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1 # *Camponotus fellah* queens are singly mated

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12

13 ## Abstract

14 The ant *Camponotus fellah* has been used in several behavioural and life-history studies.
15 An important factor that affects colony genetic structure and division of labour is whether
16 queens are singly or multiply mated. To determine whether queens are singly mated in *C.*
17 *fellah*, as is the case in some other *Camponotus* species, we developed nine polymorphic
18 microsatellite markers and sequenced 16 workers from each of 20 colonies at six loci.
19 Data in all colonies were compatible with queen monoandry. All the workers of one of the
20 colony had identical genotypes suggesting that they were clonally produced or that the
21 queen was inbred. We therefore genotyped the mother queen as well as 31 more workers
22 of the same colony at the same six loci plus the three remaining loci. These data revealed
23 that the queen was homozygous at eight of the nine loci and that she mated with a male
24 having an identical locus at all but one of the loci. Thus, the queen was apparently not only
25 inbred but also probably mated with a brother.

26

27 ## Keywords
28 *Camponotus fellah*, microsatellites, monoandry, ant, genetic structure

29 ## Acknowledgements

Introduction

The genetic structure of ant colonies varies widely between species and can impact colony immunity, reproductive output and division of labor (Crozier and Page 1985; Julian and Cahan 1999; Heinze and Keller 2000; Julian and Fewell 2004; Rheindt *et al.* 2005; Oldroyd and Fewell 2007; Smith *et al.* 2008). In colonies where queens are multiply mated several studies have shown that workers from different patrilines vary in their tendency to specialize in different tasks thereby potentially affecting the efficiency of division of labor and colony homeostasis (Robinson 1992; Hughes *et al.* 2003; Rheindt *et al.* 2005; Schwander *et al.* 2005; Wiernasz *et al.* 2008; Waddington *et al.* 2010). Increased within-colony genetic diversity as a result of the presence of multiple patrilines and/or matrilines may also improve resistance to parasites and pathogens (Hughes and Boomsma 2004; Reber *et al.* 2008; Schmidt *et al.* 2011) thereby enhancing the lifetime and reproductive output of a colony. Thus, knowledge on the genetic makeup of colonies provides insights on what factors might shape colony functioning.

Studies in several species of the genus *Camponotus* (*C. herculeanus*, Gertsch et al. 1995; *C. ligniperdus*, Gertsch et al. 1995, Gadau et al. 1998; *C. floridanus*, Gadau et al. 1996; *C. nawai*, Satoh et al. 1997; *C. consobrinus*, Fraser et al. 2000; *C. ocreatus*, Goodisman and Hahn 2004 and *C. festinatus*, Goodisman and Hahn 2005) revealed that queens are generally singly-mated with occasional instances of polyandry. An interesting species of the genus Camponotus that has not yet been investigated is C. *fellah*. This species has been extensively used to study nestmate recognition (Boulay et al. 2000a, b; Katzav-Gozansky et al. 2004; Katzav-Gozansky et al. 2008), and more recently to unravel the impact of social isolation (Boulay et al 1999; Koto et al. 2015) and decipher the organizational principles of division of labor (Mersch et al. 2013; Greenwald et al. 2015; Kafsi et al. 2016). The colonies exhibit pronounced division of labor with workers organized in distinct social groups that specialize in different tasks (Mersch *et al*. 2013). The propensity of a worker to join a social group, and execute preferentially some tasks might thus be dependent on a worker's patriline. To determine whether *Camponotus fellah* queens are singly-mated, or whether workers come from different fathers, we developed and tested a set of nine microsatellites.

Material and methods

Collection of ants

Adult workers were collected from 20 lab-reared colonies (A–T). Each lab colony was established from a single queen collected during a mating flight in 2007 on the campus of the Tel-Aviv University, Tel Aviv, Israel, which is within the natural range of the species (Ionescu-Hirsch 2009).

Genetic analysis

PCR primers were developed for *C. fellah* microsatellite loci from a partial genomic library that was derived from 10 µg genomic DNA extracted with the Qiagen robot from one worker, and sent to Ecogenics (http://www.ecogenics.ch/) for 454 sequencing and primer design. To determine which of the designed primers were polymorphic we tested nine primers on two workers from each of eight colonies (A-H). Six out of the nine microsatellites had five or more alleles in the workers tested (Table 1). We used these six microsatellites to assess queen-mating frequency in 20 colonies using 16 workers per colony. However, because there was a very high level of homozygosity in colony S, we increased the sample size to 47 workers and also genotyped the queen using one of her legs. In this colony we also genotyped all individuals at the three remaining microsatellites (7275, 7207, 7300).

