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Wachi, Nakatada

Matsubayashi, Kei W.

Maeto, Kaoru

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Application of next-generation sequencing to the study of non-model insects

Nakatada Wachi[1, 4], Kei W. Matsubayashi[2], Kaoru Maeto[3]

[1]Biohistory Research Hall, Takatsuki, Japan

[2]Faculty of Arts and Science, Kyushu University, Fukuoka, Japan.

[3]Graduate School of Agricultural Science, Kobe University, Kobe, Japan

[4]Present address: Department of Environmental Research, Research Institute of Environment, Agriculture, and Fisheries, Osaka Prefecture, Habikino, Japan

[1]nwachi@kyudai.jp

[2]matsubayashi.kei@artsci.kyushu-u.ac.jp

[3]maeto@kobe-u.ac.jp

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ABSTRACT

Recent developments in and widespread availability of platforms, technologies, and computer software and hardware have enhanced the use of genetic markers to address major scientific questions in non-model organisms, even by researchers with limited expertise in genomics. However, there are few studies investigating the genomes of non-model insects using these approaches. This review discusses the application of next-generation sequencing (NGS) technologies to the study of genomes of wild organisms. We first introduce currently available NGS-based methods—including restriction site-associated DNA sequencing; multiplexed inter-simple sequence repeat genotyping by sequencing; target capture; and amplicon, transcriptome, and whole genome sequencing—as useful tools for studies of non-model insects. We also provide guidelines for first-time users of NGS systems. In addition to the massive amount of information that it provides, a major advantage of NGS data is the scalability to future research projects. Studies using NGS technology can answer questions related to basic entomology by focusing on the unique nature of non-model insects in wild environments in a way that is not possible for model organisms.

35 **INTRODUCTION**

36 Genomic studies of non-model species (i.e., those for which genomic information is
37 non-existent or limited) have multiplied with the emergence of next generation sequencing
38 (NGS). There are many advantages to using NGS-based methods for investigations of
39 non-model organisms (Ekblom & Galindo 2011; McCormack *et al.* 2013; Lemmon &
40 Lemmon 2013). Indeed, the levels and patterns of genetic diversity, phylogenetic
41 relationships, and genetic regions responsible for adaptation in non-model organisms have
42 been investigated by NGS (Nadeau & Jiggins 2010; Stapley *et al.* 2010).

43 Despite the availability of technology and knowledge, there have been relatively
44 few studies of non-model insects especially in Japan in the 10 years since the development of
45 NGS. The reasons for this relate to four aspects of phylogenetic and phylogeographic studies
46 (McCormack *et al*. 2013): (1) a lack of focus on non-model species in general; (2) the need
47 for a large number of samples; (3) a lack of standardized protocols; and (4) the transitional
48 state of techniques. NGS-based methods require sophisticated bioinformatics analyses, and
49 the lack of expertise in this area has constrained the application of this technology to studies
50 in entomology.

51 In this review, we introduce studies that have used NGS technologies to a wide range of

52 entomologists who are especially interested in insect taxonomy, ecology, and natural history
53 but not necessarily genomic information. Since the general methods (Davey *et al*. 2011;
54 Ekblom & Galindo 2011; Lemmon & Lemmon 2013; Jones & Good 2016) and their
55 application to phylogenetic studies (McCormack *et al*. 2013) and genomics (Rice *et al*. 2010;
56 Stapley *et al*. 2010) have already been reviewed in detail elsewhere, we focus here on
57 describing methods that can be applied to studies of non-model insects. After a brief review
58 of these methods, we provide practical examples and address the feasibility of using these
59 techniques. The reviews and references herein (Lemmon & Lemmon 2013; McCormack *et al*.
60 2013; Jones & Good 2016) provide a more detailed analysis of the advantages and caveats of
61 each approach.
62
63 *Restriction site-associated DNA sequencing (RAD-seq)*
64 RAD-seq can detect genetic markers in limited amounts of sequencing data from individuals
65 rather than from whole genome sequences (Davey *et al*. 2011, Andrews *et al*. 2016). There
66 are several variations of RAD-seq (Andrews *et al*. 2016) that differ in terms of the type,
67 number, and restriction enzyme combinations that are used. Numerous short sequence reads
68 (< 300 bp) are clustered to 'loci' based on their similarities and analyzed using bioinformatics

