

# The Genome of the Clonal Raider Ant *Cerapachys biroi*

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## Summary

Social insects are important models for social evolution and behavior. However, in many species, experimental control over important factors that regulate division of labor, such as genotype and age, is limited [1, 2]. Furthermore, most species have fixed queen and worker castes, making it difficult to establish causality between the molecular mechanisms that underlie reproductive division of labor, the hallmark of insect societies [3]. Here we present the genome of the queenless clonal raider ant *Cerapachys biroi*, a powerful new study system that does not suffer from these constraints. Using cytology and RAD-seq, we show that *C. biroi* reproduces via automixis with central fusion and that heterozygosity is lost extremely slowly. As a consequence, nestmates are almost clonally related ( $r = 0.996$ ). Workers in *C. biroi* colonies synchronously alternate between reproduction and brood care, and young workers eclose in synchronized cohorts. We show that genes associated with division of labor in other social insects are conserved in *C. biroi* and dynamically regulated during the colony cycle. With unparalleled experimental control over an individual's genotype and age, and the ability to induce reproduction and brood care [4, 5], *C. biroi* has great potential to illuminate the molecular regulation of division of labor.

## Results and Discussion

To establish the clonal raider ant *Cerapachys biroi* (Figure 1) as a model eusocial organism, we sequenced and assembled its 214 megabase (Mb) draft genome using 33 gigabases (Gb) of Illumina reads (119.2× coverage) and 526 Mb of Roche 454 reads (2.5× coverage). Transcriptome data (11.24 Gb) from all developmental stages and different behavioral states were generated to aid genome annotation. This assembly, and the annotation of 17,263 protein coding genes, provides the first dorylomorph genome and the first genome of an asexual ant.

The *C. biroi* genome assembly is comparable in quality and completeness to the other sequenced ant genomes (Table 1; Supplemental Experimental Procedures, Sequencing and Assembly, available online). *Cerapachys biroi* has the smallest

and most compact ant genome, with genes (including introns) accounting for 36.7% of the assembled genome. Like the other sequenced ant genomes, *C. biroi* has a complete set of DNA methylation enzymes (Supplemental Experimental Procedures, DNA Methylation and Histone Modification). However, the unimodal CpG<sub>(observed/expected)</sub> distribution (Figure S1) provides no clear evidence for germline methylation of the genome [13]. *Cerapachys biroi* has the largest set of odorant receptors of any sequenced insect (Tables 1 and S1; Supplemental Experimental Procedures, Chemoreception), suggesting that the species relies heavily on chemical cues. This is consistent with its subterranean lifestyle and lack of developed eyes. *Cerapachys biroi* also has several gene expansions in the UDP glycosyltransferase and cytochrome P450 gene superfamilies (Table 1; Figures S2 and S3; Supplemental Experimental Procedures, UDP Glycosyltransferases and Cytochrome P450 Genes), which are involved in a broad array of metabolic functions.

## Clonal Reproduction

Colonies of *C. biroi* contain no queens and consist entirely of totipotent workers, all of which reproduce asexually [10, 11]. Most forms of asexual reproduction result in genomic loss of heterozygosity (LOH) each generation. This can incur a high cost: in many eusocial Hymenoptera, development of the female sex is believed to be determined by heterozygosity at one or more sex determining loci, while haploid eggs develop into males [14]. LOH at the sex-determining loci therefore leads to the production of infertile diploid males [15]. Because less than one in 10,000 diploid offspring in *C. biroi* are male (see [8] and the Experimental Procedures), we expected that either the species used a mode of reproduction with no or very little LOH (e.g., premeiotic doubling, ameiotic reproduction, or automixis with central fusion and low recombination rates) or it had evolved an alternate mechanism for sex determination.

To determine the mode of asexual reproduction in *C. biroi*, we stained ovaries and eggs to observe chromosomes during oogenesis and embryogenesis (Figures 2 and S4). Eggs undergo regular meiotic division, which initiates within the first half hour postpartum (Figures 2A and 2B). After the second meiotic division, two haploid nuclei, one from each of the reductional division daughter nuclei, fuse (Figure 2C) and migrate toward the center of the egg. This process occurs entirely within the first hour postpartum. After fusion, the diploid nucleus commences mitotic division. Meanwhile, the remaining two haploid nuclei incompletely fuse, migrate to the membrane, and eventually degenerate (Figure 2D). Therefore, rather than reproducing via premeiotic doubling or ameiotically, *C. biroi* reproduces via automixis with central fusion.

This cytological mechanism also underlies thelytoky in Cape honeybees (*Apis mellifera capensis*) [17]. It has also been inferred for several other ants [18–21], although in those cases it has not been demonstrated cytologically. Automixis with central fusion incurs LOH during meiotic recombination, because all loci distal to a chromosomal crossover event become homozygous unless a second crossover occurs to

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Figure 1. A Worker of the Clonal Raider Ant, *Cerapachys biroi*, Carrying a Pupa

Ants of the genus *Cerapachys* are myrmecophagous and raid the nests of other ants [6]. The genus belongs to the dorylomorph clade of ants, which also includes the infamous army ants [6]. Since the early 1900s, introduced populations of *C. biroi* have become established on tropical and subtropical islands around the world, probably as a consequence of human traffic and trade [7, 8]. Like in many other dorylomorphs, colonies of *C. biroi* undergo stereotypical behavioral and reproductive cycles [5, 9]. Colonies of *C. biroi* lack queens and instead consist entirely of totipotent workers, all of which reproduce asexually [10, 11].

