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Genomics and genome editing techniques of crickets, an emerging model insect for biology and food science

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Most tools available for manipulating gene function in insects have been developed for holometabolous species. In contrast, functional genetics tools for the Hemimetabola are highly underdeveloped. This is a barrier both to understanding ancestral insect biology, and to optimizing contemporary study and manipulation of particular large hemimetabolous orders of crucial economic and agricultural importance like the Orthoptera. For orthopteran insects, including crickets, the rapid spread of next-generation sequencing technology has made transcriptome data available for a wide variety of species over the past decade. Furthermore, whole genome sequences of orthopteran insects with relatively large genome sizes are now available. With these new genome assemblies and the development of genome editing technologies such as the CRISPR-Cas9 system, it has become possible to create gene knock-out and knock-in strains in orthopteran insects. As a result, orthopteran insects should become increasingly feasible for laboratory study not only in research fields that have traditionally used insects, but also in agricultural fields that use them as food and feed. In this review, we summarize these recent advances and their relevance to such applications.

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Introduction

Orthoptera is the order of insects that includes grasshoppers, locusts, crickets and katydids. The order is subdivided into two suborders, Caelifera (grasshoppers, locusts, and their relatives) and Ensifera (crickets, katydids and their relatives). Crickets sensu lato belong to the Gryllidae. More than 3000 species of crickets are known to belong to this family. Like grasshoppers, they have large hind legs for jumping and their forewings are hard and leathery. Among orthopteran insects, crickets are famous as chirping insects. Traditional cultures have long been fascinated by the sound of crickets and there are many folk tales and myths about crickets worldwide. This review introduces the most recent genomic resources and genome editing technologies for crickets, which might make crickets new model organisms for functional genomics research, and allow boosting of cricket production for commercial purposes (Tables 1 and 2).

Gryllus bimaculatus was first described in 1773 by Charles De Geer (Geer 1773), who named it *Gryllus* ('cricket' in Latin) *bimaculatus* ('two-spotted' in Latin) (Figure 1a). This species is thus commonly referred to as the 'two-spotted field cricket', which is derived from the fact that it has two pale yellow spots on the dorsal side of its forewings adjacent to the margin of the pronotum [1]. This species is found mainly in tropical and subtropical regions of Asia, Africa and Europe. *G. bimaculatus* has long been used in a wide range of research fields, including insect physiology, neurobiology, and behavior. In addition, the study of *G. bimaculatus* as a model for hemimetabolous insects has been greatly accelerated due to the discovery over a decade ago of the effectiveness of RNA interference (RNAi) methods as a tool for gene function analysis. Additionally, its informative phylogenetic position makes it a great model for evolutionary developmental studies of insects [2]. Some of the advantages of using *G. bimaculatus* as research model are the easy rearing systems [3], detailed developmental staging tables [4], established gene expression analysis methods [3], transgenic techniques [5], cell tracking analysis methods using confocal microscopy and light-sheet microscopy [6], rich transcriptome resources [7–9], assembled and annotated genomes [10••] and genome editing methods for gene knock-out and knock-in [11,12••]. These techniques have helped to make the hemimetabolous cricket *G. bimaculatus* a valuable new model organism in various fields [3,13].

Insects are also attracting attention as one of the next generations of sustainable alternative protein sources for

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Table 1

Q1 **Orthopteran genomes available in NCBI as of October 2021**

Name	Suborder	Family	Length (gb)	Scaffold n50 (bp)	Number of genes[a]	Accession number and genome database	Reference
Gryllus bimaculatus	Ensifera	Gryllidae	1.66	6 287 223	17 871	PRJDB10609 http://gbimaculatusgenome.rc.fas.harvard.edu	[10••]
Laupala kohalensis	Ensifera	Gryllidae	1.60	583 478	12 767	PRJNA392944	[53]
Teleogryllus occipitalis	Ensifera	Gryllidae	1.93	214 129	20 768	PRJDB9056	[54]
Teleogryllus oceanicus	Ensifera	Gryllidae	2.05	62 615	19 157	PRJEB24786 http://www.chirpbase.org	[55]
Apteronemobius asahinai	Ensifera	Gryllidae	1.68	27 317	19 896	PRJDB11838	[81]
Locusta migratoria	Caelifera	Acrididae	6.53	322 700	17 307	PRJNA185471 http://www.locustmine.org/	[20]
Schistocerca gregaria	Caelifera	Acrididae	8.82	157 705	18 815	PRJEB38779	[21]

[a] Annotated protein coding genes are listed.