69 approaches (e.g., STACKS, Catchen *et al*. 2011; PYRAD, Eaton & Ree 2013). These
70 methods were first applied to linkage mapping in fish (Baird *et al*. 2008), and later used to
71 reconstruct the phylogeography of the pitcher plant mosquitos *Wyeomyia smithii* (Emerson *et*
72 *al*. 2008). Major benefits of RAD-seq are its applicability to insects for which there is no
73 reference genome information available and adaptability to different types of investigation;
74 indeed, it has been used for studies on insect population genomics (*Heliconius* butterflies,
75 Nadeau *et al*. 2013, 2014; a stick insect of the genus *Timema*, Soria-Carrasco *et al*. 2014; a
76 cricket of the genus *Teleogryllus*, Pascoal *et al*. 2014; and *Blastophaga* fig wasps, Wachi *et al*.
77 2016) and phylogenetics (*Carabus* ground beetles, Cruaud *et al*. 2014, Takahashi *et al*. 2014;
78 *Adelpha* butterflies, Ebel *et al*. 2015; and *Magicicada* periodical cicadas, Koyama *et al*.
79 2016). However, one disadvantage is the issue of missing data, which can affect the results of
80 population genetic analyses (Gautier *et al*. 2013). When genomic information is available,
81 RAD-seq can provide insight into the genetic basis of adaptive differentiation (e.g., as
82 observed for *Heliconius* butterflies, Nadeau *et al*. 2014; a stick insect of the genus *Timema*,
83 Soria-Carrasco *et al*. 2014; and a fruit fly of the genus *Rhagoletis*, Egan *et al*. 2015).
84 RAD-seq and related methods require a large amount of high-quality genomic DNA (20–150
85 ng/μl concentration, 120–900 ng in total); therefore, the use of a DNA extraction kit on

86 frozen samples preserved in absolute ethanol is recommended. The requirements in terms of
87 the quality and quantity of genomic DNA are similar to those for amplified fragment length
88 polymorphism (AFLP).
89
90 *Multiplexed inter-simple sequence repeats (ISSR) genotyping by sequencing (MIG-seq)*
91 MIG-seq is a PCR-based method used to identify large numbers of genetic markers
92 throughout the genome (Suyama & Matsuki 2015). RAD-seq and its iterations require large
93 amounts of genomic DNA; MIG-seq is an alternative approach that can yield high-resolution
94 NGS data from small amounts of low-quality genomic DNA using multiplex PCR primers
95 targeting ISSRs (Suyama & Matsuki 2015). One drawback of this method is that it identifies
96 fewer single-nucleotide polymorphisms (SNPs) than RAD-seq methods; although the number
97 of loci obtained is sufficient for population genetic studies (Suyama & Matsuki 2015;
98 Takahashi *et al*. 2016). In fact, the genetic structure of *Ischnura senegalensis* and *I. asiatica*
99 damselfly (Odonata) populations have been investigated using this method, leading to the
100 identification of 231 and 270 SNPs, respectively (Takahashi *et al*. 2016). Given its successful
101 application to studies of various taxonomic groups (e.g., mushrooms, *Pholiota microspore*;
102 copepods, *Eodiaptomus japonicas*; sea cucumbers, *Apostichopus japonicus*; sea snails,

103 *Laguncula pulchella*; Carolina anoles, *Anolis carolinensis*; and orchids, *Cypripedium*
104 *macranthos* var. *rebunense*) (Suyama & Matsuki 2015), MIG-seq—like RAD-seq—can be
105 useful for genetic studies of non-model insects.
106
107 *Target capture*
108 Target capture is a solution-based hybridization method in which genomic regions of
109 interest—such as those that are phylogenetically significant or encode functional genes—are
110 enriched using specific probes. In phylogenetic analyses, target capture uses probes that
111 hybridize to and enrich regions flanking sequences that are conserved among samples
112 (Lemmon & Lemmon 2013; Jones & Good 2016) for NGS. However, genomic information
113 must be available for target capture probe design. PCR capture, de novo assembly capture,
114 and divergent reference capture provide solutions for species for which genomic information
115 is lacking (Jones & Good 2015). These strategies differ in the number of target loci, cost
116 (mainly associated with probe synthesis), applicable taxonomic range, and requirement for
117 reference genome sequences (Jones & Good 2015). Currently, divergent reference capture
118 dominates the phylogenetic study of non-model insects. In this approach, a probe set is
119 designed for genomic sequences of interest based on the reference genome assembly of a

