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ORIGINAL ARTICLE

The prospects of using retrotransposon iPBS molecular marker to characterise the genetic diversity of *Chrysochraon dispar* and *Stethophyma grossum* (Orthoptera: Acrididae)

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Abstract. Molecular markers have become indispensable tools in contemporary ecological research, offering insights into genetic diversity and structure. These parameters are pivotal for addressing fundamental questions in landscape ecology and planning effective species conservation. Grasshoppers have one of the largest genomes known. A significant portion of the grasshopper genome is composed of mobile genetic elements, with a particular abundance of retrotransposons. In this study, we utilised the iPBS (inter-primer binding sequence) PCR based fingerprinting marker system, as a novel approach based on retrotransposons for the study of Orthoptera. We evaluate the efficacy of the iPBS primers system in characterizing the genetic diversity of two large-genome grasshopper species, *Stethophyma grossum* and *Chrysochraon dispar*. Our findings demonstrate the potential of iPBS markers as a valuable tool for assessing the genetic diversity of orthopterans. This approach offers a promising avenue for future research in population genetics and conservation biology.

INTRODUCTION

Population genetics studies become increasingly important when developing practical solutions to mitigate the loss of habitats and species. Molecular studies of Orthoptera populations are aimed at both pest species (Li et al., 2010; Rosetti & Remis, 2012) and rare and endemic species (Nogureales et al., 2016). In this study, we focus on the potential of using the genetic structure of habitat specialist species as indicators for landscape-level processes. *Stethophyma grossum* (Linnaeus, 1758) and *Chrysochraon dispar* (Germar, 1834) both belong to the wetland species community, who are specialised for living in habitats like transition mires, fens, and wet grasslands (Sardet et al., 2021; Starka et al., 2022). Both species are distributed across most of Europe (GBIF, 2024a, b), and in the Baltics are fairly common in suitable habitats (Budrys & Pakalniškis, 2007; Budrys et al., 2008; Runnel, 2017a, b; Starka et al., 2022). In Latvia, all the EU-protected habitat types suitable for these species have unfavourable-inadequate (U1) or unfavourable-bad (U2) conservation status (DAP, 2019), which highlights the necessity to develop habitat functionality indicators for these habitats.

Reliable indicators are needed to assess the quality of and necessary protection measures for habitats (Dvořák et al., 2022). Changes in the occurrence or other response variables of the selected indicator organism can be microhabitat, habitat-patch or landscape-level driven, and can indicate processes in various temporal scales. Invertebrates, especially habitat specialist species, rapidly respond to changes in environmental conditions (Perner & Malt, 2003). Orthopterids make up most of the invertebrate biomass in grassland habitats (Labadessa et al., 2014), and their response to environmental changes can be detected using a relatively small sample size (Bazelet & Samways, 2011). Orthoptera are well-established indicators of habitat quality (Maes & Van Dyck, 2005; Gardiner et al., 2005; Bazelet & Samways, 2011; Fartmann et al., 2012), higher trophic levels (Senn et al., 2011), and landscape functionality (Keller et al., 2013), including the role of landscape structure on gene flow (Tinnert et al., 2016). Population genetic structure is determined by population dynamics, which, on the landscape level, is influenced by the effects of habitat amount, fragmentation and barriers to dispersal (Jackson & Fahrig, 2016). Orthoptera species have diverse wing morphology (from apterous to macropterous species),

this generally translates to dispersal abilities which allows for the assumption of the geographical scale of indication. However, there is more to dispersal than just wing morphology – locomotory behaviour, physiology, sex, wing morphs, population processes, and landscape features also play an important role (Reinhardt et al., 2005; Poniatowski & Fartmann, 2011; Tinnert et al., 2016).

