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Fluorescence *in situ* hybridization: a new method for determining primary sex ratio in ants

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Abstract

The haplodiploid sex determining system in Hymenoptera, whereby males develop from haploid eggs and females from diploid eggs, allows females to control the primary sex ratio (the proportion of each sex at oviposition) in response to ecological and/or genetic conditions. Surprisingly, primary sex ratio adjustment by queens in eusocial Hymenoptera has been poorly studied, because of difficulties in sexing the eggs laid. Here, we show that fluorescence *in situ* hybridization (FISH) can be used to accurately determine the sex (haploid or diploid) of eggs, and hence the primary sex ratio, in ants. We first isolated the homologue coding sequences of the *abdominal-A* gene from 10 species of 8 subfamilies of Formicidae. Our data show that the nucleotide sequence of this gene is highly conserved among the different subfamilies. Second, we used a sequence of 4.5 kbp from this gene as a DNA probe for primary sex ratio determination by FISH. Our results show that this DNA probe hybridizes successfully with its complementary DNA sequence in all ant species tested, and allows reliable determination of the sex of eggs. Our proposed method should greatly facilitate empirical tests of primary sex ratio in ants.

Keywords: *abdominal-A* gene, egg sex determination, fluorescence *in situ* hybridization (FISH), Formicidae, primary sex ratio

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Introduction

Over the past decades, study of the sex ratio has been the focus of much theoretical and empirical research in evolutionary ecology. Recently, an increasing number of studies in birds and mammals has addressed the issue of variations in the primary sex ratio (the proportion of each sex at oviposition or birth) in relation to factors such as environmental and parental quality, or differential dispersal of sexes. Results from these studies tend to either support adaptive deviations from a 1:1 sex ratio (e.g. Westerdahl *et al*. 2000; Oddie & Reim 2002; and references therein), or not find any consistent deviation (Radford & Blackey 2000; Grindstaff *et al*. 2001; Brown & Silk 2002). However, the proximate mechanisms of primary sex ratio control in vertebrates remain enigmatic; no physiological or genetic mechanism for skewing the sex ratio at egg-laying or birth are known (for potential mechanisms, see, e.g. Krackow 1995; Oddie 1998; Komdeur & Pen 2002; Lee 2002).

By contrast to vertebrates, Hymenoptera are characterized by a haplodiploid sex determination system, whereby males usually develop from unfertilized, haploid eggs, and females from fertilized, diploid eggs. Male haploidy gives females a powerful mechanism for controlling the sex of the brood, by deciding whether to fertilize an egg. Thus, opening or closing the spermathecal valve under the decision of the female is the controlling element of the primary sex ratio in Hymenoptera (Gerber & Klostermeyer 1970). Surprisingly, whereas the proximate mechanism controlling sex determination is well-known in Hymenoptera (Cook & Crozier 1995), primary sex ratio adjustment by laying females has been poorly studied. For instance, in parasitoid Hymenoptera, which have given ample support for sex allocation theories (Godfray 1994), primary sex

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ratio adjustment in response to ecological and genetic conditions has been usually inferred on the basis of variation in the secondary sex ratio (proportion of each sex at the adult stage; Charnov *et al*. 1981; Wrensch & Ebbert 1993; Godfray & Werren 1996; West & Sheldon 2002). Yet, sex ratios at the egg and adult stages may differ because of sex-specific differences in brood mortality during larval development (Smith & Shaw 1980; King 1993; Nagelkerke & Hardy 1994). In eusocial Hymenoptera, sex ratio studies are complicated by a possible conflict between the queens and the nonreproducing workers over the optimal sex ratio investment (Trivers & Hare 1976; Bourke & Franks 1995; Crozier & Pamilo 1996). Such conflicts arise because of the haplodiploid genetic system, which results in asymmetries of genetic relatedness among colony members with workers being more closely related to their sisters than to their brothers. Hence, workers, who reproduce indirectly by rearing the queen's offspring, should maximize their inclusive fitness by favouring female-biased broods. By contrast, queens are equally related to offspring of both sexes, and are selected to favour an even sex investment ratio. Workers may perform sex allocation biasing by killing male brood (Aron *et al*. 1994, 1995; Keller *et al*. 1996; Sundström *et al*. 1996; Chapuisat *et al*. 1997) or by controlling the female caste fate, i.e. whether a diploid larva will develop into a reproductive female (potential queen) or a sterile worker (Hammond *et al*. 2002). However, queens may gain some control over colony sex ratio by limiting the number of diploid (female) eggs laid (Passera *et al*. 2001; Reuter & Keller 2001).

A critical issue concerning sex allocation of reproductive offspring in eusocial Hymenoptera is therefore the extent each caste (the queens or the workers) controls the proportion of males and females among the dispersers. The most straightforward method to address this issue relies on a precise estimation of the primary and the secondary sex ratios. Despite its theoretical interest and consequences, little quantitative work on primary sex ratio determination in eusocial Hymenoptera has been performed, because of the difficulty in estimating the proportion of haploid and diploid eggs laid by queens. To date, data on primary sex ratio have been obtained either from karyotypes (Aron *et al*. 1994, 1999; Sundström *et al*. 1996; Aron & Passera 1999) or from genetic analyses at several microsatellite marker loci (Arévalo *et al*. 1998; Ratnieks & Keller 1998; Passera *et al*. 2001; Hammond *et al*. 2002). However, both procedures have major drawbacks.