“rescue” heterozygosity [22]. This LOH has been observed in other thelytokous eusocial insects: a clonal lineage of the Cape honeybee had lost 19.1% of its ancestral heterozygosity after 10 years [23] (~146 generations [24], or 0.13% LOH per generation), and in the ants *Wasmannia auropunctata* and *Cataglyphis cursor*, single loci lose 0%–2.8% and 6%–33% (the theoretical maximum under automixis with central fusion) of heterozygosity each generation, respectively [18, 19]. As expected, recombination in these species leads to high proportions of diploid males: 15.4% of diploid eggs in the Cape honeybee [25] and 4.5% of diploid adults in *C. cursor* [26].

Using restriction-site-associated DNA sequencing (RAD-seq), we obtained 7× coverage of ~10% of 91 individual genomes from 19 colonies from four independent clonal lineages (multilocus lineages, MLLs) [8]. Polymorphic loci (including indels) that were scored in at least 80% of individuals were identified, providing 100,608 informative loci for analysis. These loci were used to reconstruct the ancestral genotype for each of the clonal lineages (Figure 3, sensu [23]), allowing us to calculate the average LOH for each clonal lineage. *Cerapachys biroi* has been established invasively for over 100 years in some locations [7], and, based on the time of first collection of a given MLL from the respective study population [8] and a generation time of 34 days [5], a minimum of 225 (MLL1 from Okinawa, Japan), 86 (MLL4 from St. Croix, USA), 43 (MLL6 from Okinawa, Japan), and 0 (MLL13 from Shenzhen, China) generations of asexual reproduction had elapsed by the time we collected samples for our analyses. Surprisingly, no more than 0.3% of ancestral heterozygosity has been lost per individual in any clonal lineage (Figure 3). This implies that the rate of LOH in *C. biroi* is as low as 0.0013% per generation, 100 times lower than in the Cape honeybee. Consequently, individuals from the same MLL are almost clonally identical across the entire genome (average within-MLL relatedness = 0.991; average within-colony relatedness = 0.996) (Figure 3).

Table 1. Summary Statistics of the *Cerapachys biroi* Draft Genome in Comparison to the Eight Sequenced Ant Genomes

	<i>Cerapachys biroi</i>	Eight-Ant Median	Eight-Ant Range
Genome assembly size (Mb)	214	269	214–353
Diploid chromosome number <sup>a</sup>	28	32	16–38
Scaffold N50 (bp)	1,291,492	944,008	598,192–5,154,504
Contig N50 (bp)	31,934	28,034	11,606–62,705
Average sequencing depth	122×	86×	19–123×
Genes with EST support (%)	75.5	66.0	40.0–84.0
CEGMA genes (%)	99.6	99.0 <sup>b</sup>	98.0–99.6 <sup>b</sup>
Repeats (%) <sup>c</sup>	13.8	24.0	11.5–28.0
GC (%)	41.7	36.3	32.6–45.2
CpG <sub>(observed/expected)</sub> (CDS) <sup>d</sup>	1.20	1.42	1.17–1.29
CDS (percent of genome)	9.76	7.52	4.87–9.76
Introns (percent of genome)	26.87	16.62	7.67–26.87
miRNAs <sup>e</sup>	63	93	63–159
Protein coding genes (with IPR domains; with GO terms)	17,263 (9,628; 7,835)	17,220	16,123–18,564
Species-specific genes	4,892	5,025	3,263–6,869
Odorant receptors <sup>f</sup>	369	347 <sup>g</sup>	337–369 <sup>g</sup>
Gustatory receptors <sup>f</sup>	27	63 <sup>g</sup>	21–117 <sup>g</sup>
Ionotropic receptors <sup>f</sup>	26	26	23–32
Cytochrome P450 genes <sup>h</sup>	69	60.5	28–84
UDP glycosyltransferases <sup>h</sup>	21	12	9–21

The draft genome includes official gene set 1.8. The statistics for the eight sequenced ant genomes includes *C. biroi*. Methods are described in the Supplemental Experimental Procedures. See also Figures S1–S3 and Table S1.

<sup>a</sup>From [12].

<sup>b</sup>Data from *H. saltator* and *C. floridanus* are not available.

<sup>c</sup>See the Supplemental Experimental Procedures, Repeats.

<sup>d</sup>Calculated for coding sequences only; for additional CpG analyses, see Figure S1 and the Supplemental Experimental Procedures, DNA Methylation and Histone Modification.

<sup>e</sup>See the Supplemental Experimental Procedures, miRNAs.

<sup>f</sup>Annotated manually and includes only putatively functional genes (for more-detailed results, see Table S1).

<sup>g</sup>Data from *S. invicta*, *A. echinator*, and *A. cephalotes* are not available.

<sup>h</sup>Annotated manually (for more-detailed results, see Figures S2 and S3).