The Orthoptera genome has been extensively studied in recent years. The Orthoptera order, especially the short-horned grasshopper family Acrididae, is generally characterised by their large genome size (Chapuis et al., 2011; Cong et al., 2022; Hawlitschek et al., 2023). The diploid chromosome number for *S. grossum* is 22+XX in females and 22+X0 in males and for *C. dispar* 16+XX in females and 16+X0 in males respectively (Hawlitschek et al., 2023). Both species have some of the largest genome so far recorded in insects, with female genome size measuring up to 1C = 18.48 pg for *S. grossum* and 1C = 19.43 pg for *C. dispar* (Husemann et al., 2020; Hawlitschek et al., 2023). One of the reasons for the large genome is believed to lie in the large amounts of repetitive elements: satellite DNA, long terminal repeats (LTR), and transposons (Majid & Yuan, 2021; Cong et al., 2022; Liu et al., 2022; Hawlitschek et al., 2023; Nie et al., 2024). Orthoptera, compared to other insect orders, have relatively long microsatellites (Chapuis et al., 2011). Transposable elements (TE) make up to 75% of the grasshopper genome (Palacios-Gimenez et al., 2020). Due to their high copy number, repetitive elements, from which retrotransposons dominate, cause issues in interpreting sequencing data used for evolution research (Majid & Yuan, 2021). Additionally, these sequences can cause problems in PCR. Repetitive DNA can interfere with the reaction by causing non-specific annealing of primers or the formation of hairpin loops, which may result in incomplete amplification or multiple product bands instead of a single, clear band (Hommelsheim et al., 2014). While adjusting PCR protocols can sometimes mitigate these problems (Riet et al., 2017), they remain a considerable obstacle. However, since environmental stress can induce transpositions that result in genetic diversity, retrotransposon markers can be used to indicate recent population processes (Suh et al., 2018; Milyaeva et al., 2023).

Molecular markers have a central role in capturing the genetic diversity in a time of overall biodiversity loss. Therefore, there is an increasingly wide range of molecular markers and their applications. Nuclear DNA markers are useful for researching gene flow (Kartavtsev et al., 2021), which is a critical metric for assessing habitat functional connectivity at the landscape level. These markers are very useful in conservation biology. Retrotransposons, that are found universally and in high numbers within eukaryotic genomes (Amiteye, 2021), are Class I mobile genetic elements. They increase their genomic copy number through a 'copy-and-paste' process, where an RNA intermediate is reverse-transcribed into a cDNA copy that is integrated elsewhere in the genome (Kalendar et al., 2010; Finnegan, 2012; Bourque et al., 2018). Retrotransposons are divided into three subclasses: LTR (Long Terminal Repeats), DIRS (Dictyostelium repetitive sequence), and non-LTR retrotransposons (Finnegan, 2012; Bourque et al., 2018). LTR and non-LTR subclasses are widely distributed across all eukaryotic organism's genomes (Havecker et al., 2004). LTR retrotransposons are characterised by tandem repeats, typically a few hundred base pairs long, located at each end (Kalendar et al., 2010), and integration that occurs by means of a cleavage and strand-transfer reaction (Bourque et al., 2018). A highly conserved PBS region is located adjacent to the 5′ LTR sequence in LTR retrotransposons, which is essential for initiating reverse transcription (Monden et al., 2014). DIRS elements are unique among retrotransposons because they use tyrosine recombinase (YR) for integration into the host genome, unlike other retrotransposons that typically use an integrase (Gazolla et al., 2022). Non-LTR retrotransposons, such as LINEs (Long Interspersed Elements) and SINEs (Short Interspersed Elements), are a diverse group of mobile elements that lack terminal repeats and employ a mechanism called target-primed reverse transcription (TPRT) for insertion (Christensen & Eickbush, 2005; Fambrini et al., 2020). While some non-LTR retrotransposons, like the R2 element, insert copies into specific conserved sites such as the 28S ribosomal DNA (Christensen & Eickbush, 2005; Lee et al., 2024), most LINEs integrate into various, less-conserved genomic locations (Arvas et al., 2023).

The LTR retrotransposon iPBS (inter-primer binding sequence) is a dominant molecular marker with high reproducibility, specificity, and polymorphism (Amiteye, 2021). The principal of the iPBS method is based on retrotransposons being typically located in the non-coding regions of DNA (Kalendar et al., 2010). LTR retrotransposons consist of evolutionary stable PBS sequences (Arvas et al., 2023). These sequences are highly specific, conserved DNA segments, typically 10–20 nucleotides long, located at the 5′ end of LTR retrotransposons (Kalendar et al., 2010; Arvas et al., 2023). The unique feature of PBS sequences is their complementarity to a host cell's transfer RNA (tRNA) molecule. During natural retrotransposon replication, the PBS sequence serves as a binding site for a specific tRNA, which initiates the reverse transcription process (Finnegan, 2012). Because different types of retrotransposons utilise various tRNA molecules, there are several distinct groups of PBS sequences. However, within each group, the sequences are highly similar and evolutionarily conserved (Arvas et al., 2023). This conservation allows iPBS primers, designed to match these specific PBS groups, to simultaneously bind to numerous different retrotransposon elements. This enables the amplification of DNA fragments between two closely located, inward-facing retrotransposon elements without prior knowledge of the full genome sequence, which makes this method well-suited for species with underdeveloped DNA marker systems (Kalendar et al., 2010, Arvas et al., 2023). This is a considerable improvement compared to other retrotransposon-based markers, making the method increasingly popular (Amiteye, 2021).