1 Karyotype analyses are tedious and time-consuming. The number of chromosomes must be known for each species and the optimal 'age' of eggs (the time since they were laid) must be determined because younger eggs may not have enough cells in metaphase and older ones may have too many. More importantly, reliable estimates of the primary sex ratio require large samples, as sex determination is successful for ≈ 50–60% of the eggs at best (Aron, personal observation).

2 The use of microsatellite marker loci is certainly more accurate. However, the isolation and characterization of such markers require much effort and are costly. Moreover, egg sex determination may be limited when genetic variation is reduced, which may occur when populations have been through bottlenecks (e.g. invasive species) or in species with a high level of inbreeding (e.g. social parasites).

Here, we propose the use of fluorescence *in situ* hybridization (FISH) as a new, large-scale method for primary sex ratio determination in the Formicidae. This method of cytogenetics involves hybridizing a specific DNA probe, labelled with a fluorochrome, with its complementary target DNA sequence (Muleris *et al*. 1996; Stanley 1996). Our aim was to isolate a unique and highly conserved genomic DNA sequence, which can be used as a generally applicable DNA probe for sexing ant eggs using FISH analyses. Because of male haploidy, the target DNA sequence is in a single copy in nuclei from haploid eggs and in a double copy in nuclei from diploid eggs, so that hybridization should reveal one or two fluorescent spots per nucleus, respectively. The homeotic gene *abdominal-A* (*abd-A*), known to specify the identity of the most abdominal segments in insects (Hughes & Kaufman 2002), has previously been shown to present a high level of sequence conservation between a coleopteran, *Tribolium castaneum*, and a hymenopteran, *Myrmica rubra* (Niculita *et al*. 2001). We first isolated the homologue coding sequences of the *abd-A* gene from 10 species from 8 subfamilies of Formicidae. Our data show that the nucleotide sequence of this gene is highly conserved among the different subfamilies of Formicidae. This led us to select a sequence of 4.5 kbp from this gene as a DNA probe for primary sex ratio determination by FISH. Our results show that this probe hybridizes successfully with all ant species tested, and allows reliable determination of the sex of the eggs.

Materials and methods

Samples

The samples of ant species used are given in Fig. 1. For *abdominal-A* homologue isolation, we selected 10 species taken from 8 of the 16 recognized ant subfamilies. Samples were kept in ethanol (100%) at –20 °C until nucleic acid extraction. For primary sex ratio determination by FISH, haploid and diploid eggs were collected from laboratory-reared ant colonies, belonging to 15 species and 4 subfamilies. Haploid eggs were obtained from workers reared in the absence of queens in species where workers are not completely sterile. Diploid eggs originated from

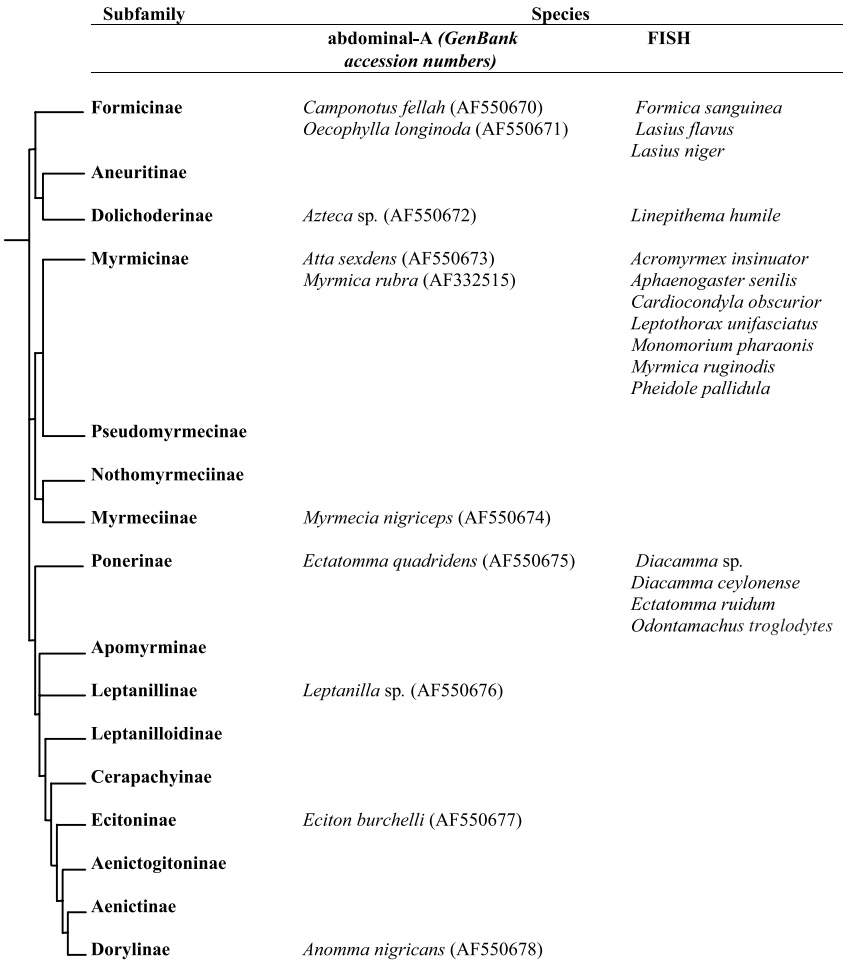

Fig. 1 Cladogram of ant subfamilies (according to Baroni Urbani *et al*. 1992). The species studied for *abdominal-A* homologue analyses and primary sex ratio determination by FISH are indicated.