We extracted DNA from each individual using the BioSprint 96 DNA Blood Kit for tissue sample from QIAGEN and dissolved the DNA in a final volume of 200µl. We performed PCR amplifications for each ant sample in a total volume of 20µL. To reduce the amount of work and sequencing, we multiplexed primers 1284, 6826 and 5134 in one reaction, and primers 3186, 3675, 4380 in the other. Each reaction consisted of: 4µL of genomic DNA, 4µL of 10x PCR buffer (QIAGEN), 1.92µL of 25 mM MgCl2, 0.32µL of 25 mM dNTPs, 1µL of each forward and reverse primer (10 µM), 0.3µL of 5U/µL Taq polymerase (QIAGEN), 3.2µL of 5x enhancers (Qsolution from QIAGEN), and 0.26µL H2O. The PCR consisted of an initial denaturing step of 5 min at 95°C, followed by 35 cycles that each consisted of 30s of denaturation at 95°C, 30 s of annealing at 55°C, and 60 s elongation at 72°C. A final elongation step was performed at 72°C for 10 min. We analyzed PCR products with the ABIprism3100 DNA sequencer and the GeneMapper Software (Applied Biosystems).

100 **Data analysis**

101 We estimated linkage disequilibrium between each pair of loci using the Genepop

102 software (Raymond and Rousset 1995; Rousset 2008). For each colony, we determined

103 the number of worker patrilines. This was simplified by the fact that all colonies were lab-

104 raised with all workers being the offspring of a single queen. Further, in ants males are

105 haploid because of the haplodiploid sex determination system of Hymenoptera. Thus, at

106 each locus, a worker inherits one allele from her father and one of the two alleles of the

107 queen. If two males share the same alleles at all genotyped loci, it is impossible to

108 distinguish their offspring. The theoretical probability of such non-detection errors was

109 estimated as:

110

111 $$\prod\sum q_i^2$$

112

113 with q_i as the frequency of the ith allele (Boomsma and Ratnieks 1996). The sum is over

114 all alleles at a locus, and the multiplication is over all loci.

115

116 We discarded from our patriline analyses eight out of the 351 workers genotyped. Two

117 samples from colony B were contaminated during the PCR and six workers from four

118 different colonies (colony B: 2 workers, colony G: 2 workers, colony L: 1 worker, colony

119 R: 1 worker) had genotypes incompatible with the genotype inferred for their queen.

120 Because colonies were all lab-raised and headed by a single queen, we suspect that these

121 workers were intruders that had escaped from other colonies. Aggression toward non-

122 nestmates is often attenuated in the lab for two reasons. First, colonies, which are kept in

123 proximity to each other, habituate to olfactory volatiles of other colonies, which reduce

124 aggression (Katzav-Gozansky *et al.* 2008). Second, lab colonies are typically fed with the

125 same diet, which can reduce inter-colony differences in hydrocarbon profiles (Richard *et*

126 *al.* 2004, Ichinose *et al.* 2009).

127

128 **Results**

129 The six microsatellite were very polymorphic with an average of 7.11 alleles per locus

130 (range: 2-14, Table 1), as estimated from all genotyped workers (n = 365). There was no

131 sign of gametic linkage among the loci (p > 0.05 for all pair-wise comparisons of loci after

132 sequential Bonferroni corrections), and all loci were in Hardy-Weinberg equilibrium (P

133 >0.4). Overall, the probability of not detecting a second male was extremely low ($4.89*10^{-4}$

134).

135

136   Our patriline analysis showed that for all colonies and all loci, except one (locus 3675 in

137   colony I), worker genotypes were consistent with workers originating from a queen

138   mated to a single male (Table 2). In colony I, eight workers were homozygote for the allele

139   224 at locus 3675 and the remaining worker homozygous for allele 240 (Table 2). Given

140   that workers of this colony had normal genotypes at other loci, this pattern is most likely

141   explained by the queen having a 224/240 genotype, and the male having a null allele at

142   this locus.