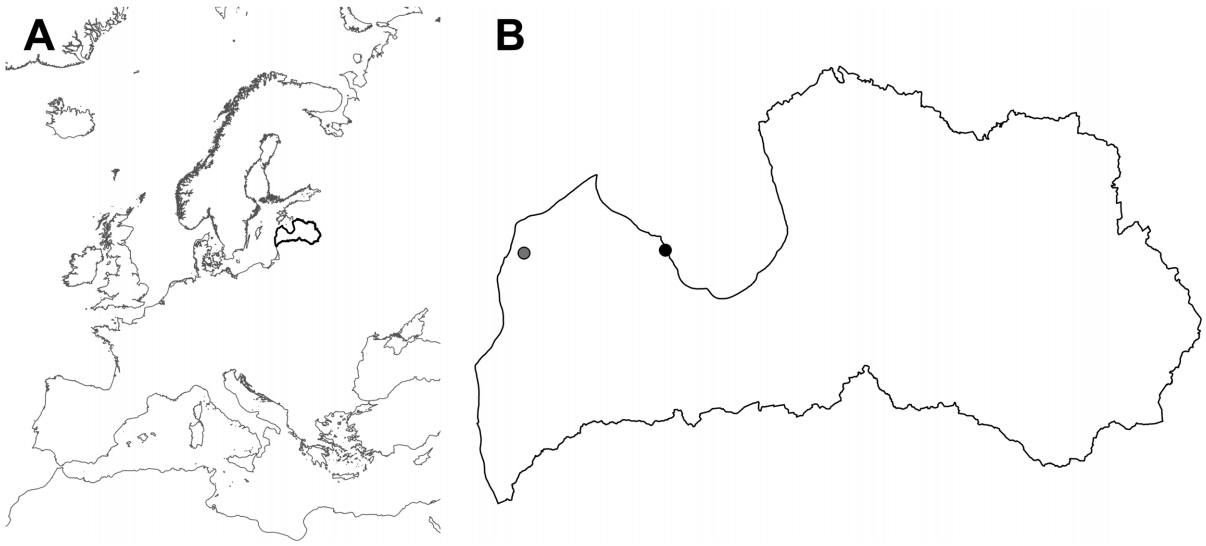

Fig. 1. Study sites (base map © EuroGeographics). A – Latvia in Europe; B – insect collection sites in Latvia, where nature park "Engure" is marked with a black dot whereas nature reserve "Platenes purvs" – with a grey dot.

The iPBS marker technique can be used for quantitative comparison of multiple population genetic variability (Fokina et al., 2020). It shows about the same level of polymorphism as other retrotransposon-based markers, such as IRAP (inter-retrotransposon amplification polymorphism) and REMAP (retrotransposon microsatellite amplification polymorphism) (Kalendar & Schulman, 2014). IRAP amplifies the intervening region between the LTRs of two TE sequences while REMAP amplifies the DNA fragment between an LTR sequence and a simple sequence repeat (SSR) (Monden et al., 2014). The iPBS primers identify the insertion point of two inwards facing LTR retrotransposons (Kalendar et al., 2010) which allows for the amplification of diverse LTR sequences (Monden et al., 2014). These characteristics suggest that iPBS could perform well on a genetically challenging insect group such as Orthoptera. The aim of this study is to determine whether iPBS can be used as a DNA fingerprinting technique for studies on the genetic structure of grasshopper species populations.

MATERIAL AND METHODS

Research site and specimen collection

Chrysochraon dispar and *Stethophyma grossum* specimens were collected in alkaline fens (EU habitat 7230) in nature park "Engure" (WGS84: 57.284802, 23.149825) and nature reserve "Platenes purvs" (WGS84: 57.399825, 21.725283) (Fig. 1). The Euclidean distance between both sampling sites is ~85 km. Both sample sites were isolated from their surrounding open habitats by forest. The specimen collection was conducted on August 2023. Each habitat patch was slowly walked through in a random pattern and individuals were caught using a sweep net. In each habitat patch 30 individuals per species were caught, with an equal number of males and females. All individuals were immediately euthanised in ether vapor and later stored in 96% ethanol at 4°C. Each individual was assigned a unique ID code.