young freshly mated queens which mostly lay diploid worker-destined eggs in their founding stage (Aron & Passera 1999). It should be stressed, however, that our sample contained a social parasite (*Acromyrmex insinuator*) and several (facultatively) polygynous species (*Formica sanguinea*, *Linepithema humile*, *Cardiocondyla obscurior*, *Monomorium pharaonis*, *Myrmica ruginodis*), where queens do not found independently and can start laying both haploid and diploid eggs quickly after mating (Aron & Passera 1999). However, despite that these species could contribute to blur the segregation between haploid worker-laid eggs and diploid queen-laid eggs, this imprecision did not affect our data (see result, Table 2). Once collected, the eggs were used immediately for FISH analyses, or stored in a freezing milieu (10% DMSO/BME) in liquid nitrogen or at −80 °C for subsequent analyses.

Comparison of the coding sequences of the abd-A *homologue in ants*

Primer development for cloning of the abd-A *homologue.* A comparative analysis of the complete *abd-A* gene previously revealed that sequences are highly conserved between a beetle (*Tribolium castaneum*) and an ant (*Myrmica rubra*) (Niculita *et al*. 2001). This gene has three exons (Fig. 2). The coding region of the first exon (the largest: 750 bp) has several motifs conserved between Coleoptera and Hymenoptera; the second exon, although very small (36 bp), is 100% identical between these insects; the coding region of the third exon (396 bp) contains the homeobox and the Ubx-*abd-A* signature, which are also highly conserved. The *abd-A* homeobox is very similar to that of *Ultrabithorax* and *Antennapedia* (Niculita *et al*. 2001). Therefore, to eliminate possible cross-hybridization between *abd-A* and other homeobox genes we isolated the coding region of the first exon.

To compare the nucleotide sequence of the gene *abd-A* between the different ant species we designed two pairs of primers from published *abd-A* sequences of *Myrmica rubra* (Niculita *et al*. 2001) and *Tribolium castaneum* (Shippy *et al*. 1998) (Fig. 2). For the first pair, primer M (positive strand: 5′-CGGCACCGGCGATATGAGTACGAAATTC-3′) was at the beginning of the coding sequence and primer J (negative strand: 5′-GGGTTGTTGGCAGGATGTCAAAGGATG-3′) was at the end of the first exon. The M–J primer pair

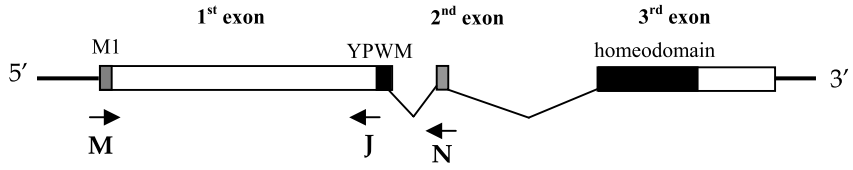

Fig. 2 Schematic structure of the *abd-A* locus in *Myrmica rubra*. The coding sequences are shown as bars, the introns as thin black lines, the 5′-UTR and the 3′-UTR as thick black lines. The hexapeptide (YPWM) and homeodomain positions are indicated by filled black bars; filled grey bars correspond to conserved sequences, with M1 at the 5′ extremity of the coding region and the small second exon (from Niculita *et al*. 2001). The position of primers M, J and N is indicated by arrows.

amplifies a 700-bp sequence corresponding to the coding region of the first *abd-A* exon. An additional primer, primer N (negative strand: 5′-CACACCACTCTGTCAAATGGA CTCATCC-3′) was designed to anneal in the highly conserved second exon. This primer, when paired with primer M, amplifies a 4.5 kbp sequence corresponding to the coding region of the first exon, the first intron and the second exon. Primer pairs M–J and M–N were used to amplify genomic DNA. The polymerase chain reaction (PCR) products from M–N and M–J were then sequenced for 2 species (*Myrmica rubra* and *Camponotus fellah*) and all 10 species, respectively.

DNA extraction and PCR. The equivalent of ≈ 10 mg of fresh tissue, corresponding to one or more workers, was crushed for 5 min in 100 µL of extraction buffer (0.1 M EDTA, 1% SDS, 0.1 M Tris–HCl, pH 9.0) in an Eppendorf tube using a rotating pestle. 25 µL of 5 M potassium acetate were added and the tube placed at 4 °C for 5 min. After 15 min of centrifugation at 10 000 *g* the supernatant was collected and precipitated with 0.5 volume of isopropanol. The DNA precipitate was dissolved in 100 µL of 1 mM EDTA, 10 mM Tris–HCl, pH 7.5. The yield was ≈ 10 µg of genomic DNA per sample. Between 10 and 20 ng were used in each PCR. Long PCR were performed from high molecular mass genomic DNA isolate with Qiagen Genomic-tip 20/G kit. DNA was diluted in 100 µL of TE. PCR were performed, in a total volume of 25 µL containing 10–20 ng of genomic DNA, 2.5 µL reaction buffer 10× (Appligene), 10 pmol of each primer, 10 nmol of each dNTP and 0.25 U of *Taq* DNA polymerase (Appligene). The PCR temperature profile comprised an initial denaturation for 1 min at 95 °C followed by 40 cycles of denaturation (30 s at 95 °C), annealing (30 s at a temperature adapted for each species between 56 and 68 °C), extension (1 min 30 s at 72 °C). A 3-min extension step at 72 °C ended the reaction.