143

144   In colony S, all 16 workers had identical genotypes. They were homozygous at five of the

145   six loci while at locus 6826 they were all heterozygotes. This pattern could be explained

146   by workers being clonally produced or by the mother being homozygous at the six loci

147   and mated with a male having an identical allele at five of the six loci. To discriminate

148   between these two hypotheses we genotyped all workers at three additional loci,

149   analysed another 31 workers from the same colony at the nine loci and also genotyped

150   the queen. The 31 additional workers had identical genotypes as the initial 16 workers

151   at the six first loci. For two of the additional loci all 47 workers had again identical

152   homozygous genotypes, but for the third additional locus (locus 7275) 24 workers were

153   homozygote and 23 were heterozygote. The queen was homozygote for all loci except

154   locus 7275 for which she was heterozygote. Because workers were heterozygote at locus

155   6826 where the queen was homozygote, and varied in their genotypes for locus 7275,

156   they were apparently sexually-produced. The most parsimonious explanation for the

157   genotypic composition of this colony is that the queen was inbred and that she mated with

158   a brother that shared the same allele at all but locus 6826.

159   **Discussion**

160   The genotypic data from a total of 343 workers from 20 colonies suggest that *C. fellah*

161   queens mate with a single male, and thus that all workers produced by the queen are full

162   sisters. This, together with previous studies in *C. herculeanus* (Gertsch et al. 1995), *C.*

163   *ligniperdus* (Gertsch et al. 1995; Gadau et al. 1998), *C. floridanus* (Gadau et al. 1996), *C.*

164   *nawai* (Satoh et al. 1997), *C. consobrinus* (Fraser et al. 2000), *C. ocreatus* (Goodisman and

165   Hahn 2004) and *C. festinatus* (Goodisman and Hahn 2005) indicate that queens are mostly

166   singly-mated in the genus *Camponotus*.

167

168   Estimates of patrilines can be inaccurate due to non-detection errors and small sample

169   size (Pedersen and Boomsma 1999). In this study, errors due to non-detection of a second

170   father were very low (P< 0.001). Our sample size of 16 workers per colony also enabled

171   us to detect a second patriline with P > 0.95 if the second male contributed to about 20%

172   of the workers (Boomsma and Ratnieks, 1996). In colony S for which we genotyped 47

173   workers enabling us to detect a second patriline with P>0.9 even if the second male

174   fathered only 5% of the workers (Boomsma and Ratnieks, 1996), we also only detected

175   one male.

176

177   In one of the 20 colonies analysed all 16 workers had identical genotypes at the six loci

178   analysed. To determine if this pattern was due to workers being clonally produced or

179   from the mother being homozygous at the six loci and mated with a male having an

180   identical allele at five of the six loci we genotyped all workers at three additional loci,

181   analysed another 31 workers from the same colony at the nine loci and also genotyped

182   the queen. These analyses revealed that the queen was homozygous at eight of the nine

183   loci and that she mated with a male having an identical allele at eight of the nine loci. Given

184   the high polymorphism of the microsatellites, the most likely explanation for this pattern

185   is that the queen was inbreed and that she mated with a brother. In ants, there is usually

186   a low level of inbreeding due to the high synchronization of mating flight among colonies

187   of the same populations (Tabot 1945; McCluskey1965; Boomsma and Leusink 1981).

188   Queens and males from *C. fellah* colonies typically leave their nest in the evenings of the

189   first warm spring days where individuals of many nests take part to the mating flight

190   (Hefetz A, personal communication). However, instances of inbreeding due to sib-matings

191   have been reported in two *Camponotus* species (C. *ocreatus*, Goodisman and Hahn 2004

192   and C. *yamaokai*, Satoh *et al.* 1997) as well as several species of several other genera (e.g.,

193   *Cardiocondyla batesii*, Schrempf *et al.* 2005); *Cardiocondyla nigra*, Schrempf 2014);

194   *Cardiocondyla shuckardi*, Heinze *et al.* 2014) and *Formica exsecta*, Sundström *et al.* 2003;

195   Vitikainen *et al.* 2015). Interestingly, the inbred queen that we found had apparently

196   mated with a brother. A similar pattern has been uncovered in *Formica exsecta* where