DNA extraction and iPBS primer screening

Hind-leg muscle tissue was extracted from the exoskeleton and air-dried before DNA extraction to avoid amplification of gut microbiota, food items, and pollen. The DNA was extracted using the protocol described by Moller et al. (1992) with the following modifications: in the lysis buffer we used 10 µL proteinase K (100 mg/mL), for DNA purification we used 25 : 24 : 1 phenol : chlorophorm : isoamyl alcohol instead of 24 : 1 chlorophorm : isoamyl alcohol, and we added a step to remove RNA – adding 5 µL RNase to supernatant in a fresh tube, followed by 30 min incubation at 37°C. DNA quality was tested on 1% agarose gel. These results were used to pick the 10 individuals per species (sex ratio 1 : 1) that had the most successful DNA extraction. The DNA concentration of the selected samples was measured using a NanoPhotometer® N60/N50.

The PCR reactions for iPBS analyses were performed in a 25 µL reaction mixture (16.1 µL molecular water; 3 µL (10×) Thermo Scientific DreamTaq Green Buffer; 3 µL (4 µM) iPBS primer; 0.6 µL (10 mM); Thermo Scientific dNTP Mix; 0.3 µL (5u/µL) Thermo Scientific DreamTaq DNA Polymerase; 2 µL (25–50 ng/µL) DNA). The PCR was performed on a GeneAmp® PCR System 9700 thermocycler and consisted of 35 cycles. PCR temperatures were tailored to each primer according to the optimal annealing temperatures (Kalendar et al., 2010, Table S1). The PCR results were separated using 1.7% agarose gel electrophoresis for 5 h at 100 V, stained with ethidium bromide, and visualised on UV light for scanning. The Thermo Scientific GeneRuler DNA Ladder Mix was used as a reference for fragment length quantification.

PCR efficiency was interpreted on a scale from 0–5, as in Kalendar et al. (2010): 0, no bands; 1, few, weak bands; 2, few, strong bands; 3, ~10 strong bands; 4, many bands (good primer); 5, many strong and equally amplified bands. The retrotransposon iPBS method was developed and mostly used for plant population genetics (Kalendar et al., 2010, Doungous et al., 2020). We used plant DNA as a positive control (Krasņevska et al., 2022) in PCR reactions to avoid false-negative results (0 efficiency) in the screening. For the negative control, PCR mix with no DNA was used. Additionally, for randomly chosen primers, and all primers that initially showed low results, the screening was repeated to ensure band consistency. If differences between repetitions were detected, the highest number of bands was considered for evaluating primer efficiency. Out of all the 83 developed iPBS primers (Kalendar et al., 2010) 73 primers were tested in this study.

iPBS fragment analysis

After the electrophoresis of the PCR product, the presence or absence of amplified bands was scored for each individual's genetic profile in a data frame, using GelAnalyzer 23.1.1 software (available at www.gelanalyzer.com). To compare the primer efficiency across the two species, the Wilcoxon signed-ranked test for paired samples was used. To visualise an example of the distribution of amplified fragment lengths for *S. grossum* across the two populations, the raw data from the GelAnalyzer was rounded to levels according to the used gene ladder and the frequency of fragments detected within each level were summarised. Statistical tests were performed in RStudio version 2025.04.0 (Posit team, 2025) using R version 4.4.2 (R Core Team, 2024). The graphics were created using R package "ggplot2" (Wickham, 2016).

For the primers with the highest efficiency, allele frequency and polymorphism information content (PIC) was evaluated. Allele frequency was calculated for each population and for both populations in total. Polymorphism was estimated as a % proportion of polymorphic loci versus all loci. Primer PIC values were calculated as an average of the individual allele PIC values, as follows: $PIC = 2 \cdot f_a(1 - f_a)$, where f_a is the individual allele fequency.

RESULTS

At least three primers showed high efficiency for both species, amplifying 30–45 bands in PCR (Fig. 2). The percentage of polymorphic loci for the most efficient primers varied between 86% and 100% and the primer PIC values varied between 0.32–0.39. Overall, most primers show average efficiency for both species, with slightly more efficient primers for *S. grossum* (Fig. 2). The Wilcoxon signed-rank test showed a statistically significant difference in primer efficiency between the two species (V = 330.5, p-value = 0.0004, α = 0.01). Interestingly, the most efficient primers do not overlap between the species (Fig. 2). For example, the primer 2218 is non-informative for *C. dispar* while being an effective primer for *S. grossum*. The efficiency of all the tested primers for each species can be seen in Table S1. Additional pictures of gel electrophoresis can be found in Fig. S1.