Table 2

Transcriptome data reported in the literature as of October 2021 for crickets of the family Gryllidae

Name	Source	Reference
Acheta domesticus	Embryos, nymphs and adult of males and females	[56]
	Head and thorax	[57]
Allonemobius fasciatus	Male accessory gland	[58]
Allonemobius socius	Embryos	[59]
Apteronemobius asahinai	Female heads	[60]
	Male adult whole-body	[61]
Dianemobius nigrofasciatus	Ovaries	[62]
Gryllodes sigillatus	Male accessory glands	[63]
Gryllus assimilis	Male and female heads, testis, and ovary	[64]
Gryllus bimaculatus	Blastema	[65]
	Female mid-gut, male mid-gut, testes and ovaries	[66]
	Ovaries and embryos	[8]
	Ovaries and embryos	[67]
	Prothoracic ganglion	[9]
Gryllus firmus	Fat body, flight muscles	[68]
	Male accessory gland	[69]
Gryllus pennsylvanicus	Hindgut and malpighian tubules	[70]
	Male accessory gland	[69]
Gryllus rubens	Eggs, 1–6 instar nymphs, adult male and female	[71]
Gryllus veletis	Male nymph fat body	[72]
Laupala cerasina	Male and female, juveniles and adults	[73]
Laupala kohalensis	Nerve cord	[74]
Teleogryllus commodus	Male and female brain	[75]
Teleogryllus emma	Whole-body adult	[76]
Teleogryllus occipitalis	Muscle tissues	[54]
Teleogryllus oceanicus	Developing wing buds	[77]
	Heads, accessory glands, testes, and the remaining muscles and tissues	[78]
	Testis, accessory gland, male adult remaining tissue	[79]
	Male and female neural, thoracic and gonads	[80]

90 humans and livestock. Food security is becoming a global issue, as climate change and other factors devastate many previously stable agricultural industries. In addition, the world's population is projected to grow to nine billion by 2050, which will require a 70% increase in food production. The farming of crickets and other insects is seen as 96 an excellent opportunity to enhance food security and meet the growing demand for animal protein, while reducing greenhouse gas emissions and the use of land, water, and feed compared to conventional livestock farming. Crickets are already traditionally consumed in many countries [14], and their market is growing as a next-

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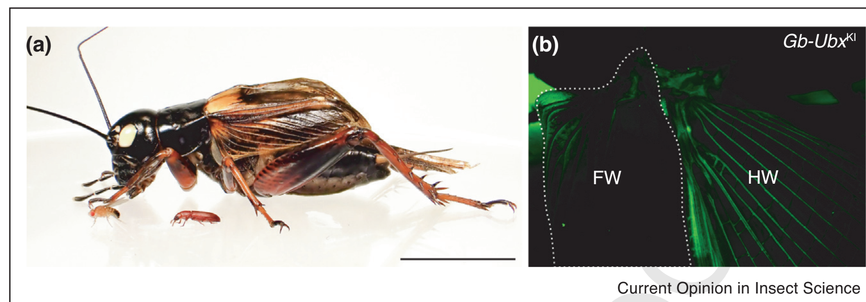
Figure 1



(a) Size comparison of adult *G. bimaculatus* male with *D. melanogaster* and *T. castaneum*. Scale bar: 1 cm. **(b)** GFP expression in the hindwing of a *Gb-Ubx* KI heterozygous adult female. FW: forewing ; HW: hindwing.

generation food because of their high nutritional value, general safety for human consumption, and the multiple health benefits of incorporating crickets into the diet.