120 closely related species (Jones & Good 2015). An average of 863 and 721 regions from 12
121 genome-available and 30 newly investigated species of Hymenoptera, respectively, were thus
122 captured (Faircloth *et al*. 2015) using 1,510 ultra-conserved elements (UCEs; Faircloth *et al*.
123 2012) from *Apis mellifera* and *Nasonia vitripennis* genomes, yielding phylogenies that were
124 consistent with those obtained in previous studies. Based on these 1,510 UCEs in
125 Hymenoptera, 959 loci (30% missing data) were obtained across Formicinae species
126 (Blaimer *et al*. 2015) along with 944 loci (30% missing data) for 23 species of *Acropyga* ants
127 (Blaimer *et al*. 2016). Using anchored hybrid enrichment (Lemmon *et al*. 2012) similar to the
128 UCE approach, a set of 962 probes was developed based on 4,485 protein-coding genes from
129 13 insect species (Diptera, Hymenoptera, Lepidoptera, Strepsiptera, and Coleoptera, with
130 Hemiptera and Anoplura as outgroups) (Young *et al*. 2016). To investigate phylogenetic
131 relationships among syrphid species (Diptera), these investigators obtained alignments of 343
132 loci (6% missing data) from 30 Syrphidae and five dipteran species. Target capture is
133 applicable to small insect species as it enables library construction from relatively small
134 amounts of DNA (70 ng; Faircloth *et al*. 2014). Thus, even museum collections with genomic
135 DNA of limited quality and quantity can be used for phylogenomic studies (Faircloth *et al*.
136 2015).

137 Exon capture has been used to assess sequence variations at various functional loci
138 (Smadja *et al*. 2012). Using probes targeting 1,021 exon sequences from 203 loci of both
139 candidate (chemosensory) and non-candidate (randomly selected) genes, sequences from
140 samples of *Acyrthosiphon pisum* pea aphids on three different host plants were captured,
141 revealing a signature of divergent selection consisting of 128 SNPs (95% of which were in
142 chemosensory genes). As an alternative approach to target capture, organelle enrichment is a
143 feasible method for ordinary entomology using mitochondrial DNA fragments, and has been
144 reviewed in detail elsewhere (Lemmon & Lemmon 2013).
145
146 *Amplicon sequencing (amplicon-seq)*
147 NGS of PCR amplicons is a feasible method for ordinary entomology, for instance in
148 phylogeography and phylogenetics (McCormack *et al*. 2013). There are two general
149 approaches to amplicon-seq:
150 1. Uniplex PCR amplification of target regions within a sample, with tagging by several
151 base indices as in other methods and pooling of amplicons from each loci (parallel
152 tagged sequencing, Meyer *et al*. 2008, targeted amplicon-seq: Bybee *et al*. 2011).
153 Based on six loci from 44 samples, phylogenies of Pancrastacea (including Diptera,

two Coleoptera, Hymenoptera, Lepidoptera, Blattodea, Plecoptera, and Hemiptera) with well-supported nodes and few missing data were reconstructed (Bybee *et al*. 2011). The drawback of this method is that PCR is required for each locus and sample, which is time-consuming and labor-intensive when analyzing multiple samples.

2. Multiplex PCR using multiple primer sets for different loci in the same reaction. Limitations of multiplex PCR include biased amplification of specific amplicons and DNA sequence chimerism (Mamanova *et al*. 2009).