Cloning and sequencing. PCR products were cloned using the TOPO TA cloning system (Invitrogen). Clones were sequenced by the Applied Biosystems dye terminator method and run on an ABI automated sequencer. Sequences were analysed using the Wisconsin™ Package Version 9.1, Genetics Computer Group (GCG).

Primary sex ratio determination by FISH

Preparation of the DNA probe. FISH requires a long probe for detection of fluorescent signals. Therefore, the sequence of 4.5 kbp of the gene *abd-A* amplified by the primers M–N from the myrmicine ant *Myrmica rubra* was used as a DNA probe (see Results). The probe was labelled indirectly with biotinylated-16-dUTP using a nick-translation kit (Roche). The enzymatic reaction was kept for 3 h at 15 °C to obtain probe fragments of an average size of 500 bp. The reaction was stopped by adding 1.2 µL EDTA 0.5 M and heating at 65 °C for 10 min.

FISH analysis. Eggs were spread out on a prewashed (acetic acid and ethanol) slide in 15 µL of KCl 0.075 M. After 45 min, the slides were immersed in a bath of Carnoy's fixation solution (1 acetic acid: 3 methanol) for 30 min, and air-dried before storage at −80 °C until ready for FISH. The hybridization protocol used was a modification of the standard FISH method applied in human cytogenetic laboratories (Knoll & Lichter 1994). Slide preparations were treated with RNase A 100-µg/mL (Roche) for 45 min in a moist chamber at 37 °C, and rinsed three times for 5 min in 2× SSC at room temperature. After 15 min treatment at 37 °C in a pepsin 0.05% (Serva)/0.01 M HCl solution, the slides were consecutively immersed for 5 min in a bath of 1× PBS, prefixative buffer 1 (1× PBS/$MgCl_2$ 0.05 M), fixative buffer 2 (1× PBS/$MgCl_2$ 0.05 M/paraformaldehyde 4%) and 1× PBS at room temperature. Slide preparations were dehydrated in an ethanol series baths (70, 90 and 100%) for 3 min each at room temperature, then denatured with a 70% formamide/2× SSC solution in a hotplate at 75 °C for 3 min and immediately dehydrated through a cold (–20 °C) ethanol series. The hybridization mixture consisted of a final concentration of biotin-labelled DNA probe at 100 ng/µL, of 50% formamide, 2× SSC, 12.5% dextran sulphate. This mix was denatured by heating at 70 °C for 5 min, and quick-cooled on ice for 3 min prior to use. Preliminary experiments revealed that addition of repetitive DNA sequence (Cot-I DNA) did not improve hybridization. Nevertheless, prehybridization was performed because it substantially decreased aspecific hybridization. After this prehybridization phase of the

probe mix (40–90 min at 37 °C), an aliquot of 10 μL was applied onto the nuclei preparations, the slides were covered with a cover slip and incubated overnight in a moist chamber at 37 °C. The following day, slides were washed at 45 °C successively in three changes of 50% formamide/2× SSC for 7 min each time, in 0.1× SSC for 10 min, in 2× SSC for 5 min, and rinsed at room temperature in BIOT-washing solution (4× SSC/Tween-20 0.05%) for 5 min. Blocking buffer containing 5% nonfat dry milk/4× SSC was added to each slide and slides were incubated for 20 min in a moist chamber at 37 °C. Slides can not be dried in any point during detection and washing steps. After blocking treatment, hybridization signals were detected by avidin conjugated to fluorescein isothiocyanate (FITC) (Roche) and biotinylated goat antiavidin (BgAA) antibodies (Vector Laboratories). Slides were incubated in a moist chamber at 37 °C with 1:200 dilution of avidin–FITC in blocking buffer followed by 1:100 dilution of BgAA in blocking buffer for 15 min each. Between each layer of antibodies, slides were washed three times in BIOT-washing solution for 7 min each at 45 °C in darkness. The intensity of biotin-linked fluorescence is amplified by adding in the end a layer of avidin–FITC. Nuclei were counterstained by adding 15 μL of a staining solution [DAPI 125 μM/Vectashield antifade solution (Vector)] under a cover slip. Hybridized slides were observed under a Zeiss Axioskop epifluorescence microscope (×1000) equipped with FITC (λ_{exc} = 493 nm, λ_{em} = 523 nm) and DAPI (λ_{exc} = 367 nm, λ_{em} = 452 nm) bandpass filters. Images were captured and analysed by using CYTOVISION software (Applied Imaging).

Egg sex determination. The ploidy level of eggs (haploid or diploid) was based on the number of fluorescent (FL) spots per nucleus. Several nuclei were observed for each egg (range: 5–27). The proportion of single spot nuclei per egg (PSSN) was used as the main variable to differentiate a haploid male from a diploid female egg.

Statistical analyses

A two-way ANOVA was carried out using the arcsine-transformed PSSN in order to test for the effect of sex and ant subfamily. Samples with PSSN values of 1 or 0 (n = 3), not suitable for the arcsine transformation, were removed from the analysis.