Under automixis with central fusion, we expect to find LOH events spanning large genomic regions and a bias of LOH toward the telomeres, as is the case for Cape honeybees [23, 27]. The exceptionally rare occurrence of haploid males in *C. biroi* [8] precluded obtaining a linkage map to assemble the scaffolds and determine their proximity to the telomeres. However, 18 of the 1,077 assembled scaffolds contained the insect telomeric repeat sequence [28], and these scaffolds were significantly enriched for LOH events (binomial probability distribution,  $p < 0.05$ ). We also found LOH events larger than 1 Mb, as expected. Consistent with a low rate of LOH, no more than 13 large LOH events were present in any single clonal lineage (Figure 3).

Although all clonal lineages show little LOH from their heterozygous lineage ancestor, there is variation in the number of ancestrally heterozygous loci between clonal lineages (Figure 3). The uniform distribution of ancestral heterozygosity across the genomes of the less heterozygous lineages (Wald-Wolfowitz runs test for MLL1 and MLL4,  $p = 0.19$  and  $p = 0.34$ , respectively) is not consistent with localized LOH arising from recombination, but it is consistent with homozygosity arising from inbreeding in a sexual population. We therefore speculate that *C. biroi* underwent inbreeding in a

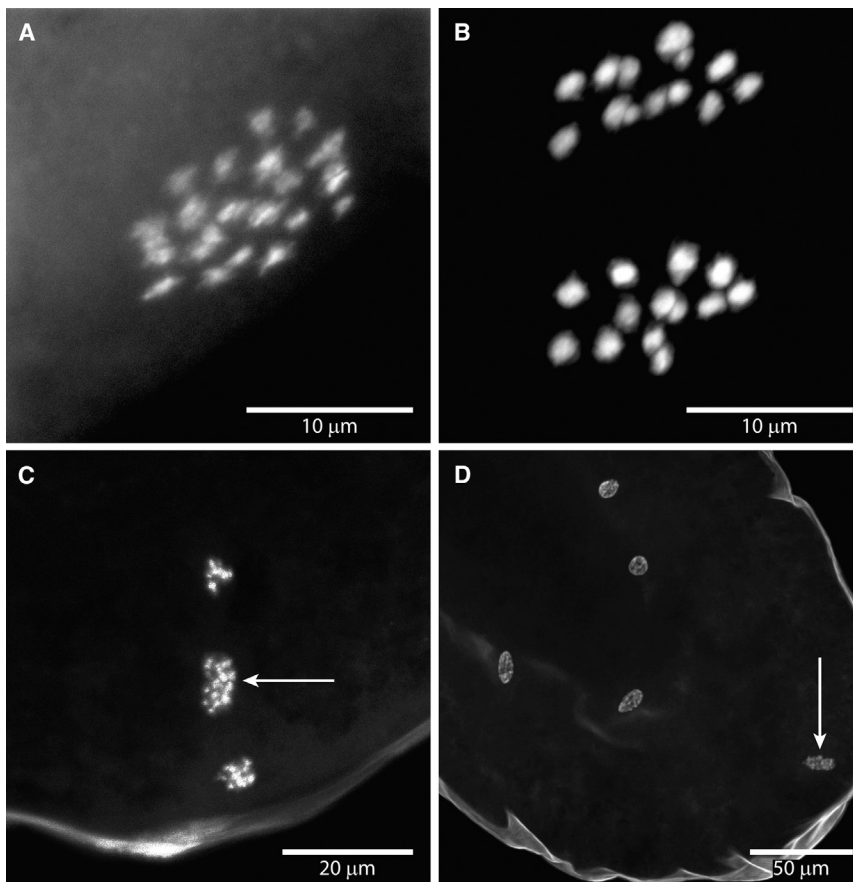


Figure 2. Three-Dimensional Projections of DAPI-Stained Chromosomes in <2-hr-Old Eggs Showing that *C. biroi* Reproduces through Automixis with Central Fusion

Embryos were prepared according to [16]. Details are given in the [Supplemental Experimental Procedures](#), Cytology. The diploid chromosome number in *C. biroi* is  $2n = 28$  [12].

(A) Prophase I immediately postpartum showing a single diploid nucleus close to the posterior pole of the egg.

(B) Meiosis I (reductional division) at approximately 30 min postpartum. Two nascent haploid nuclei can be seen.

(C) Fusion of central products of meiosis II (indicated by arrow) within 1 hr postpartum.

(D) Embryo after two rounds of mitotic division with four diploid nuclei, within 2 hr postpartum. The polar bodies in (C) have fused (arrow) and migrated to the cell membrane, where they degenerate.

See also [Figure S4](#) for additional stages.

subsocial to eusocial, the physiology and behavior expressed during the reproductive phase of the subsocial lifecycle became robustly expressed in the queen caste, while the physiology and behavior expressed during the brood care phase became expressed in the worker caste ([Figure 4A](#)). This hypothesis was later applied explicitly to the molecular regulation of division of labor in honeybee workers by

(possibly facultatively) sexual population, before asexuality became fixed in MLL1 and MLL4.