An example of a good primer is shown in Fig. 3. Here, a dataset of 45 loci can be obtained between the two populations using a single iPBS primer. The length of the amplified fragments varies between 400–6000 bp, most of them being 1000–3000 bp long (Fig. 4). Distinguishing loci in shorter fragments is problematic, because overlapping fragments cannot be separated using gel electrophoresis (Fig. 3). Numerous polymorphic loci (86%) can be observed both within and between the two sampled populations. Loci that are not polymorphic can be used as anchor points for data scoring to ensure comparative data input when scoring PCR results from multiple agarose gels (Fig. 3). When compiling the information obtained from a small set of primers, more than 120 loci with over 90% polymorphism can be analysed.

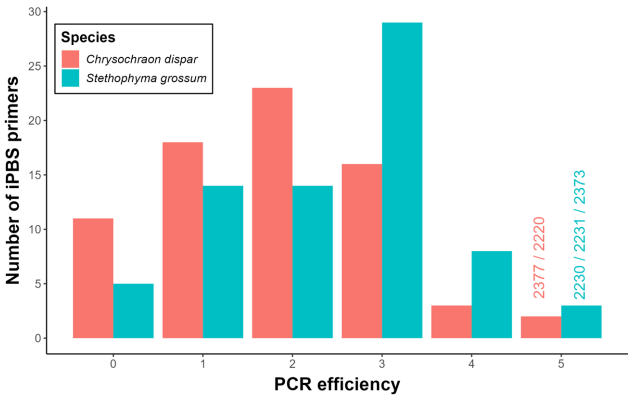

Fig. 2. Number of iPBS primers in each PCR efficiency group. Primers that showed the best results for each species are indicated above the bars. Primer efficiency scale (Kalendar et al., 2010): 0 – no bands; 1 – few, weak bands; 2 – few, strong bands; 3 – ~10 strong bands; 4 – many bands (good primer); 5 – many strong and equally amplified bands.

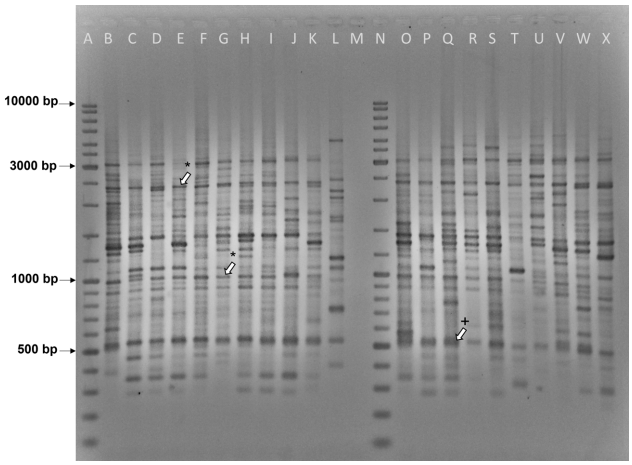

Fig. 3. Results of iPBS primer 2231 for 10 genotypes of *Stethophyma grossum* from each population. A negative image of ethidium bromide-stained agarose gel after electrophoresis is shown. Lanes are A, N – gene ladder; B–K – Engure nature park (B–F – females, G–K – males); L – positive control; M – negative control; N – gene ladder; O–X: Platene nature reserve (O–S – females, T–X – males). Non-polymorphic loci are marked with white arrows. An asterix "*" indicates loci useful as anchor points, whereas "+" indicates blurred loci where distinguishing between single and multiple loci is problematic.