A binomial model was used to evaluate the probability of mistakenly classifying an egg as haploid or diploid for a given number of observed nuclei per egg. In this model, each observation constituted an independent trial in which a nucleus from a haploid or a diploid egg has a probability P_M or P_F of presenting a single spot, respectively. P_M and P_F were estimated independently by the observed average PSSN for haploid and diploid eggs. The probability of error was estimated by summing up probabilities predicted by the binomial model corresponding to cases in which the egg would be wrongly classified using a PSSN classification threshold of 0.5 for a total number of observed nuclei of 5, 10, 15 and 20.

Results

Comparison of the coding sequences of the abd-A *homologue in ants*

A single *abd-A* gene was found in the 10 ant species studied. The comparison of the coding region of the *abd-A* locus between these phylogenetically divergent species showed an unusually high level of similarity of the nucleotide sequences. The identity observed for the first exon of the *abd-A* (700 bp sequenced out of the 750 bp of the whole exon) ranged from 0.806 to 0.927 (Table 1).

The high molecular mass of the DNA required to allow amplification of the 4.5 kbp sequence was obtained for only 2 of the 10 species sampled: *Myrmica rubra* and *Camponotus fellah*. The alignment of the nucleotide sequences for the 3.8 kbp of intronic sequence revealed that they were 60% identical. Because of the high similarity of the *abd-A* homologue between many ant species, we selected the 4.5 kbp sequence of *abd-A* from *Myrmica rubra* as a DNA probe for FISH analyses.

Primary sex ratio determination by FISH

The 4.5 kbp DNA probe hybridized successfully with all 15 ant species in our sample (Table 2 and Fig. 3). A two-way ANOVA showed that the proportion of nuclei containing a single fluorescent spot (PSSN) differed significantly with the sex of the eggs (sex: $F_{1,44}$ = 239.8, P < 0.001), with diploid eggs showing a lower PSSN than haploid eggs, but not with ant subfamily (ant subfamilies: $F_{3,44}$ = 0.388, P = 0.762; sex × ant subfamily: $F_{2,44}$ = 1.375, P = 0.263). The distribution of the PSSN in haploid and diploid eggs across all species is summarized in Fig. 4. In diploid eggs, the proportion of single FL-spot nuclei ranged from 0.0 to 0.4 and was on average 0.157. In haploid eggs, this proportion ranged from 0.5 to 1.0 and was on average 0.728. Restricting the data to cases in which a number of nuclei analysed per egg is n > 10, the extreme value of 0.5 disappears and both distributions are even more separated.

Table 3 gives the probability of error in determining the 'sex' of the eggs as a function of sample size, as predicted by the binomial model using probabilities of presenting a single spot of P_M = 0.728 and P_F = 0.157 for haploid and diploid eggs, respectively. When the number of nuclei analysed per egg n = 10, the probability of error in sexing eggs reaches the acceptable value of 0.0298 for haploids

	M.r.	A.s.	M.n.	C.f.	O.l.	A.sp.	E.q.	L.sp.	E.b.	A.n.
M.r.	1.000	0.901	0.903	0.881	0.850	0.893	0.927	0.840	0.881	0.893
A.s.	—	1.000	0.887	0.866	0.860	0.870	0.891	0.818	0.856	0.860
M.n.	—	—	1.000	0.872	0.868	0.901	0.925	0.832	0.893	0.875
C.f.	—	—	—	1.000	0.856	0.852	0.877	0.806	0.887	0.844
O.l.	—	—	—	—	1.000	0.846	0.866	0.834	0.848	0.858
A.sp.	—	—	—	—	—	1.000	0.909	0.838	0.911	0.875
E.q.	—	—	—	—	—	—	1.000	0.846	0.899	0.887
L.sp.	—	—	—	—	—	—	—	1.000	0.828	0.840
E.b.	—	—	—	—	—	—	—	—	1.000	0.887
A.n.	—	—	—	—	—	—	—	—	—	1.000

M.r. *Myrmica rubra*, A.s. *Atta sexdens*, M.n. *Myrmecia nigriceps*, C.f. *Camponotus fellah*, O.l. *Oecophylla longinoda*, A.sp. *Azteca* sp., E.q. *Ectatomma quadridens*, L.sp. *Leptanilla* sp., E.b. *Eciton burchelli*, A.n. *Anomma nigricans*.

Table 1 Sequence identity matrix for the first exon *abdominal-A* nucleotidic sequences isolated from 10 ant species. The lowest identity (80.6%) observed inside the ant family is between *Leptanilla* sp. (L.sp.) and *Camponotus fellah* (C.f.)