Amplicon-seq has the advantage that DNA fragments can be sequenced from multiple loci or samples without cloning; as such, it is more time- and cost-effective than Sanger sequencing. An early application of amplicon-seq to non-model insects used sequenced amplicons covering the whole mitochondrial genomes of 30 Coleoptera species (Timmermans *et al*. 2010). Instead of labeling individuals, raw reads were assembled to reconstruct contigs, which were assigned to each species using 'baits' (*cox1*, *cytb*, and *nad5*) as queries for homology searches. In this manner, near-complete mitochondrial genomes of 21 species and partial sequences from the remainder were obtained. These sequences were used to reconstruct phylogeny at the family level (Timmermans *et al*. 2010). Amplicon-seq of DNA barcode sequences provides ordinary entomologists with novel opportunities for

171 taxonomic identification and analyses of host-parasite and predator-prey interactions. General
172 approaches to DNA barcoding using NGS for ecological network analyses have been
173 previously reviewed (Toju 2015; Kishimoto & Ito 2015). For the identification of 1,010
174 individual specimens caught by Malaise trap, at least one sequence from 983 samples
175 (97.3%) was obtained (Shokralla *et al*. 2015); the order-level identification based on
176 sequence homology from 757 individuals (75.0%) was matched to morphological
177 characteristics. These proportions were greater than for sequences obtained by the Sanger
178 method (53.2% and 50.4%, respectively). To evaluate the utility of molecular data from a
179 quantitative analysis of food web structure in host-parasitoid interactions, a set of
180 lepidopteran hosts and their parasitoids (Ichneumonidae, Braconidae, and Eulophidae:
181 Hymenoptera; Tachinidae: Diptera) were examined by Sanger sequencing-based DNA
182 barcoding (Wirta *et al*. 2014); 14 of 21 host-parasitoid relationships were revealed by
183 ordinary rearing methods, and an additional 40 were newly detected interactions. Thus, DNA
184 barcoding with NGS can reveal interactions among insect species with high resolution.
185
186 *Transcriptome sequencing (RNA-seq)*
187 RNA-seq is more suitable for detecting functional genes since all obtained sequences are

188 from coding genes. The advantage of this method is that it directly connects genomic
189 information and function, unlike other genome-based methods. However, its requires
190 high-quality RNA, which poses a challenge when working with non-model insects, especially
191 field samples and from museum collections. There are various methods for obtaining
192 high-quality RNA from wild insect specimens—e.g., direct extraction from living samples;
193 flash-freezing in liquid nitrogen followed by storage at −80°C; and use of RNA-stabilizing
194 solutions such as RNAlater (Thermo Fisher Scientific) that enable extraction of high-quality
195 RNA even from samples that have been stored at 0°C for several months after collection.
196 However, even with the availability of such solutions, specimens for RNA extraction should
197 not be keep at room temperature for more than one night.
198 A database of transcriptome data from thousands of insects has been constructed
199 (1K Insect Transcriptome Evolution; http://www.1kite.org). Based on analyses of orthologous
200 loci in transcriptome sequences, several satisfactory phylogenies have been reconstructed
201 (Hymenoptera: Johnson *et al*. 2013; Insecta: Misof *et al*. 2014). The first robust phylogenetic
202 tree of Lepidoptera was reconstructed using newly obtained and available transcriptome data
203 (13 and 33 species, respectively, for a total of 2,696 genes) (Kawahara & Breinhost 2014)
204 and revealed that butterflies are a monophyletic group nested within macromoths.

205 Additionally, sequences obtained by RNA-seq can be used as references to design probes and
206 PCR primers for target capture and amplicon-seq, respectively.
207
208 *Whole genome sequencing (WGS)*
209 WGS yields very long (sometimes nearly complete) genomic DNA sequences of an organism
210 through the assembly of numerous shotgun reads that cover the genome multiple times. WGS
211 is always useful as a reference for any of the NGS techniques described above, and its
212 application to non-model organisms is garnering considerable interest, with at least four
213 suppliers in Japan currently distributing WGS platforms for non-model insects
214 The PacBio RS II sequencing system (Pacific Bioscience of California) has been
215 applied to various materials, including non-model insects (e.g., a *Belgica* antarctic midge,
216 which has one of the smallest insect genomes at 99 Mbp; Kelley *et al*. 2014). This system
217 enables the construction of adequately long scaffolds without a reference genome; however,
218 one drawback is the requirement for large amounts of genomic DNA (concentration > 50
219 ng/μl and over several micrograms in total). WGS depends on comparison to closely related
220 individuals (i.e., within the same family or ideally, genetic clones), but it is difficult to obtain
221 the required amounts of genomic DNA from small insects. Although novel platforms such as