Large genome size in orthoptera

The genome size of orthopteran insect species is generally larger and more variable than that of other insects and the suborder Ensifera is the most diverse group. Measurements of genome sizes of 32 species belonging to the Ensifera using flow cytometry showed that the difference between the largest and smallest genome size was more than 20-fold, from the male of *Oecanthus sinensis* (Gryllidae), 1C = 0.952 pg to the female of *Deracantha onos* (Tettigoniidae), 1C = 19.135 pg [15]. Phylogenetic comparative analysis using genome size and mitochondrial genome data of 32 ensiferan species showed no correlation between genome size and body size or flight ability in the Tettigoniidae [15]. Reconstruction of ancestral genome sizes showed that the ensiferan genome size has evolved such that the genome size of the grylloid clade (the infraorder Gryllidea) tends to decrease and the genome size of the non-grylloid clade tends to increase [15].

The genome sizes of Polyneoptera tend to be relatively larger than those of holometabolous species [10••,16]. The question of when and how variation in genome size is acquired during evolution has been a perennial concern for biologists. Major mechanisms contributing to genome size variation include whole-genome duplication, chromosomal aneuploidy, indels, gene duplications/deletions, and repetitive DNA such as transposable elements (TEs) [17,18]. There is no evidence to our knowledge supporting the occurrence of whole-genome duplication events in the Orthoptera [19•]. Therefore, the contribution of tandem repeat DNA and transposable elements is more likely to be an important factor in both genome size and variation in orthopterans. Indeed, evidence of high content of repeat elements in the grasshopper genomes is provided by the recent sequencing of the entire genome of the migratory locust *Locusta migratoria* [20] and the desert locust *Schistocerca gregaria* [21]. Recently, the sequencing, assembly and annotation of the genomes of *G. bimaculatus* [10••], the Hawaiian cricket *Laupala kohalensis* [22] and *Teleogryllus occipitalis* [54] were reported. Approximately 35–45% of the genomes of those cricket species were occupied by repetitive DNA. Although the genomes of *L. migratoria* and *S. gregaria* are among the largest insect genomes ever sequenced at 6.5 Gb and 8.8 Gb respectively, the number of annotated genes (17 307 and 18 815, respectively) is almost the same as that of *G. bimaculatus* (17 871). This suggests that the significant genome size difference between these orthopteran species is due to TE content, which is also correlated with genome size in several eukaryotic species [23]. Comparing the genomes of these two crickets with those of 14 other insect species supports the hypothesis that relatively small ancestral insect genomes were expanded to larger sizes in many lineages by TE activation [10••].

The genetic basis of cricket chirping

Adult male crickets produce songs by rubbing their forewings, where they have sound-producing apparatus: files and a scraper. The songs have three types of calls: a calling song to attract females, a courtship song to court approaching females and an aggressive song when males fight with each other, each with a different rhythm and pitch [24,25]. To date, much has been learned about the shape of the file organ, the structural properties of the wings involved in sound amplification, and the neural activity patterns and behaviors that drive the wings. However, the genes involved in the control of sound production and how these genes are regulated to form the sound-producing organs, have not been elucidated. The analysis of the cricket genome revealed an expansion in the number of *pickpocket* (*ppk*) class V genes, which belong to the Degenerin/epithelial Na^+ channel (DEG/ENaC) family [10••]. In *Drosophila melanogaster*, the *pickpocket* gene regulates neural mechanisms such as courtship behavior [26]. *D. melanogaster* abdominal ganglia

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elicit courtship behavior through sensory neurons expressing *fruitless* (*fru*) and *ppk* genes, which are determinants of male sexual behavior. In crickets, the abdominal ganglion plays a role in determining song rhythm. Interestingly, a transcriptome of the ganglion of the pronotal segment showed enriched expression of *ppk* and *fru* genes [10••]. This suggests that the expression of ppk and fru genes in the ganglion could be involved in the rhythmic control of sound production, a courtship behavior. Furthermore, in *L. kohalensis*, where quantitative trait locus (QTL) analysis has successfully identified genomic regions associated with song rhythm during mating behavior, the *ppk* gene is also included within the QTL peak [22]. Taken together, the extended *pickpocket* gene family in the cricket genome may play a role in controlling the rhythmic wing movements and sound perception required for mating.