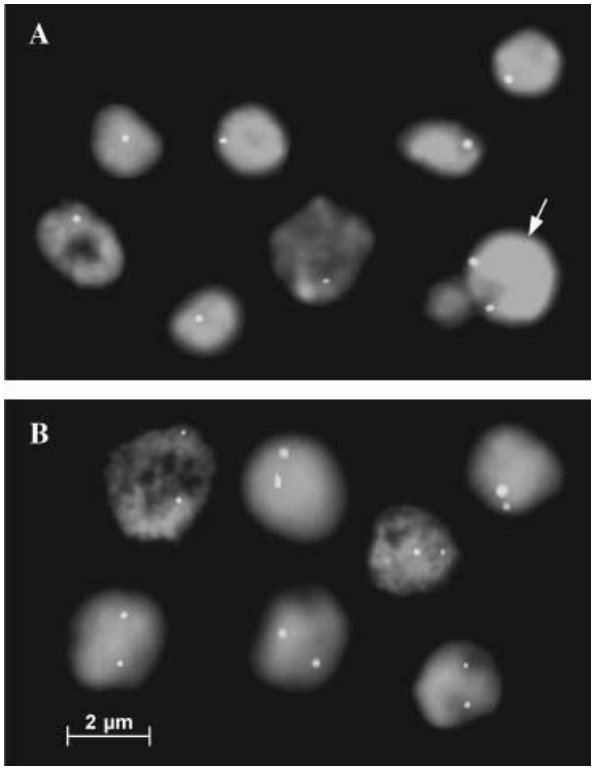

Fig. 3 Fluorescence *in situ* hybridization of egg nuclei using the *abdominal-A* sequence of 4.5 kbp as a probe. (A) Nuclei from a worker-laid (haploid) egg of *Diacamma ceylonense*. Arrow: nucleus with two FL-spots at the G_2 stage of the cell cycle. (B) Nuclei from a queen-laid egg (diploid) of *Pheidole pallidula*. Hybridization signals are detected by FITC fluorescence (green), whereas the nuclei are counterstained with DAPI (blue).

and 0.0120 for diploids. Tables of predicted probability associated with all possible combinations of results from $n = 5$ to $n = 20$ for haploid and diploid eggs are reported in the Appendix.

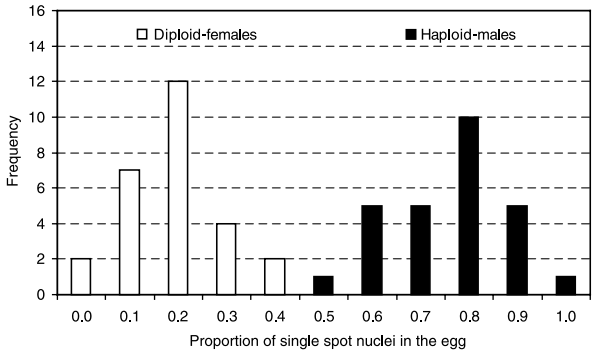

Fig. 4 Distribution of the proportion of single spot nuclei (PSSN) among haploid and diploid eggs. Overall, the average PSSN is 0.728 for haploid eggs and 0.157 for diploid eggs.

Discussion

According to the most recent estimates (Grimaldi & Agosti 2000), divergence from the basal hymenopteran lineage of the family Formicidae is thought to have occurred ≈ 120 Ma. Approximately 20 Myr later, the subfamilies Formicinae, Myrmicinae and Ponerinae split from each other and the origin of the more modern subfamilies dates from the Eocene. These phylogenetical data support our belief that the eight subfamilies we studied are representative of the diversity of ants in general.

Our results show a high identity between all nucleotide sequences from the first exon of the gene *abd-A* in 10 representative species of the 8 subfamilies of ants, with an identity ranging from 0.806 to 0.927. Moreover, the nucleotide sequences from the first intron of 3.8 kbp are 60% identical in at least two ant species. These results, together with the high similarity of the second exon (Niculita, personal observation) and the third exon (Astruc *et al*. manuscript in preparation) of the *abd-A* in Hymenoptera, indicates that the gene *abdominal-A* is highly conserved in the Formicidae.

Table 2 Ploidy-level determination of eggs by FISH, for 15 species from 4 ant subfamilies. Each line corresponds to a single egg. S: expected egg ploidy level; H: haploid (worker-laid egg); D: diploid (queen-laid egg); n: total number of nuclei observed per egg; n_1 to n_4: number of nuclei with 1, 2, 3 or 4 fluorescent spots. PSSN: proportion of single spot nuclei (n_1/n)

Species	S	n	n_1	n_2	n_3	n_4	PSSN
Lasius niger	D	14	1	**10**	1	1	0.071
	D	20	6	**12**	0	2	0.300
	D	15	1	**11**	1	2	0.067
	D	16	2	**9**	4	1	0.125
Lasius flavus	H	20	**16**	4	0	0	0.800
	H	10	**8**	2	0	0	0.800
	H	12	**9**	3	0	0	0.750
	H	5	**5**	0	0	0	1.000
	H	14	**8**	6	0	0	0.571
Formica sanguinea	H	22	**15**	7	0	0	0.682
Myrmica ruginodis	D	18	2	**8**	2	6	0.111
	D	24	7	**15**	1	1	0.292
	D	26	9	**11**	2	4	0.346
	H	15	**9**	6	0	0	0.600
Leptothorax unifasciatus	H	17	**13**	4	0	0	0.765
	H	27	**18**	9	0	0	0.667
	H	20	**17**	3	0	0	0.850
	H	10	**7**	3	0	0	0.700
	H	21	**12**	9	0	0	0.571
	H	6	**5**	1	0	0	0.833
Monomorium pharaonis	D	20	3	**15**	1	1	0.150
	D	18	2	**16**	0	0	0.111
	D	18	0	**14**	2	2	0.000
	D	21	4	**16**	1	0	0.190
Aphaenogaster senilis	H	17	**13**	4	0	0	0.765
	H	21	**14**	7	0	0	0.667
	H	21	**14**	6	1	0	0.667
	H	21	**17**	4	0	0	0.810
	H	6	**3**	3	0	0	0.500
	H	22	**13**	9	0	0	0.591
Pheidole pallidula	D	25	6	**16**	3	0	0.240
	D	19	2	**14**	3	0	0.105
	D	10	4	**5**	1	0	0.400
Acromyrmex insinuator	D	15	1	**13**	0	1	0.067
	D	10	1	**9**	0	0	0.100
Cardiocondyla obscurior	D	10	2	**8**	0	0	0.200
	D	14	4	**9**	1	0	0.286
Diacamma ceylonense	H	27	**22**	4	1	0	0.815
	H	21	**17**	4	0	0	0.810
	H	21	**15**	6	0	0	0.714
	H	20	**17**	3	0	0	0.850
Diacamma sp.	H	20	**15**	5	0	0	0.750
	H	19	**11**	7	1	0	0.579
Ectatomma ruidum	D	20	2	**17**	1	0	0.100
	D	20	3	**13**	2	2	0.150
	D	20	4	**14**	0	2	0.200
	D	9	0	**8**	0	1	0.000
Odontomachus troglodytes	H	15	**11**	3	1	0	0.733
	H	5	**4**	1	0	0	0.800
Linepithema humile	D	13	1	**9**	2	1	0.077
	D	20	3	**14**	2	1	0.150
	D	17	2	**12**	1	2	0.118
	D	10	2	**6**	1	1	0.200
	D	12	1	**9**	1	1	0.083