197   more inbred queens are also more likely to have mated with a brother (Vitikainen *et al.*

198   2015). An association between the level of inbreeding of queens and their likelihood of

199   mating with siblings could occur if there is a genetic basis influencing dispersal

200   propensity. Thus, females that are genetically less inclined to disperse may be more

201   inbred and also more likely to mate with males of the same nest. Alternatively, the

202   propensity to disperse may be directly influenced by the level of inbreeding (Vitikainen

203   *et al.* 2015). Because more inbred queens may be smaller and less fit than outbred queens

204   (Coltman et al. 1999; Keller and Waller 2002; McQuillan et al. 2012), they might disperse

205 less (Wolf and Seppa 2016), thereby increasing their likelihood to mate with a sibling. I

206 WOULD SUGGEST TO FINISH HERE.

207

208 If the number of colonies participating in the same mating flight is in addition small like

209 it is in a few *Formica exsecta* and *Pogonomyrmex barbatus* populations (Suni and Gordon

210 2010, Vitikainen *et al.* 2015), or if inbred colonies produce more sexuals like *Hypoponera*

211 *opacior* colonies do (Kureck et al. 2012), then the likelihood to mate with a sibling are

212 further increased.

213

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387

388 ## Tables
389
390 **Table 1** Characteristics of the nine microsatellite loci from *Camponotus fellah* colonies.
391 All forward primers are preceded with an 18bp M13-tag: 5'-TGTAAAACGACGGCCAGT-3'.
392

Locus	Primer sequence (5'–3') (F: forward; R: reverse)	T_a (°C)	Repeat type	Size of cloned amplicon (bp)	No. of alleles	Amplicon size range
1284	**F:** GTCGGAATGTTGCACCTACG **R:** CAGACCGCGAGAGGAGATAC	53.8	(AG)	226	5	230-237
3186	**F:** AATTGAACGTTTCGTCCGCC **R:** AGTCTTTACGCCCTCCTACG	51.8	(AC)	190	8	194-206
3675	**F:** TTGTAGAGCGACGACGAGAG **R:** TGAGAGGTGAGAGCACGAAG	53.8	(CGT)	203	8	221-252
4380	**F:** TGGCTACAGTTGTTGTGCAG **R:** TTTGTCGCGGCTCGTAAATC	54.8	(AC)	115	10	126-148
5134	**F:** TACAATCGAGTGGACGGGAG **R:** CAGATGAAAGGCGAACGGTG	53.8	(CGA)	247	9	246-299
6826	**F:** GTACGCGGTCAGAATTACGC **R:** ACCTGATTTCTGGTTGGCAC	53.8	(AG)	176	14	178-207
7207	**F:** AGGCATTCGATACTGTCGGC **R:** TACGACAAGGATGCTACCGC	53.8	(TGC)	163	2	184-187
7275	**F:** CGCATCGGGAGCATAAACAG **R:** AAAGCTGGCATGCAGTTACG	53.8	(CA)	102	5	118-123
7300	**F:** AATTTGCGGAGAATCCGTGG **R:** CATCGGCAAAGTCGTCCTTC	51.8	(CT)	185	3	201-205

393
394
395

396 **Table 2:** Summary of all worker genotypes and inferred male and queen genotypes.
397 Symbols indicate the following: **§** Two samples were discarded because they had been contaminated. ****** Worker genotype
398 is incompatible with the queen genotype and thus the worker(s) likely come from another nest. **†(X)** Number of samples
399 that did not amplify. **^^** All workers are homozygote for this locus, but there are 2 alleles in the colony, thus we could not
400 infer the queen and male genotype. **°°** We genotyped 47 workers and the queen for all nine primers. **?** Genotype profile is
401 ambiguous, possibly homozygote.
402