Genome editing of crickets

The rapid spread of next-generation sequencing technology has led to the sequencing of entire genomes of organisms that had not been previously analyzed. Scientists now have access to larger amounts of genomic sequences than ever before [27,28]. These genomic data allow scientists to develop technologies that enable genome editing to explore gene functions for a mechanistic understanding of genomes and phenotypes [29]. RNA interference (RNAi)-mediated gene silencing is a powerful tool for functional gene study in non-model organisms. Within Orthoptera RNAi has been established in *G. bimaculatus*, in which it is a very efficient and convenient method to effectively, rapidly (within a few hours) and stably reduce the expression of target genes by injecting double-stranded RNA (dsRNA) into fertilized eggs or animals [2]. The RNAi method to knock down gene expression is also effective in orthopteran insects other than *G. bimaculatus*. RNAi, however, has its limitations because it cannot completely eliminate all the transcripts of the targeted genes, which often hinders researchers from comprehensively understanding gene functions.

Advanced functional genetic techniques commonly used to modify genomes at specific sites *in vivo* include the CRISPR/Cas9, TALEN and ZFN systems [30]. These methods produce double-strand breaks in target DNA sequences that trigger cellular DNA repair mechanisms, such as non-homologous end joining (NHEJ) and homology-directed repair (HDR). DNA end-joining via NHEJ is prone to mutations at the junction, resulting in insertions or deletions at the break point [31]. All these techniques are currently available and functioning in crickets. The use of ZFNs and TALENs in crickets was established in 2012, and was reported to successfully create homozygous gene knock-outs [11]. CRISPR/Cas9 has also been used to develop homozygous gene knock-outs of cricket genes [32]. The generation of gene knock-out lines via NHEJ using the CRISPR/Cas9 system has also been reported in another orthopteran species, the locust *L. migratoria*. Some locust species change from a cryptic solitary behavioral stage to a swarming collective behavioral stage when the local population density increases, resulting in catastrophes and serious agricultural damage. Olfactory stimuli play an essential role in guiding insect behavior including swarm formation in locusts. Accordingly, locust gene knock-out mutants made with the CRISPR/Cas9 system targeting Orco, an olfactory receptor co-receptor [33] and the odorant receptor gene Or35 [34], lost their attraction response to aggregation pheromones. These results open the door to new ideas for pest control through the use of genome editing to avoid swarm formation.

In recent years, gene knock-in using the CRISPR/Cas9 system has also been developed for *G. bimaculatus*. Methods based on the HDR system can accurately integrate donor sequences into the genome, and thus are well known for generating gene knock-in mutants. However, due to the low efficiency of HDR in eukaryotes, gene knock-in has only been reported in a few insect species [35], including *D. melanogaster* [36], several mosquitoes [37,38], *Tribolium castaneum* [39••] and the Mediterranean fruit fly [40]. Unfortunately, to our knowledge, it has never been reported to be successfully applied in Orthoptera. An alternative efficient gene knock-in method using NHEJ was developed in zebrafish [41]. In this method, both the genome and the donor vector are cleaved *in vivo*, and then the terminal genome sequence and the donor sequence are bound by NHEJ. This method can efficiently integrate long constructs into the genome [41], and it has been used in *D. melanogaster* to insert donor plasmid into the target genome locus [42,43]. A similar method has also been developed for the cricket *G. bimaculatus*. In this case, the donor vector containing an expression cassette with the *G. bimaculatus* actin promoter followed by the eGFP coding sequence was knocked into an exon of the Hox genes *Ultrabithorax* and *abdominal-A*. The resulting animals displayed GFP expression that recapitulated the endogenous Hox gene expression (Figure 1b). This approach has revealed that gene knock-in can function efficiently by a homology-independent NHEJ method in *G. bimaculatus* [12••]. Furthermore, this homology-independent method is cost effective and simpler than the homology-dependent method, as a donor plasmid does not need to be newly made for each target region [12••].