*Cerapachys biroi* has the lowest LOH rate of any thelytokous eusocial insect studied, losing less heterozygosity in 21 years (MLL1) than other species lose in a single generation. It is not yet clear whether LOH in *C. biroi* arises through reduced recombination during meiosis, via selection against homozygous individuals, or both, as is the case in Cape honeybees [25]. Minimizing LOH allows asexual species with complementary sex determination to avoid the production of diploid males. Intriguingly, however, *C. biroi* lacks an ortholog of the honeybee complementary sex determiner gene *CSD* (see the [Supplemental Experimental Procedures](#), *Transformer Genes*), which, except for the fungus-growing ant *Acromyrmex echinatiflor*, is conserved among all other sequenced eusocial Hymenoptera [29]. Further work is required to determine whether the novel mode of sex determination in *C. biroi*, in concert with low recombination rates, helps prevent the frequent production of diploid males.

### Behavioral and Reproductive Colony Cycles

Many eusocial Hymenoptera are derived from subsocial, progressive provisioning wasps [30–32]. The lifecycle of a progressive provisioning wasp is divided into a reproductive phase, during which the wasp constructs a brood cell, has activated ovaries, and lays an egg, as well as a brood care phase, during which the wasp forages and provisions the larva while her ovaries are inactive ([Figure 4A](#)). Mary Jane West-Eberhard's ovarian ground plan hypothesis (OGPH) [31, 32] states that during the transition from

Amdam et al. [33, 34] as the reproductive ground plan hypothesis (RGPH).

Colonies of *C. biroi* consistently cycle between reproductive and brood care phases that last for 18 and 16 days, respectively ([Figure 4A](#)) [5, 9]. The transitions between phases are synchronized with the development of the brood, which mature in discrete age cohorts. Larvae hatch at the end of the reproductive phase and trigger the transition to brood care by inducing foraging behavior and suppressing ovarian activity in the adults [4]. The reproductive phase, during which workers lay eggs and no foraging occurs, begins when the cohort of larvae pupates. The colony cycle of *C. biroi* and other phasic dorylomorphs, which is more recently derived from nonphasic eusocial ancestors, thereby recapitulates the phasic lifecycle of the ancient subsocial ancestor ([Figure 4A](#)). If the OGPH and RGPH are correct, we would predict that the molecular mechanisms underlying division of labor in nonphasic eusocial insects and, presumably, their subsocial ancestors should also be involved in regulating the *C. biroi* colony cycle. In that case, *C. biroi* could become a powerful model system to establish causality between different molecular mechanisms underlying the fundamental aspects of reproductive physiology and behavior pertinent to the evolution of eusociality. This is because reproductive and brood care behavior in *C. biroi* can be induced via simple brood swap experiments [4, 11], while the analogous experiment is not possible in most other ants, because the equivalent behaviors are fixed in queen and worker castes: induction of caste development at the larval stage does not allow isolation and evaluation of the salient

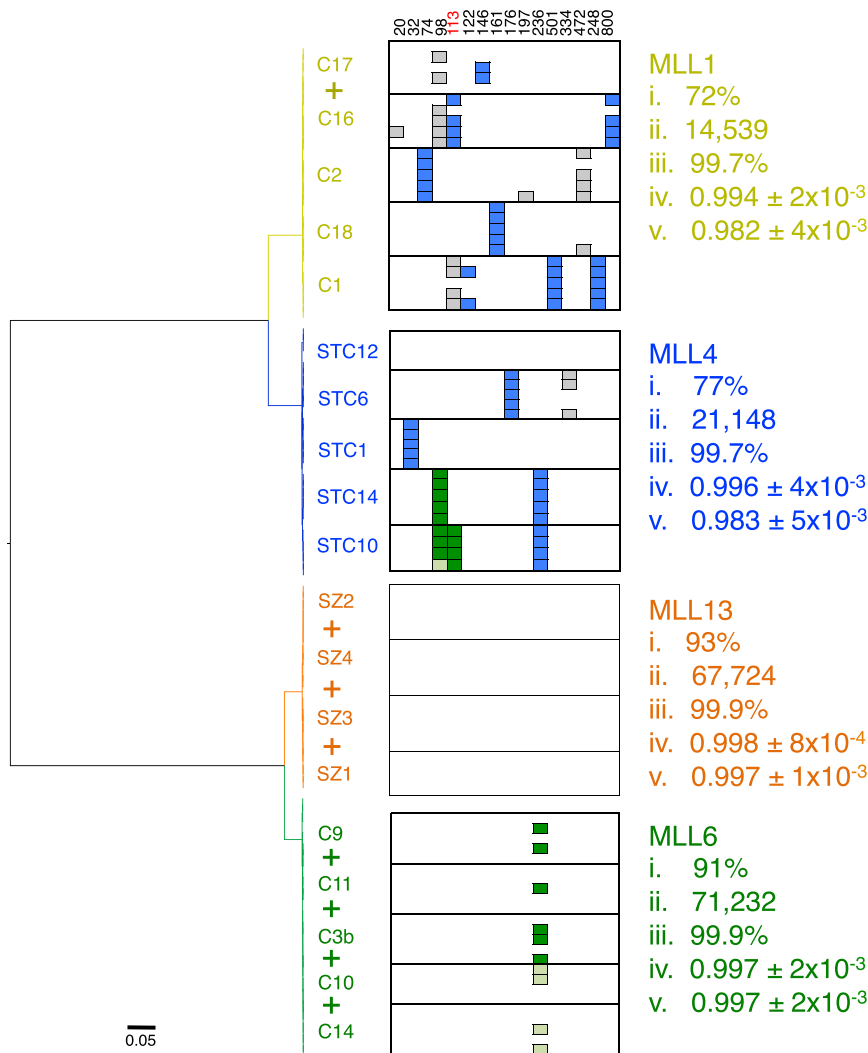


Figure 3. Phylogenetic and Genomic Relationships between *C. biroi* Individuals, Colonies, and Clonal Lineages