222 with PacBio Sequel (Pacific Bioscience of California) have been developed, their efficiency
223 has not yet been validated in various non-model organisms.
224 Dovetail's Chicago Method (Dovetail Genomics LLC) consists of chromosome-scale
225 shotgun assembly based on chromatin interactions (Putnam *et al*. 2016). This system
226 provides extraordinarily long (possibly chromosome-level) scaffolds. The system is
227 completely serviced by the manufacturers from DNA extraction to genome assembly. It
228 requires relatively small amount of genomic DNA (concentration > 100 ng/μl and < 2 μg in
229 total), which can be obtained from a single individual. WGS based on the Chicago Method
230 has been applied to various non-model animals (the vampire bat *Desmodus rotundus*; the
231 prairie chicken *Tympanuchus cupido*; and the cichlid *Amphilophus citrinellus*) and plants (the
232 common onion *Allium cepa*; and the dwarf cashew tree *Anacardium occidentale*)
233 (https://dovetailgenomics.com/services/case-studies/, cited 30 Jan 2017). Although only a few
234 non-model insects have been analyzed with the system, high-resolution WGS (N50 > 3.8
235 Mbp) has been performed for Japanese *Henosepilachna* ladybird beetle (K. Matsubayashi,
236 unpublished data)..
237 The GemCode system (10X Genomics), which also yields chromosome-length
238 scaffolds based on linked reads by emulsion PCR has the advantage of requiring a small

239 quantity of genomic DNA for library preparation (< 5 ng in total). This system was optimized
240 for the human genome, but a novel Chromium platform from the same manufacturers is
241 available for general use and can be useful even for non-model insects. WGS of the Sitka
242 Spruce chloroplast genome (Coombe *et al*. 2016) has been achieved with this system.
243 The Irys system (Bionano Genomics) yields 1 Mbp-long scaffolds based on
244 single-molecule fluorescence detection by high-resolution imaging with a charge-coupled
245 device camera. The system allows whole genome mapping data to be rapidly obtained from a
246 small amount of DNA (less than several ng), and has been used for genome assembly of the
247 common bed bug *Cimex lectularius* (Rosenfeld *et al*. 2016). Additional details for each
248 system can be found on the manufacturers' web pages.
249
250 *Advantages of NGS for ordinary entomology*
251 Although it may be of interest to researchers in ordinary entomology, NGS is costly in terms
252 of equipment and time required for data analysis. Nonetheless, NGS has some advantages
253 such as rapid data acquisition, lower cost and time investment than Sanger sequencing, and
254 the large amount of genomic information obtained from the source material. Among the
255 papers published in *Entomological Science* between 2004 to 2016, 66 contained DNA

256 sequence data obtained by Sanger sequencing for various applications including taxonomy
257 (18), molecular identifications (15), population genetics (14), phylogeny (10), marker
258 development (4), phylogeography (2), molecular biology (2), and genotyping (1). Many of
259 these (44/68) used a small number of loci and samples and partial mitochondrial DNA
260 sequences. For such cases, NGS and Sanger sequencing may be similar in terms of total cost
261 (Nelson *et al*. 2012; Hodel *et al.* 2016), although less time is required to obtain sequence data
262 by NGS. Amplicon-seq or PCR capture may be more suitable than Sanger sequencing for a
263 small number of loci and/or samples, whereas RAD-seq, MIG-seq, or target capture may be
264 more appropriate for a large number of loci and/or samples (~100 and more). RNA-seq and
265 even low-coverage WGS has been useful for identifying genetic markers (e.g., a wasp of the
266 genus *Sirex*, Santana *et al*. 2009; a scuttle fly of the genus *Megaselia*, Rasmussen & Noor
267 2009; a water strider of the genus *Gerris*, Perry & Rowe 2011; and *Melitaea* butterflies,
268 Nakahama *et al*. 2015). Sequences obtained by these NGS methods can also be used to
269 design probes for target capture or primers for amplicon-seq. NGS data for specific insect
270 species acquired in current research projects can also be used as a basis for future research
271 topics (Table 1).
272 Ordinary entomology often relies on specimens in museum collections. NGS can

potentially be applied to genomic studies based on these relatively old specimens, which then become useful repositories of DNA sequences for future research (i.e., museomics; Buerki & Baker 2016 and references therein).