DISCUSSION

Many molecular markers have been developed for studying population genetic structure: IRAP, REMAP, RAPD, AFLP, SNPs, and microsatellites. All genetic markers re-

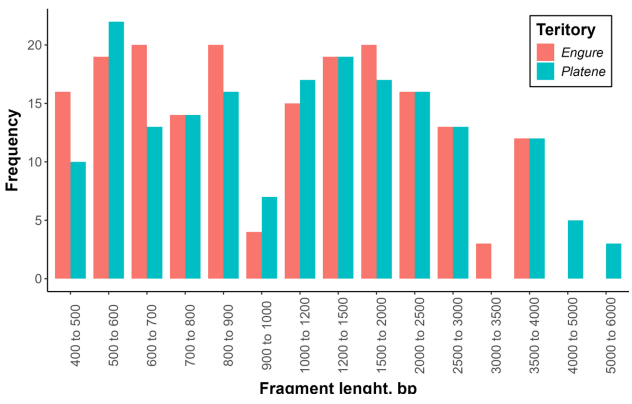

Fig. 4. Fragment length frequency from iPBS primer 2231 for 10 genotypes of *Stethophyma grossum* from each population.

flect differences in DNA sequences, with a trade-off between precision and convenience (Sunnucks, 2000). Similarly to RAPD, AFLP and microsatellites (Selkoe & Toonen, 2006), the iPBS is a multilocus marker. The iPBS method offers some advantages in the study of genetic diversity. In comparison to other retrotransposon-based markers, this method does not require prior sequence knowledge or the development of species-specific primers – all retrotransposons that do not have internal coding domains serve as targets (Arvas et al., 2023), allowing the adaptation of the method to new species fast and cheap. The iPBS primers can directly visualise polymorphisms in retrotransposon loci in the genome (Kalendar et al., 2010). Additionally, iPBS is a dominant marker, so the binary matrices, that can be obtained, are simple to analyse. However, it needs to be addressed that iPBS primers, similarly to AFLP, are not species specific, and will amplify DNA from any species (Selkoe & Toonen, 2006). It is therefore important to take precautionary measures to avoid cross-contamination. In our study, we addressed this issue by extracting hind-leg muscle tissue and hence avoiding amplification of gut microbiota, parasites or pollen.

The previously mentioned advantages of this method allow for universal use, such as picking habitat specialist species and researching population genetic diversity, how population demographics influence population viability, gene flow in the landscape, and other aspects that are crucial to landscape ecology research. Although the two sampled populations were only 80 km apart, and only 10 individuals per population were used for the primer screening, differences in genetic profiles between individuals and populations can be observed (Figs 3, 4). This suggests, that when increasing the number of individuals per population and combining the matrices obtained from multiple primers, iPBS can be a very informative tool for studying population genetics.

The results show that grasshoppers tend to have both long and short amplified fragments. To visualise the longer fragments, a crucial precondition for a successful PCR is to use high quality DNA. The retrotransposon iPBS method works better for some species than others (Fig. 2), therefore before population genetics analysis, a small-scale primer screening is still necessary, when adapting this method to new Orthoptera species.

The iPBS molecular marker has been used to characterise genetic diversity of animals before. In research on perch *Perca fluviatilis* genetic diversity, the iPBS method yielded 35–50 loci per iPBS primer with 64% polymorphism (Fokina et al., 2015). In swan *Cygnus olor* population research 18–25 fragments per primer with 28% polymorphic loci was obtained (Kolodinska-Brantestam et al., 2015). When analysing the genetic structure of benthic clams *Limecola balthica* in the Baltic Sea 17–23 loci per iPBS primer with 53–85% polymorphism per site was observed (Fokina et al., 2020). Similar to the previous studies on adapting iPBS to new species (Fokina et al., 2015, 2020; Kolodinska-Brantestam et al., 2015; Krasņevska et al., 2022), we suggest that using 2–3 iPBS primers that show high efficiency (4 or 5 on the efficiency scale) is optimal for population genetic diversity comparison for Orthoptera. We also recommend using a single primer per PCR – analysing fragments from multiple primers on single agarose gel could cause imprecisions due to fragment overlapping and, hence scoring difficulties.

The resolution of PCR scoring directly translates to the estimated genetic diversity. The iPBS is a multiple-band fingerprinting technique and bands are often localised near each other (Kalendar & Schulman, 2014). A similar pattern was observed in our results (Fig. 3). DNA is high quality and the gel electrophoresis has stretched the PCR product sufficiently, errors in reading the gel will be minimalised. If the DNA is degraded (broken down to smaller fragments) the amplification of longer fragments (e.g. between 1000–3000 bp) is not possible and information is lost (Fig. S1 A). Some short fragment loci appear blurred when visualizing on agarose gel (Fig. 3) and distinguishing between single and multiple loci becomes nearly impossible. A similar issue arises when the time of electrophoresis is too short – primer efficiency can be estimated, but scoring the fragments in a data frame would be compromised (Fig. S1 B). We suggest, that limiting the data input to certain fragment lengths (e.g. to 900–3000 bp) can eliminate such situations, at the cost of a small amount of imprecise information, the loss of which can be substituted by adding another primer to the analysis.