The UPGMA tree shows the average number of substitutions per site for 100,608 informative sites, between 91 ants from four clonal lineages and 19 colonies. Colonies that are not recovered as monophyletic in the phylogeny are indicated by a “+” between colony names (there is no colony-level resolution for MLL6 and MLL13). Contiguous regions of homozygous loci  $\geq 1$ Mb in size are shown in the map on the right: each colored square represents a putative single loss of heterozygosity (LOH) event. Columns indicate the 16 different scaffolds that contain LOH spanning  $\geq 1$ Mb (scaffold number given above each column). Scaffold 113 (marked in red) contains the telomeric repeat sequence. Identical block colors within a column indicate homozygous fragments with identical beginning and end positions, which most likely arose from a single ancestral LOH event. Lighter-colored blocks indicate individuals that had only unscored loci in the focal positions and are therefore consistent with either sharing the LOH of the darker-colored individuals or having the ancestral heterozygous genotype. Regions marked in gray contained unscored loci that were heterozygous in the individuals lacking the LOH fragment and may therefore actually represent two or more smaller homozygous fragments, each  $< 1$  Mb. Figures beneath each lineage name are as follows (from top to bottom): (i) the percentage of 1,077 scaffolds that contain some heterozygosity in the ancestral state; (ii) the number of loci that are heterozygous in the ancestral genotype; (iii) the average heterozygosity per individual, calculated as the percentage of heterozygous loci among the loci that were inferred to be heterozygous in the lineage ancestor (for calculations, see the [Supplemental Experimental Procedures, RAD-Seq Analysis](#)); (iv) the average within-colony relatedness ( $\pm$ SD); and (v) the average between-colony relatedness ( $\pm$ SD).

behaviors, and reversible activation of worker reproduction and queen-like behavior is usually only possible in highly contrived situations and not amenable to precisely controlled time-course analysis.

We therefore tested whether conserved genes that have previously been implicated in division of labor in other eusocial insects are dynamically regulated during the *C. biroi* colony cycle. We chose to study juvenile hormone (JH) synthesis, *Vitellogenin* (*Vg*), *Foraging* (*For*), and *Malvolio* (*Mvl*). The RGPH explicitly links JH and *Vg* to the evolution of division of labor [33]. Similarly, *For* and *Mvl* gene expression have been shown to influence worker division of labor in social insects [35–37].

*Vitellogenin* (*Vg*) is an egg yolk precursor protein produced for egg provisioning, while JH is a gonadotropic hormone associated with ovary activation, vitellogenesis, and egg laying in solitary insects. In honeybee workers, decreasing *Vg* and increasing JH levels are associated with the transition to foraging behavior [38]. In ants, JH inhibits egg laying and increases foraging in founding queens and workers [39–44], but stimulates egg laying in established queens [44, 45].

*Vitellogenin* was duplicated in the ancestor of the Formicoid ants, creating two clades that exhibit queen/nurse-specific

and forager-specific expression [46, 47] (hereafter referred to as *Vgq* and *Vgw* clades, respectively). To investigate the evolution and expression of *Vg* in *C. biroi*, we used the previously identified *Vg* genes from ants [46–51] and *A. mellifera* to identify and reannotate *Vg* sequences for all eight ants (see the [Supplemental Experimental Procedures, Vitellogenin Annotation and Phylogeny](#)). Incomplete genome assembly in *Camponotus floridanus* at the *Vg* tandem duplication locus (Figure 5A) prevented identification of more than one *Vg* gene. *Cerapachys biroi* has two copies of *Vg*, one in the *Vgq* clade and one in the *Vgw* clade (Figure 5B). Unlike all other sequenced ant *Vgw* genes, *CbirVgw* does not have a 3' truncation in the final exon relative to *Vgq*.

*CbirVgq* expression was significantly higher in workers in the reproductive phase immediately prior to egg laying (Figure 4B). *CbirVgw* expression was significantly higher in workers during the brood care phase (Figure 4B). Both *C. biroi* *Vgs* were more expressed in abdomens than in heads (Figure 4C). While in the harvester ant *Pogonomyrmex barbatus* the expression levels of *Vgq* were consistently higher than those of *Vgw* [53], the reverse was the case in *C. biroi*, where *CbirVgw* expression was higher than *CbirVgq* expression in 94% of samples.