Colony	Locus	Worker genotypes (# workers)	Inferred male	Inferred queen
A	3675	224::230 (7) 221::224 (9)	224	221::230
	1284	230::236 (8) 231::236 (8)	236	230::231
	4380	128::128 (6) 128::132 (10)	128	128::132
	5134	261::261 (9) 246::261 (7)	261	246::261
	6826	188::188 (7) 188::190 (9)	188	188::190
	3186	197::197 (11) 197::203 (5)	197	197::203
B §	3675	221::221 (7) 221::228 (5) 221::224 (2) **	221	221::228
	1284	232::236 (10) 236::236 (4)	236	232::236
	4380	132::146 (6) 132::132 (7) 128::132 (1)** 128::132 (1)**	132	132::146
	5134	246::261 (12) 246::246 (1) ** 246::272 (1)**	246 or 261	246::246 or 261::261
	6826	178::188 (8) 178::184 (4) 180::184 (2) **	178	184::188
	3186	201::203 (5) 199::201 (7) 203::205(2)**	201	199::203
C	3675	221::224 (16)	221 or 224	221::221 or 224::224
	1284	230::230 (8) 230::236 (8)	230	230::236
	4380	130::144 (8) 144::148 (8)	144	130::148
	5134	246::270 (9) 249::270 (5)	270	246::249
	6826 †(1)	186::190 (7) 186::196 (8)	186	190::196
	3186	197::203 (9) 203::203 (7)	203	197::203
D	3675 †(2)	240::240 (5) 224::240 (9)	240	224::240

	1284 †(1)	232::237 (10) 237::237 (5)	237	232::237
	4380 †(1)	130::146 (9) 130::142 (6)	130	142::146
	5134 †(1)	249::299 (5) 249::249 (10)	249	249::299
	6826	184::194 (12) 194::203 (4)	194	184::203
	3186 †(1)	196::203 (7) 196::196 (8)	196	196::203
E	3675	228::252 (11) 224::228 (5)	228	224::252
	1284	230::232 (12) 232::236 (4)	232	230::236
	4380	130::130 (4) 130::146 (12)	130	130::146
	5134	246::246 (10) 246::249 (6)	246	246::249
	6826	188::196 (7) 196::196 (9)	196	188::196
	3186	197::205 (5) 197::197 (11)	197	194::205
F	3675	221::249 (9) 224::249 (7)	249	221::224
	1284	231::231 (4) 231::236 (12)	231	231::236
	4380	130::130 (10) 126::130 (6)	130	126::130
	5134	246::249 (10) 246::261 (6)	246	249::261
	6826 †(1)	194::198 (7) 184::198 (8)	198	184::194
	3186	203::205 (7) 203::203 (9)	203	203::205
G	3675	221::228 (14) 221::224 (2) **	221 or 228	221::221 or 228::228
	1284	231::236 (8) 232::236 (6) 230::230 (2) **	236	231::232
	4380	128::128 (6) 128::130 (9) 130::130 (1) **	128	128::130
	5134	246::272 (10) 246::249 (6)	246	249::272
	6826	186::198 (8) 186::207 (6) 188::188 (1) ** 188::199 (1) **	186	198::207
	3186	201::203 (10) 203::203 (4) 197::201 (1) ** 201::205 (1) **	203	201::203
H	3675 †(1)	221::221 (15)	221	221::221
	1284	231::236 (10) 231::231 (6)	231	231::236
	4380	128::130 (6) 130::132 (10)	130	128::132
	5134	246::261 (8) 246::246 (8)	246	246::261
	6826	186::186 (16)	186	186::186
	3186 †(1)	203::203 (11) 203::205 (4)	203	203::205
I	3675 ^^	240::240 (8) 224::224 (8)	^^	^^
	1284 †(2)	230::236 (12) 232::236 (2)	236	230::232
	4380	128::135 (16)	128 or 135	128::128 or 135::135
	5134 †(1)	246::246 (6) 246::267 (9)	246	246::267
	6826	192::192 (7) 192::199 (9)	192	192::199
	3186 †(1)	201::203 (9) 203::206 (6)	203	201::206
J	3675	221::224 (16)	221 or 224	221::221 or 224::224
	1284	230::231 (10) 231::236 (6)	231	230::236
	4380	130::133 (9) 133::148 (7)	133	130::148
	5134	246::246 (16)	246	246::246
	6826	190::198 (7)	198	186::190