Table 3 Probability of classification error of haploid (male) eggs and diploid (female) eggs, with a threshold of the proportion of single spot nuclei (PSSN) of 0.5 (classification rule: haploid when PSSN = 0.5; diploid when PSSN < 0.5), as predicted by a binomial model

	P error	
n	Males	Females
5	0.1281	0.0302
10	0.0298	0.0120
15	0.0286	0.0008
20	0.0079	0.0004

The high level of conservation of *abd-A* was critical for generating a unique probe for FISH analysis. Our data show that the probe of 4.5 kbp from *Myrmica rubra* hybridizes successfully with the DNA of all ant species tested. Moreover, its use in FISH allows the 'sex' (haploid or diploid) of eggs, and hence primary sex ratio to be reliably determined on the basis of the number of fluorescent spots per nucleus. Particularly, the proportion of nuclei containing a single fluorescent spot per egg (PSSN) obviously is an accurate measure for differentiating haploid male from diploid female eggs, the latter always showing a lower PSSN value than the former. When the number of nuclei/egg observed was greater than 10, the probability of erroneously determining the sex of eggs was < 0.0298 for haploid eggs and < 0.0120 for diploid eggs. Compared with the other methods used to date for primary sex ratio determination, the FISH method is more accurate than karyotypes and cheaper than microsatellites. Moreover, compared with microsatellite marker loci, FISH does not require to design species-specific primers and is not affected by genetic variability. However, if microsatellite markers are designed for a given species, primary sex ratio determination by FISH in this species is more time-consuming than genetic analyses.

At least three explanations may account for the difference between the observed and expected level of ploidy of the nuclei (i.e. nuclei from haploid eggs sometimes revealing more than a single FL-spot and nuclei from diploid eggs sometimes revealing more or fewer than two FL-spots, see Table 2). First, cells in developing eggs undergo active divisions so that they have nuclei at all stages of the cell cycle, including at the G_2 stage (with a double DNA content preceding the actual nuclear division) which will have twice the expected number of spots. In addition, homologous chromosomes may not duplicate synchronously, leading to nuclei with three FL-spots in diploid eggs. Second, because of the 3D structure of the nuclei, the probe does not always successfully hybridize with the target DNA, resulting in a lower than expected number of FL-spots. Third, nonspecific hybridization, as reported in ants (Lorite *et al*. 2002) and other hymenopteran species (Matsumoto *et al*. 2002), may also account for deviations from the number of FL-spots expected. This could explain, for example, the presence of three FL-spots in some haploid nuclei. However, despite these potential confounding effects, our data show that these factors did not significantly affect egg sex determination.

One factor that may potentially limit the application of FISH for sexing eggs is the existence of diploid males. Diploid males arise from fertilized eggs with homozygosity at the sex-determining locus (Cook & Crozier 1995). FISH does not enable discrimination between diploid male and diploid female eggs. However, because diploid males arise from fertilized eggs, they were 'intended' to be females and could reasonably be entered as such in determining the sex ratio established by selection. It should be mentioned that none of the species selected for our FISH analyses is known to have diploid males.

In conclusion, our results show that FISH can be applied successfully to determining the sex of eggs in ants. Importantly, the use of a unique probe made of a DNA sequence that is highly conserved between the different subfamilies of Formicidae means it can be used for egg sex determination in phylogenetically diverse species. The ability to determine reliably the primary sex ratio laid by queens has important implications for testing key issues in evolutionary biology such as queen–worker conflict over sex ratio in ants (Trivers & Hare 1976) and adaptive primary sex ratio adjustment in response to ecological and/or genetic conditions, e.g. the mode of colony foundation (Aron & Passera 1999), the number of queens per colony (Keller *et al*. 1996) or local competition (Hamilton 1967; Clark 1978). Our proposed method should greatly facilitate empirical tests of these hypotheses.

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This work is part of Ludivine de Menten's doctoral thesis on queen control over sex ratio in ants, granted by the Belgian FRIA. Hélène Niculita works on molecular evolution of the homeotic gene *abd-A* and morphological diversity of abdomen in ants. HN is a post-doc at the Université de Lausanne (Switzerland). Danièle Delneste heads the Laboratory of Cytogenetics (Hospital Erasme), and is specialized in human prenatal diagnosis of chromosomal anomalies. Serge Aron and Marius Gilbert are Research Scientists of the Belgian FNRS. MG. works on the spatial ecology of insect pest species. SA works on kin conflicts and sex ratio determination in ants.