We conclude that the iPBS molecular marker can be used to study the genetic diversity and genetic differences of acridid species populations, especially ones with underdeveloped marker systems. Using the genetic diversity information of grasshopper species, together with their role as bioindicators for habitat quality and landscape functionality, we can potentially learn about the functional connectivity of highly valuable and protected wetland habitats.

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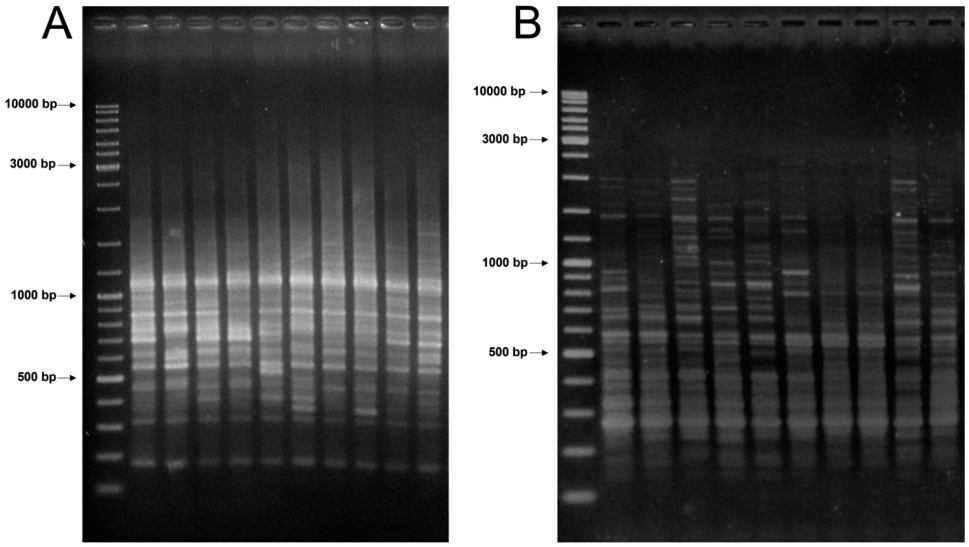

Fig. S1. Gel electrophoresis images from primer screening. First lanes are gene ladder. A – iPBS Primer 2373, *Stethophyma grossum*, 10 genotypes from Platene nature reserve. Primer shows polymorphism, multiple equally amplified fragments, but there there are signs of possible DNA degradation – lanes are smeared, longer fragments are less visible. B – iPBS Primer 2220, *Chrysochraon dispar,* 10 genotypes from Engure nature park. Primer shows polymorphism, many amplified fragments, but due to insufficient time in gel electrophoresis fragments are not well separated, making scoring difficult.

Table S1. PBS primers and their efficiency in single-primer iPBS amplification. Primers that showed the highest efficiency are marked with bold.