Preparations required for NGS

We suggest the following preparations for projects involving NGS of non-model insects (Fig. 1).

1. Consider the benefits and disadvantages of using genomic approaches for the species of interest. The greatest merit of using non-model insects is the unique nature of their presence in wild environments, which is never achievable for model organisms. Since NGS and data analysis are costly and time consuming, researchers should maximize the scientific advantages of valuable materials. The study of material-specific ecology, physiology, behavior, and natural history is a good basis for applying NGS. Similarly, there may be challenges associated with the material such as small body size, limited amounts of fresh organs, rarity of specimens, polyploidy, and large genome size. These factors may negatively affect DNA extraction, sequencing, and de novo sequence assembly. Specifically, target genome

290 size is important information to consider prior to initiating any NGS method;
291 sequencing and sequence assembly of a large genome is currently prohibitively
292 expensive. One large database of animal genome size (http://www.genomesize.com/)
293 is available (Gregory *et al*. 2007); in addition, the genome size of a specific material
294 can be easily measured by flow cytometry (Sota *et al*. 2013; Matsubayashi &
295 Ohshima 2015).
296 2. Choice of NGS method. There is no method capable of capturing data for all
297 purposes of a genomic study. Therefore, the first step is to identify an appropriate
298 NGS method and platform to address the research question. The platforms used in
299 the studies cited herein are shown in Table 2. Early case studies that applied NGS to
300 non-model insects (Ekblom & Galindo 2011) primarily used 454 (Roche FLX)
301 sequencers, but more recently, Illumina sequencers (Hiseq and Miseq) have been
302 favored (Table 2). There are several types of sequencers varying in terms of length
303 and number of reads, cost, and running time (briefly reviewed in Toju 2015).
304 3. Consider the collection and storage of tissues to be used for DNA extraction. In
305 general, DNA should be extracted from the freshest possible tissue. Although
306 extraction from living or flash-frozen specimens is the best approach, specimens

fixed with absolute ethanol are also suitable for RAD-seq, target capture, amplicon-seq, and MIG-seq. Specimens should be stored between −20°C and −80°C before extraction. Genomic DNA extraction should be performed with muscle or other clean tissue after degutting the specimen to avoid potential contamination from symbiotic microorganisms and food contents. Bioinformatics techniques are available for the removal of contaminating sequences. For examples, symbiont or contaminant sequences were identified in the genomes of *Dirofilaria* and *Caenorhabditis* nematodes (Kumar & Blaxter 2011; Kumar *et al*. 2013; Percudani 2013), a heartworm of the genus *Dirofilaria* (Godel *et al*. 2012), and a tardigrade of genus *Hypsibius* (Koutsovoulos *et al*. 2016; Delmont & Eren 2016). Storage and extraction of RNA and genomic DNA for WGS require special attention to sample quality and quantity; RAD-seq and target capture require careful handling, as do AFLP and other methods used for genomic DNA analysis; and amplicon-seq and MIG-seq require normal handling for Sanger sequencing of mitochondrial DNA.