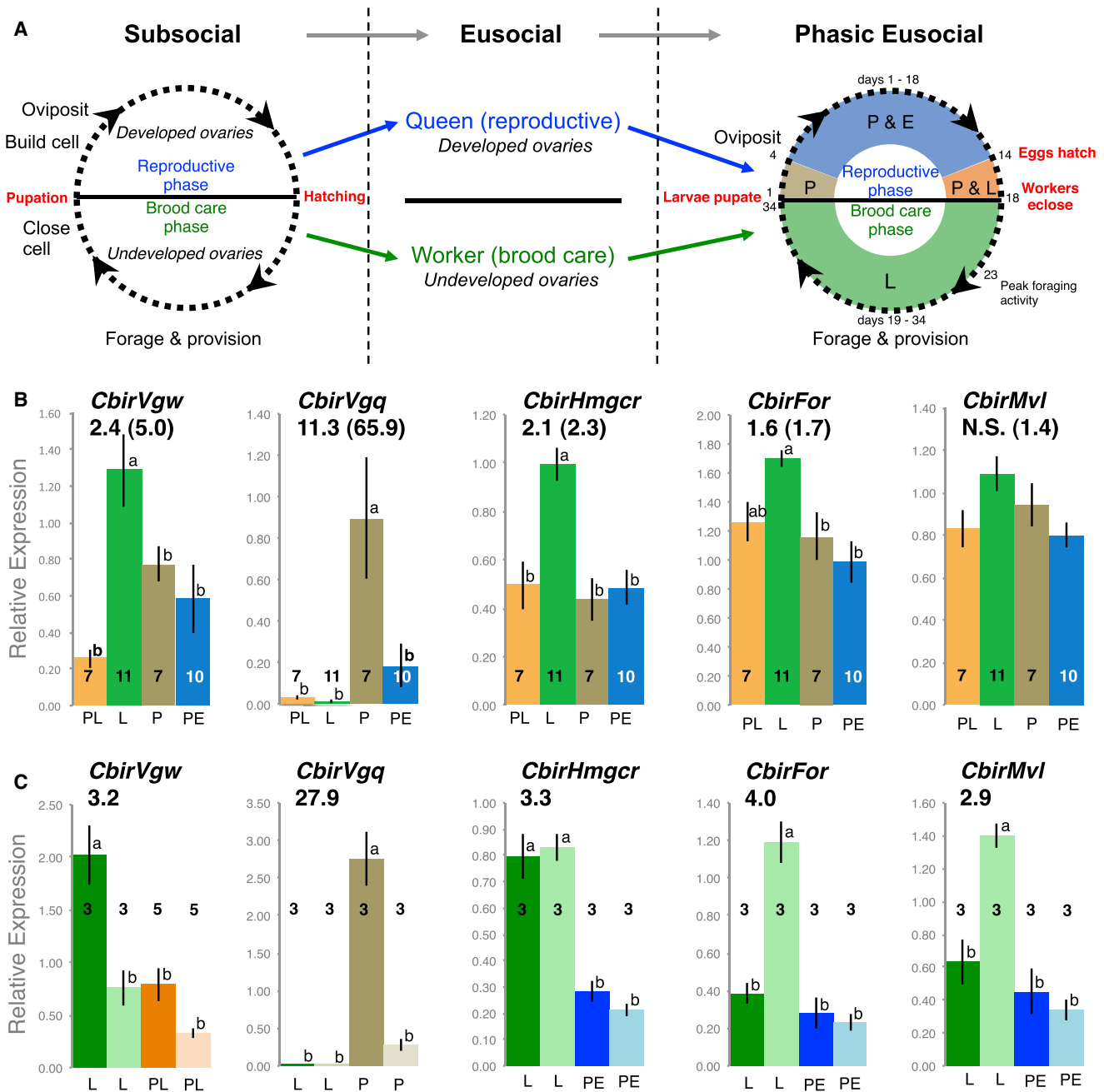


Figure 4. Schematic of the Hypothesized Evolutionary Transitions from Subsocial to Eusocial to Phasic Eusocial, with the Phase-Specific Expression of Candidate Genes throughout the *C. biroi* Colony Cycle

(A) Schematic showing the compartmentalization of subsocial behaviors into eusocial queen and worker castes and reintegration into the phasic colony cycle of *C. biroi*. The timing of behaviors and corresponding brood stages are indicated on the subsocial and phasic eusocial cycle. The *C. biroi* reproductive phase is subdivided into three stages based on the brood present: gray, pupae (P) only; blue, pupae and eggs (E); orange, pupae and larvae (L).

(B) Whole-body gene expression for *C. biroi* *Vgw*, *Vgq*, *Hmgcr*, *For*, and *Mvl* during the four stages described in (A). Graphs show relative expression (mean  $\pm$  SEM). Colors correspond to the different stages of the colony cycle in (A). Brood stages present are also indicated on the x axis of each graph and correspond to the colony cycle in (A). Samples for the brood care phase (green) were collected at day 23, when foraging activity has reached its highest level. Sample size is indicated inside or above each column in bold. Letters above columns indicate significantly different groups (Bonferroni-corrected ANOVA [ $p < 0.05$ ] with Tukey's post hoc tests [ $p < 0.05$ ]). Numbers beneath gene names show average fold change in expression between significantly different groups. Maximum fold change for each gene is indicated in parentheses.

(C) Tissue-specific gene expression in some of the behavioral stages showing differences in (B). Head and abdomen expression are indicated with light and dark colors, respectively. Graphs show mean  $\pm$  SEM gene expression as described for (B).

*Cerapachys biroi* *Vg* evolution and expression is consistent with Formicoid *Vg* duplication and functional specialization [53]. The timing and abdominal expression of *CbirVgw*

expression is consistent with a conserved role in egg yolk provisioning in ants [45], while the role of *CbirVgw* has yet to be determined.