iPBS primer	Sequence	Melting (T_m, °C)	Optimal annealing (T_a, °C)	Average PCR efficiency*	
				Chrysochraon dispar	*Stethophyma grossum*
2074	GCTCTGATACCA	40.5	49.6	3	3
2075	CTCATGATGCCA	42.1	51.2	4	4
2076	GCTCCGATGCCA	50.4	59.2	3	3
2077	CTCACGATGCCA	46.1	55.1	2	2
2078	GCGGAGTCGCCA	54.2	62.8	2	3
2079	AGGTGGGCGCCA	56.6	65.2	3	3
2080	CAGACGGCGCCA	54.6	63.3	2	2
2081	GCAACGGCGCCA	56.5	65.0	2	4
2083	CTTCTAGCGCCA	45.7	54.6	2	3
2085	ATGCCGATACCA	43.8	52.8	3	2
2087	GCAATGGAACCA	43.5	52.5	2	1
2095	GCTCGGATACCA	44.8	53.7	1	2
2374	CCCAGCAAACCA	47.1	53.5	2	3
2375	TCGCATCAACCA	45.1	52.5	2	1
2376	TAGATGGCACCA	43.1	52.0	3	3
2377	ACGAAGGGACCA	47.2	53.0	**5**	1
2378	GGTCCTCATCCA	44.2	53.0	1	1
2379	TCCAGAGATCCA	41.5	49.2	1	3
2380	CAACCTGATCCA	41.4	50.5	3	3
2381	GTCCATCTTCCA	40.9	50.0	1	1
2382	TGTTGGCTTCCA	44.9	50.5	1	1
2383	GCATGGCCTCCA	50.5	53.0	1	1
2384	GTAATGGGTCCA	40.9	50.0	0	3
2385	CCATTGGGTCCA	45.7	51.2	0	0
2386	CTGATCAACCCA	41.4	50.1	0	3
2387	GCGCAATACCCA	47.3	51.5	0	3
2388	TTGGAAGACCCA	43.4	51.0	1	1
2389	ACATCCTTCCCA	43.0	50.0	2	3
2390	GCAACAACCCCA	47.6	56.4	2	3
2391	ATCTGTCAGCCA	43.6	52.6	1	0
2392	TAGATGGTGCCA	43.1	52.2	2	1
2393	TACGGTACGCCA	47.1	51.0	2	1
2394	GAGCCTAGGCCA	48.5	56.5	1	3
2270	ACCTGGCGTGCCA	56.9	65.0	4	3
2271	GGCTCGGATGCCA	54.3	60.0	3	2
2272	GGCTCAGATGCCA	50.5	55.0	3	2
2273	GCTCATCATGCCA	47.6	56.5	2	4
2274	ATGGTGGGCGCCA	57.1	65.8	3	3
2276	ACCTCTGATACCA	42.7	51.7	0	1
2278	GCTCATGATACCA	42.3	51.0	0	1
2279	AATGAAAGCACCA	43.0	52.0	1	2
2218	CTCCAGCTCCGATTACCA	56.1	51.0	0	4
2220	ACCTGGCTCATGATGCCA	59.0	57.0	**5**	4
2221	ACCTAGCTCACGATGCCA	58.0	56.9	2	3
2222	ACTTGGATGCCGATACCA	55.7	53.0	4	4
2224	ATCCTGGCAATGGAACCA	56.6	55.4	3	4
2228	CATTGGCTCTTGATACCA	51.9	54.0	1	2
2230	TCTAGGCGTCTGATACCA	54.0	52.9	3	**5**
2231	ACTTGGATGCTGATACCA	52.9	52.0	2	**5**
2232	AGAGAGGCTCGGATACC	56.6	55.4	3	1
2237	CCCCTACCTGGCGTGCCA	65.0	55.0	2	3
2238	ACCTAGCTCATGATGCCA	55.5	56.0	3	2
2239	ACCTAGGCTCGGATGCCA	60.4	55.0	2	3
2240	AACCTGGCTCAGATGCCA	58.9	55.0	2	4
2241	ACCTAGCTCATCATGCCA	55.5	55.0	2	3
2242	GCCCCATGGTGGGCGCC	69.2	57.0	1	2
2243	AGTCAGGCTCTGTTACCA	54.9	53.8	1	1
2244	GGAAGGCTCTGATTACCA	53.7	49.0	0	0
2245	GAGGTGGCTCTTATACCA	53.1	50.0	0	0
2249	AACCGACCTCTGATACCA	54.7	51.0	1	3
2251	GAACAGGCGATGATACC	54.3	53.2	2	3
2252	TCATGGCTCATGATACCA	52.7	51.6	1	0
2253	TCGAGGCTCTAGATACCA	53.4	51.0	1	3
2255	GCGTGTGCTCTCATACCA	57.1	50.0	0	2
2257	CTCTCAATGAAAGCACCA	52.4	50.0	0	2
2295	AGAACGGCTCTGATACCA	55.0	60.0	1	3
2298	AGAAGAGCTCTGATACC	51.6	60.0	1	3
2373	GAACTTGCTCCGATGCCA	57.9	51.0	2	**5**
2395	TCCCCAGCGGAGTCGCCA	66.0	52.8	2	3
2398	GAACCCTTGCCGATACCA	57.1	51.0	2	3
2399	AAACTGGCAACGGCGCC	63.4	52.0	3	2
2400	CCCCTCCTTCTAGCGCCA	61.6	51.0	3	3
2415	CATCGTAGGTGGGCGCC	62.5	61.0	3	2

* Primer efficiency scale (Kalendar et al., 2010): 0 – no bands; 1 – few, weak bands; 2 – few, strong bands; 3 – ~10 strong bands; 4 – many bands (good primer); 5 – many strong and equally amplified bands.