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559

560 **Figure legend**

561 **Figure 1.** Work flows of NGS methods. PCR-based methods (MIG-seq and amplicon-seq)

562 can be carried out with small amounts of low-quality genomic DNA, whereas

563 fragmentation-based methods (RAD-seq, target capture, RNA-seq, and WGS) require large

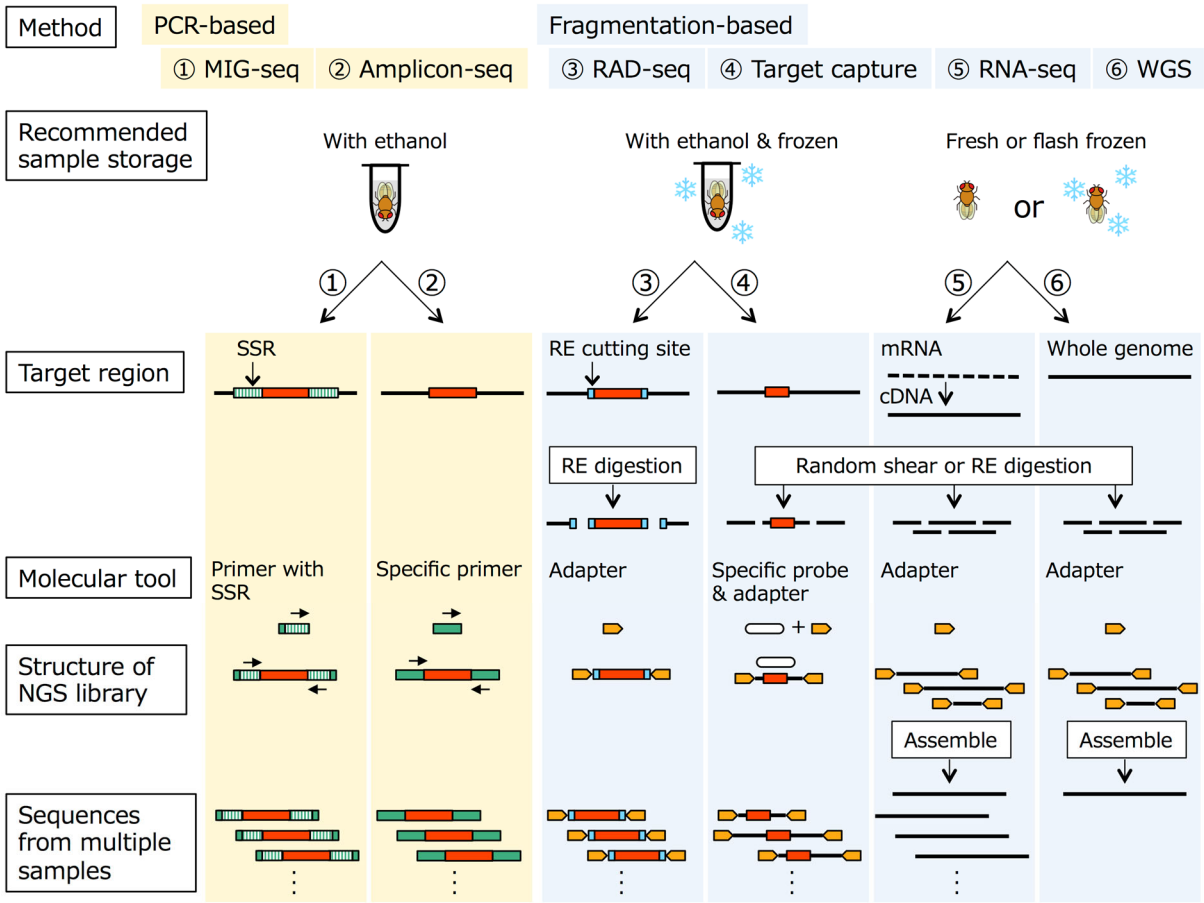
564 amounts of high-quality genomic DNA. “RAD-seq” represents double-digest RAD-seq. SSR:

565 simple sequence repeat; mRNA: messenger RNA; cDNA: complementary DNA; RE:

566 restriction enzyme.

567

568


Method
PCR-based
Fragmentation-based
① MIG-seq
② Amplicon-seq
③ RAD-seq
④ Target capture
⑤ RNA-seq
⑥ WGS
Recommended sample storage
With ethanol
With ethanol & frozen
Fresh or flash frozen
or
①
②
③
④
⑤
⑥
Target region
SSR
RE cutting site
mRNA
cDNA
Whole genome
RE digestion
Random shear or RE digestion
Molecular tool
Primer with SSR
Specific primer
Adapter
Specific probe & adapter
Adapter
Adapter
Structure of NGS library
Assemble
Assemble
Sequences from multiple samples

569

570 Table 1. The applicability of next generation sequencing methods for each research topic. Relative suitability of each application is denoted as

571 +++ (very high), ++ (high), + (moderate), and - (low), respectively. [*1] WGS can be useful for all NGS methods as a reference.