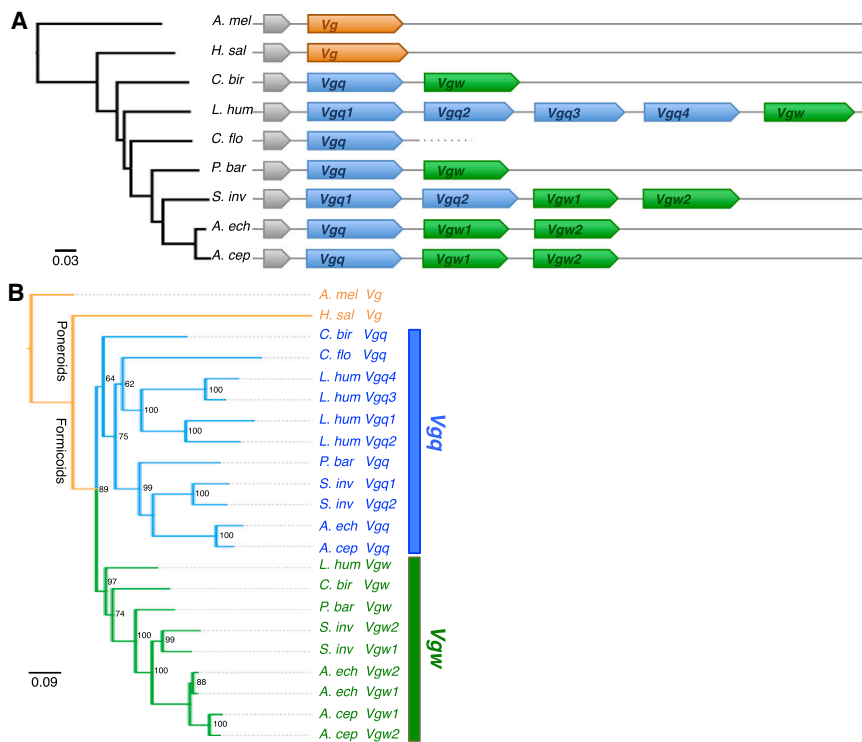


Figure 5. Vitellogenin Sequence Analysis

Previously annotated Vg genes were used to identify existing and novel Vg genes in all eight ant genomes (see the Supplemental Experimental Procedures, Vitellogenin Annotation and Phylogeny).

(A) Phylogeny of all sequenced ant species with their corresponding Vg loci mapped. The maximum-likelihood tree is based on first and second codon positions constructed with RAxML (GTR+G model) [52] using 3,164 orthologous single-gene families present in all ants, *A. mellifera*, *N. vitripennis* (not shown), and the outgroup *D. melanogaster* (not shown) (see the Supplemental Experimental Procedures, Phylogeny Reconstruction and Gene Expansions). Bootstrap support values (100 replicates) for all nodes are 100%. *A. mel*, *Apis mellifera*; *H. sal*, *Harpegnathos saltator*; *C. bir*, *Cerapachys biroi*; *L. hum*, *Linepithema humile*; *C. flo*, *Camponotus floridanus*; *P. bar*, *Pogonomyrmex barbatus*; *S. inv*, *Solenopsis invicta*; *A. ech*, *Acromyrmex echinator*; *A. cep*, *Atta cephalotes*. Arrows indicate direction of transcription. Colors correspond to the reproduction-associated and brood care-associated Vg genes (Vgq [blue] and Vgw [green], respectively). Grey genes indicate an orthologous lipid transport protein immediately upstream of all hymenopteran Vgs. A tandem duplication occurred at the base of the Formicoid clade, followed by several independent duplications in different Formicoid lineages.

(B) Maximum-likelihood phylogram of ant Vg genes. Colors and abbreviated species names correspond to those in (A). Vgq and Vgw clades are indicated with solid bars. Bootstrap values based on 1,000 replicates are given for each node. Branch lengths indicate substitutions per site.

3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*) is the rate-controlling enzyme of the mevalonate pathway responsible for JH synthesis [54]. In *C. biroi*, *Hmgcr* was upregulated during foraging (Figure 4B) and positively correlated with *CbirVgw* expression ( $R = 0.39$ ,  $p < 0.05$ ), but not *CbirVgq* expression ( $R = -0.10$ ,  $p = 0.6$ ), consistent with the nongonadotropic function of JH found in workers and foundress queens of other ant species [39–44].

*Malvolio* is a manganese transporter involved in sucrose responsiveness in *Drosophila* [55] and honeybees [35]. In honeybee workers, *Mvl* is upregulated in foragers compared to nurses and implicated in worker division of labor [35]. *Foraging* is a conserved protein kinase that regulates feeding-related behavior in Metazoans [56, 57]. The expression of *For* is specifically associated with foraging behavior in ant and bee workers, although its mode of action is varied and expression in queens has not been studied explicitly [36, 37, 58]. We found that *Mvl* and *For* are most expressed in foraging *C. biroi* workers (Figures 4B and 4C; differences in expression based on whole bodies were only significant for *For*), with both genes significantly more expressed in heads than in abdomens during the brood care phase (Figure 4C). This expression pattern is comparable to that in honeybee workers [35, 37], but inverse to the pattern observed in harvester ants for *For* expression [36]. In honeybees, *Mvl* is upregulated in response to brood pheromone [59]. It is therefore possible that the brood-mediated regulation of *C. biroi* phasic behavior [11] is similarly influenced by *CbirMvl* expression.

