite species (*V. destructor* and *V. jacobsoni*), at a range of PNA clamp concentrations. Shaded regions represent standard deviations.

References

- Elbrecht V, Braukmann TWA, Ivanova NV, Prosser SWJ, Hajibabaei M, Wright M, Zakharov EV, Hebert PDN, Steinke D (2019) Validation of COI metabarcoding primers for terrestrial arthropods. *PeerJ* 7: e7745. <https://doi.org/10.7717/peerj.7745>
- Vamos E, Elbrecht V, Leese F (2017) Short COI markers for freshwater macroinvertebrate metabarcoding. *Metabarcoding and Metagenomics* 1: e14625. <https://doi.org/10.3897/mbmg.1.14625>