Research field	NGS method					
	RAD-seq	MIG-seq	Target capture	Amplicon-seq	RNA-seq	WGS[*1]
Phylogenomics	++	++	+++	++	+++	++
Phylogeography	+++	+++	+	+	+	++
Population genomics	+++	+++	++	+	+	++
Functional genomics	++	++	+++	++	+++	++
Museomics	+	+	++	+++	-	++
DNA barcoding	-	-	++	+++	-	++

572

573 Table 2. Case studies for application of NGS in non-model insects. RAD: Restriction site associated DNA sequencing; MIG: Multiplexed ISSR

574 genotyping by sequencing; TC: Target capture; AMP: Amplicon sequencing; RNA: RNA sequencing; LWGS: Low coverage whole genome

575 sequencing; WGS: whole genome sequencing.

Insect samples	Method	Platform	Author	Year
Wyeomyia mosquito; Diptera	RAD	GAIIx	Emerson *et al*.	2010
Heliconius butterfly; Lepidoptera	RAD	GAIIx & Hiseq	Nadeau *et al*.	2013
Heliconius butterfly; Lepidoptera	RAD	Hiseq	Nadeau *et al*.	2014
Teleogryllus cricket; Orthoptera	RAD	Hiseq	Pascoal *et al*.	2014
Timema stick insect; Phasmatodea	RAD	Hiseq	Soria-Carrasco *et al*.	2014
Carabus ground beetle; Coleoptera	RAD	Hiseq	Takahashi *et al*.	2014
Carabus ground beetle; Coleoptera	RAD	Hiseq	Cruaud *et al*.	2014
Adelpha butterfly; Lepidoptera	RAD	Hiseq	Ebel *et al*.	2015
Rhagoletis fruit fly; Diptera	RAD	GAII & Hiseq	Egan *et al*.	2015
Blastophaga fig wasp; Hymenoptera	RAD	Miseq	Wachi *et al*.	2016
Magicicada periodical cicada; Hemiptera	RAD	Hiseq	Koyama *et al*.	2016
Ischnura damselfly; Odonata	MIG	Hiseq	Takahashi *et al*.	2016
Acyrthosiphon pea aphid; Hemiptera	TC	454	Smadja *et al*.	2012
Hymenoptera	TC	Miseq	Faircloth *et al.*	2015
formicine ant; Hymenoptera	TC	Hiseq	Blaimer *et al.*	2015

Acropyga ant; Hymenoptera	TC	Hiseq	Blaimer *et al.*	2016
syrphid flower fly; Diptera	TC	Hiseq	Young *et al.*	2016
Coleoptera	AMP	454	Timmermans *et al*.	2010
Pancrastacea (including Diptera, two Coleoptera, Hymenoptera, Lepidoptera, Blattodea, Plecoptera, Hemiptera)	AMP	454	Bybee *et al*.	2011
Coleoptera, Diptera, Hemiotera, Hymenoptera, Lepidoptera, Psocoptera etc.	AMP	Miseq	Shokralla *et al*.	2015
Hymenoptera	RNA	Hiseq	Johnson *et al.*	2013
Lepidoptera	RNA	Hiseq	Kawahara & Breinhost	2013
Insecta	RNA	Hiseq	Misof *et al.*	2014
Sirex wasp; Hymenoptera	LWGS	454	Santana *et al*.	2009
Megaselia scuttle fly; Diptera	LWGS	454	Rasmussen & Noor	2009
Gerris water strider; Hemiptera	LWGS	454	Perry & Rowe	2011
Melitaea butterfly; Lepidoptera	LWGS	Ion PGM	Nakahama *et al*.	2015
Belgica antarctic midge; Diptera	WGS	Hiseq & PacBio RSII	Kelly *et al*.	2014
Cimex bed bug; Hemiptera	WGS	Hiseq, Moleculo & Irys	Rosenfeld *et al*.	2016

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