To obtain genome edited offspring, it is necessary to efficiently deliver Cas9 and the gRNA ribonucleoprotein (RNP) complex to the cells of the germ line. In *D. melanogaster*, this is now readily achieved by injecting sgRNA into transgenic embryos that express Cas9 under a germ cell-specific promoter. Gene editing of arthropods

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in most cases, however, has been performed by microinjection of gRNA with Cas9 mRNA or protein into a fertilized egg [44,45]. Microinjection is a difficult skill to acquire and its feasibility depends on the species' egg physical characteristics, including egg size, resistance to injection, presence of ootheca (the egg case of cockroaches and mantises), and robustness of subsequent embryonic development. To overcome these microinjection drawbacks, the Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) technique was recently developed [46]. This technique allows researchers to perform easy injections into the adult female hemolymph and take advantage of a small ovary-targeting peptide to introduce RNP directly into the developing ovary. Yolk proteins are synthesized in the *D. melanogaster* fat body and secreted into the hemolymph, then transported to the ovaries during vitellogenesis by receptor-mediated endocytosis. Fusing a peptide ligand named P2C, derived from *D. melanogaster* Yolk Protein 1, to Cas9 protein can allow delivery of the RNP complex into the ovary. The reagents chloroquine or saponin help the P2C-RNP complex be released from endosomes and reach the oocyte cytoplasm. This method can enable targeted gene modification bypassing difficult microinjection into eggs. To date, gene modification via ReMOT Control has been tested widely in many insect species including *Aedes aegypti* [46], *Anopheles stephensi* [47], *Bemisia tabaci* [48•], *T. castaneum* [49] and *Nasonia vitripennis* [50] and even in the Chelicerate, *Ixodes scapularis* [51].

Drosophila Yolk Protein, the source of the P2C ligand, is conserved only in higher Diptera, suggesting that using *Drosophila* P2C may be less effective in other insects. Indeed, P2C ligand-based ReMOT Control in the non-dipteran insects *T. castaneum* (Coleoptera) and *B. tabasci* (Hemiptera) has been reported to be less efficient than in mosquitoes. Therefore, it is important to develop a tag that can deliver RNPs to the ovary by ReMOT Control in a wide range of insect species. One possible approach is to develop a tag based on vitellogenin, since vitellogenin is the major egg yolk protein in a wide range of animal species. It has been reported that the synthetic peptide tag from native vitellogenin allows efficient delivery of an RNP into the ovary in *B. tabasci*, a species in which the use of P2C ligand was ineffective [48•]. ReMOT Control usage is currently limited to generating knock-outs. However, by using modified Cas9 proteins, the range of applications could be greatly expanded. Recently, Aird and colleagues reported that the use of ssDNA-tethered Cas9, in which ssDNA is covalently linked to Cas9, can improve low HDR efficiency by bringing the DNA repair machinery and donor DNA into spatiotemporal proximity [52]. Furthermore, the potential applications of Cas9 range from targeted genome editing, to targeted genome regulation (by binding epigenetic effector domains to Cas9, or by competing with endogenous DNA binding factors). The combination of ReMOT Control with these applications should therefore be applicable to gene knock-outs, knock-ins and expression control.

Conclusion

Transcriptome data, genome assemblies and gene annotations for different orthopteran insect species have recently been made publicly available. In addition, the remarkable innovations in genome editing technologies have enabled functional genomics studies of orthopteran insects, which were previously challenging. These novel gene editing techniques and the genomic information available for orthopteran species might revolutionize not only those research fields that traditionally use insects, but also new fields that might become attracted to the use of orthopteran insects. Among those fields that might turn to orthopteran species are agriculture, which aims to improve the nutritional value and productivity of crickets and reduce their allergen content; biomimetics, which focuses on sound production and auditory systems; and pharmaceutical production as bioreactors using crickets.

Author contributions statement

TN wrote the first draft of the paper with subsequent input from GY and CGE.

Conflict of interest statement

Nothing declared.

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