		186::198 (9)		
	3186	197::203 (16)	197 or 203	197::197 or 203::203
K	3675 †(2)	221::221 (5) 221::236 (9)	221	221::236
	1284	231::236 (16)	231 or 236	231::231 236::236
	4380 †(2)	144::144 (9) 130::144 (5)	144	130::144
	5134	246::261 (16)	246 or 261	246::246 or 261::261
	6826	184::201 (8) 184::192 (8)	184	192::201
	3186 †(2)	203::205 (14)	203 or 205	203::203 or 205::205
L	3675 †(1)	221::224 (10) 221::221 (5)	221	221::224
	1284	236::236 (9) 232::236 (6) 230::236 (1)**	236	232::236
	4380 †(5)	128::132 (4) 128::128 (6) 132::133 (1) **	128	128::132
	5134	246::261 (16)	246 or 261	246::246 or 261::261
	6826	184::184 (8) 184:188 (7) 186::194 (1) **	184	184::188
	3186 †(1)	203::203 (6) 199::203 (8) 197::199 (1) **	203	199::203
M	3675	221::221 (7) 221::224 (8) 221::--- (1)	221	221::224
	1284 †(1)	231::236 (8) 231::231 (7)	231	231::236
	4380	130::130 (7) 130::146 (9)	130	130::146
	5134	246::275 (10) 246::246 (6)	246	246::275
	6826	194::196 (12) 196::207 (4)	196	194::207
	3186	197::201 (16)	197 or 201	197::197 or 201::201
N	3675 †(1)	221::221 (9) 221::224 (6)	221	221::224
	1284	236::237 (6) 231::236 (9) 231::--- (1)	236	231::237
	4380	128::132 (10) 128:146 (6)	128	132::146
	5134 †(1)	246::246 (15)	246	246::246
	6826	186::194 (16)	186 or 194	186::186 or 194::194
	3186 †(1)	197::203 (5) 199::203 (10)	203	197::199
O	3675 †(1)	221::221 (15)	221	221::221
	1284 †(1)	232::232 (9) 231::232 (6) ?	232	231::232
	4380 †(1)	130::142 (6) 128::130 (9)	130	128::142
	5134 †(1)	246::270 (6) 246:246 (9)	246	246::270
	6826 †(1)	178::194 (9) 178::188 (6)	178	188::194
	3186 †(1)	201::203 (3) 203::203 (12)	203	201::203
P	3675	221::221 (16)	221	221::221
	1284	231::236 (7) 231::232 (9) ?	231	232::236
	4380	128::128 (16)	128	128::128
	5134	261::275 (16)	261 or 275	261::261 or 275::275
	6826	180::196 (10) 196::207 (6)	196	180::207
	3186	197::203 (8) 203::203 (8)	203	197::203
Q	3675	221::221 (16)	221	221::221
	1284	232::232 (6) 231::232 (10) ?	232	231::232
	4380	128::130 (4) 130::130 (12)	130	128::130

	5134	249::249 (8) 249::272 (8)	249	249::272
	6826	184::203 (16)	184 or 203	184::184 or 203::203
	3186	199::201 (8) 199::199 (8)	199	199::201
R	3675	224::224 (15) 221::224 (1) **	224	224::224
	1284	230::236 (9) 230::231 (7)	230	231::236
	4380 †(1)	128::128 (7) 128::146 (7) 126::128 (1) **	128	128::146
	5134	246::246 (8) 246::263 (7) 246::275 (1) **	246	246::263
	6826	188::196 (9) 184::196 (6) 188::207 (1) **	196	184::188
	3186	197::203 (15) 197::197 (1) **	197 or 203	197::197 or 203::203
S °°	3675 †(3)	221::221 (44)	221	221::221 °°
	1284	236::236 (47)	236	236::236 °°
	4380	130::130 (47)	130	130::130 °°
	5134	246::246 (47)	246	246::246 °°
	6826	184::188 (47)	184	188::188 °°
	3186	203::203 (47)	203	203::203 °°
	7275	118::120 (23) 120::120 (24)	120	118::120 °°
	7207	184::184 (47)	184	184::184 °°
	7300	203::203 (47)	203	203::203 °°
T	3675 †(1)	221::224 (16)	221 or 224	221::221 or 224::224
	1284	236::236 (10) 230::236 (6)	236	230::236
	4380	128::132 (14) 128::128 (2)	128	128::132
	5134	246::263 (16)	246 or 263	246::246 or 263::263
	6826	180::207 (7) 180::203 (9)	180	203::207
	3186	203::203 (11) 197::203 (5)	203	197::203

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