Appendix I

Probability of having a given number of single spot nuclei according to the total number of nuclei analysed for haploid and diploid eggs. The probability associated with all possible combinations of results from $n = 5$ to $n = 20$ as predicted by the binomial model is given for males ($P_M = 0.728$) and females ($P_F = 0.157$), respectively. Tables must be used as follows. Consider a situation with five nuclei over eight revealing a single spot. The probability of such an event to occur is 0.2304 if the egg is haploid or 0.0032 if the egg is diploid. The probability of mistakenly classifying the egg as haploid is thus equal to 0.0032.

	Total number of nuclei															
Number of single spot nuclei	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0	0.0015	0.0004	0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
1	0.0199	0.0065	0.0021	0.0006	0.0002	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
2	0.1067	0.0435	0.0166	0.0060	0.0021	0.0007	0.0002	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
3	0.2855	0.1553	0.0739	0.0322	0.0131	0.0051	0.0019	0.0007	0.0002	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
4	0.3820	0.3117	0.1978	0.1076	0.0527	0.0239	0.0102	0.0042	0.0016	0.0006	0.0002	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
5	0.2045	0.3337	0.3177	0.2304	0.1410	0.0767	0.0383	0.0178	0.0079	0.0033	0.0014	0.0005	0.0002	< 0.0001	< 0.0001	< 0.0001
6		0.1489	0.2834	0.3084	0.2516	0.1711	0.1024	0.0557	0.0281	0.0134	0.0061	0.0026	0.0011	0.0005	0.0002	0.0001
7			0.1084	0.2358	0.2886	0.2617	0.1958	0.1278	0.0753	0.0410	0.0209	0.0101	0.0047	0.0021	0.0009	0.0004
8				0.0789	0.1931	0.2627	0.2620	0.2138	0.1512	0.0959	0.0559	0.0304	0.0156	0.0077	0.0036	0.0016
9					0.0574	0.1562	0.2337	0.2543	0.2248	0.1712	0.1164	0.0724	0.0418	0.0228	0.0118	0.0058
10						0.0418	0.1251	0.2042	0.2407	0.2291	0.1869	0.1356	0.0896	0.0548	0.0315	0.0171
11							0.0304	0.0994	0.1757	0.2230	0.2274	0.1980	0.1526	0.1067	0.0689	0.0417
12								0.0222	0.0784	0.1492	0.2029	0.2208	0.2042	0.1666	0.1230	0.0836
13									0.0161	0.0614	0.1253	0.1818	0.2102	0.2058	0.1773	0.1378
14										0.0117	0.0479	0.1043	0.1607	0.1967	0.2033	0.1843
15											0.0086	0.0372	0.0860	0.1404	0.1814	0.1974
16												0.0062	0.0288	0.0705	0.1214	0.1651
17													0.0045	0.0222	0.0573	0.1040
18														0.0033	0.0170	0.0464
19															0.0024	0.0131
20																0.0017

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Appendix I *Continued*

Number of single spot nuclei	Total number of nuclei															
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0	0.4257	0.3589	0.3025	0.2550	0.2150	0.1812	0.1528	0.1288	0.1086	0.0915	0.0772	0.0650	0.0548	0.0462	0.0390	0.0329
1	0.3964	0.4010	0.3944	0.3800	0.3604	0.3376	0.3130	0.2879	0.2629	0.2387	0.2156	0.1938	0.1736	0.1550	0.1379	0.1224
2	0.1477	0.1867	0.2204	0.2477	0.2685	0.2829	0.2915	0.2949	0.2938	0.2889	0.2810	0.2707	0.2587	0.2453	0.2311	0.2165
3	0.0275	0.0464	0.0684	0.0923	0.1167	0.1405	0.1629	0.1831	0.2006	0.2152	0.2268	0.2353	0.2409	0.2437	0.2439	0.2419
4	0.0026	0.0065	0.0127	0.0215	0.0326	0.0458	0.0607	0.0767	0.0934	0.1102	0.1267	0.1424	0.1570	0.1702	0.1817	0.1915
5	< 0.0001	0.0005	0.0014	0.0032	0.0061	0.0102	0.0158	0.0229	0.0313	0.0411	0.0519	0.0637	0.0760	0.0887	0.1015	0.1141
6		< 0.0001	< 0.0001	0.0003	0.0008	0.0016	0.0029	0.0050	0.0078	0.0115	0.0161	0.0217	0.0283	0.0358	0.0441	0.0531
7			< 0.0001	< 0.0001	< 0.0001	0.0002	0.0004	0.0008	0.0014	0.0024	0.0039	0.0058	0.0083	0.0114	0.0153	0.0198
8				< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0002	0.0004	0.0007	0.0012	0.0019	0.0029	0.0043	0.0060
9					< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0001	0.0002	0.0004	0.0006	0.0010	0.0015
10						< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0001	0.0002	0.0003
11							< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
12								< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
13									< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
14										< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
15											< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
16												< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
17													< 0.0001	< 0.0001	< 0.0001	< 0.0001
18														< 0.0001	< 0.0001	< 0.0001
19															< 0.0001	< 0.0001
20																< 0.0001

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