These gene expression patterns in *C. biroi* are consistent with roles associated with foraging and reproductive behavior, just as in other eusocial insects. Importantly, the phase-specific expression patterns in *C. biroi* match the behaviorally and physiologically equivalent caste-specific

expression patterns seen in nonphasic eusocial insects. The gene networks underlying reproduction and brood care in *C. biroi* are therefore likely to be the same conserved networks underlying caste-specific behavior in other eusocial insects. Furthermore, this raises the possibility that the phasic colony cycle in *C. biroi* and other phasic dorylormorphs represents a partial evolutionary reversal to the ancestral subsocial lifecycle.

## Conclusions

*Cerapachys biroi* maintains genomic heterozygosity, despite meiotic reproduction, at a level that is surpassed only by ameiotic species. The virtually clonal colony structure that results from this allows for experimental control and replication of individual genotypes and the genetic composition of social groups, to a degree that is unattainable in most other social Hymenoptera. The totipotency of workers and the absence of queens allows for easy colony propagation and the composition of arbitrarily sized experimental colonies, while the colony cycle allows for precise selection of age-matched workers and experimental control over colony demography. Because reproductive and brood care states can be experimentally induced in *C. biroi* [4, 11], reproductive caste-equivalent identity can be manipulated at the adult stage, facilitating the establishment of causality between different molecular mechanisms and caste differences. *Cerapachys biroi* therefore has great potential to provide novel insights into the regulation of gene networks that underlie reproductive division of labor in insect societies. Although a few other social insects share some of these favorable traits, none of them has become a well-established model species. In conclusion, the unique biology of *C. biroi* makes the species a promising new model system to study the molecular underpinnings of social evolution and behavior.

## Experimental Procedures

### Genome Sequencing, Assembly, and Annotation

Illumina HiSeq 2000 sequencing was performed on five paired-end libraries from individuals of MLL4. Roche 454 FLX sequencing was performed on individuals from MLL1 and MLL6. Illumina reads were assembled into scaffolds using SOAPdenovo [60], and the scaffolds were gap-filled using Illumina and Roche 454 data. Transcriptome data were obtained from pooled cDNA from all life stages from MLL4. Gene annotations were obtained using homology, de novo, and transcriptome methods. We manually curated 803 genes. For more details, see the Sequencing, Assembly, Annotation and Functional Annotation section in the [Supplemental Experimental Procedures](#).

### Estimating the Rate of Diploid Male Production in *C. biroi*

We collected five diploid males over three years from ~30 colonies, with each colony producing approximately 6,000 diploid workers over that period. Five diploid males among 180,000 diploid offspring corresponds to one in 36,000, well below one in 10,000 diploid offspring.

### Restriction-Site-Associated DNA Sequencing

Of the original 95 individuals, 91 provided a minimum genome-wide average sequencing depth of 1.3× and were included in all subsequent analyses (Figure 3). For inference of the ancestral lineage genotypes (sensu [23]), all reads at a given locus from all individuals in a lineage were pooled, and the two most frequent alleles taken as the ancestral alleles (sensu [61]), as long as both had read depths greater than 20 (no locus had more than two such alleles). To account for type II errors arising from library construction and allele sequencing bias, we did not score homozygous loci with a heterozygous ancestral genotype and a single-allele sampling probability greater than 0.001 (see [62]). Additionally, homozygous loci that were present in only one individual and were unlinked to other homozygous loci were excluded as probable genotyping errors. Pairwise relatedness between individuals was calculated for each clonal lineage as in [8] using all loci that were heterozygous in the ancestral genotype of the respective lineage. See the RAD-Seq Analysis section in the [Supplemental Experimental Procedures](#) for more details.

### Quantitative RT-PCR

For each sample, eight 1-month-old workers from colonies at the appropriate stage in the cycle were collected and pooled. RNA was extracted using a modified Trizol/phenol chloroform protocol. Quantitative PCR was performed on a Roche LightCycler using SYBR green. *RPS3*, *RPS6*, and *RPL13α*, which showed invariable expression levels during the colony cycle, were used as reference genes. Gene expression was calculated according to [63]. MIQE [64] quantitative PCR protocol details are given in the Real-Time Quantitative RT-PCR section in the [Supplemental Experimental Procedures](#).

### Accession Numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under accession number JAS100000000. The version described in this paper is JAS101000000.

### Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2014.01.018>.

### Acknowledgments

This work was supported by grant 1DP2GM105454-01 from the NIH and a grant from Rockefeller University to D.J.C.K., as well as a grant from the Leon Levy Foundation for Mind, Brain, and Behavior to P.R.O. I.F.-P. was supported by a grant from the G. Harold & Leila Y. Mathers Foundation to Cori Bargmann. S.K.M. was supported by NRSA Training Grant number GM066699 to Sidney Strickland. We would like to thank the laboratory of Hermann Steller for use of their confocal microscope. Three anonymous reviewers provided helpful comments on an earlier version of this manuscript.

Received: August 30, 2013

Revised: November 26, 2013

Accepted: January 9, 2014

Published: February 6, 2014

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