

Research



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Evolutionary biology

Wolbachia-induced meiotic drive and feminization is associated with an independent occurrence of selective mitochondrial sweep in a butterfly

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Maternally inherited *Wolbachia* endosymbionts manipulate arthropod reproduction in various ways. In the butterfly *Eurema mandarina*, a cytoplasmic incompatibility-inducing *Wolbachia* strain *w*CI and the associated mtDNA haplotypes are known to originate from the sister species *Eurema hecabe*, which offered a good case study for microbe-mediated hybrid introgression. Besides *w*CI, some females with the Z0 karyotype harbour a distinct *Wolbachia* strain *w*Fem, which causes all-female production by meiotic drive and feminization. We report that a considerable proportion of *E. mandarina* females (65.7%) were infected with both *w*CI and *w*Fem (CF) on Tanegashima Island. While females singly infected with *w*CI (C) produced offspring at a 1 : 1 sex ratio, CF females produced only females. Although Z-linked sequence polymorphism showed no signs of divergence between C and CF females, mtDNA split into two discrete clades; one consisted of C females and the other CF females, both of which formed a clade with *E. hecabe* but not with uninfected *E. mandarina*. This suggests that CF matriline also, but independently, experienced a selective sweep after hybrid introgression from *E. hecabe*. Distinct evolutionary forces were suggested to have caused C and CF matriline to diverge, which would be irreversible because of the particular phenotype of *w*Fem.

1. Introduction

Wolbachia, a maternally inherited, ubiquitous endosymbiont of arthropod and nematodes, is well known for its ability to manipulate host reproduction in order to facilitate its transmission in various ways, which can be categorized into two groups: cytoplasmic incompatibility (CI) and female-biased sex-ratio distortion [1]. CI is a lethal phenotype in embryos produced by mating between infected males and uninfected females, thus providing a reproductive advantage for infected females compared with uninfected ones. Besides CI, *Wolbachia* can bias its host sex ratio towards females by inducing thelytokous parthenogenesis, male-killing or feminization [1]. It was recently demonstrated that *Wolbachia* can also cause meiotic drive (MD), leading to all-female offspring [2].

Because of its reproductive manipulations, *Wolbachia* infection spreads and persists in host populations, together with maternally inherited mitochondria. This hitchhiking effect of mitochondria through *Wolbachia* selective sweep was first identified in *Drosophila simulans* [3], followed by similar findings in other insects [4]. Importantly, *Wolbachia* and its associated mitochondria can cross species boundaries; cytoplasmic elements that move into sibling species

via hybridization may persist and spread in the new host due to the effect of *Wolbachia*-induced reproductive manipulations (such as CI), which would otherwise disappear easily due to the genetic drift [4]. Importantly, the hybrid introgression followed by *Wolbachia* selective sweep can confound molecular phylogenetic analyses based on mitochondrial DNA (mtDNA) sequences [4].

The common grass yellow, *Eurema mandarina* (Lepidoptera: Pieridae), offered a good model case wherein hybrid introgression and selective sweep caused by CI-inducing *Wolbachia* (strain *wCI*) resulted in the fixation of mtDNA haplotypes derived from *Eurema hecabe* (i.e. a sister species of *E. mandarina*) in most populations, except those on the northernmost part of the main island of Japan, where uninfected individuals remain [5].

Independently, some individuals in particular populations of *E. mandarina* and *E. hecabe* were found to harbour a sex-ratio-distorting *Wolbachia*, the strain *wFem*, in addition to *wCI* [6,7]. In both species, females singly infected with *wCI* (C females) produce offspring at a 1 : 1 sex ratio, whereas females doubly infected with *wCI* and *wFem* (CF females) produce all-female offspring. On subtropical Okinawa-jima Island, two out of ten *E. mandarina* females were considered CF females and produced only females [6,8]. Exclusive production of females by CF females was also reported from temperate Tanegashima Island [9,10], but systematic analyses on their *Wolbachia* infection status and offspring sex ratio have yet to be performed.

Recent studies revealed that *E. mandarina* C females have the WZ sex chromosome system, whereas CF females have the Z0 sex chromosome system [2,11]. *Wolbachia* was found to affect female meiosis and prevent the production of Z-bearing oocytes, thereby producing exclusively Z0 embryos that carry a paternal Z. Moreover, *Wolbachia* is responsible for female sex determination in Z0 individuals, which would otherwise be determined as males owing to the absence of the female-determining W chromosome [2]. Therefore, the *Wolbachia*-induced coordinated dual effects (i.e. MD and feminization) are considered responsible for the all-female production in *E. mandarina*.

We speculated that the distinct phenotypes exerted in C and CF matrilines are subjected to different selective forces. We aimed to establish (i) how common *Wolbachia*-induced MD is and (ii) whether the two distinct *Wolbachia* infection statuses (i.e. C and CF) affect mtDNA diversity in *E. mandarina*.

2. Material and methods

(a) Insects

In May 2015, adults of *E. mandarina* (33 females and 29 males) were collected on Tanegashima Island, Kagoshima Prefecture, Japan (figure 1). Offspring obtained from each female were reared in the laboratory (electronic supplementary material, S1).

(b) Diagnostic PCR and sequencing of *Wolbachia wsp* gene

Infection with either *wCI* or *wFem* strain of *Wolbachia* was examined by specific PCR. The PCR products were then sequenced to confirm the identity of *Wolbachia* strains (electronic supplementary material, S1).

(c) Phylogenetic analysis of host nuclear and mtDNA genes

An intron region of the Z-linked *triose phosphate isomerase* (*Tpi*), mitochondrial *cytochrome c oxidase subunit I* (*COI*) and mitochondrial *cytochrome c oxidase subunit III* (*COIII*) and adjacent *tRNA* (referred to as *COIII* hereafter) were amplified by PCR and directly sequenced. For phylogenetic analyses, we also included previously published sequences of *COI* and *COIII* of *E. hecabe* collected from Southeast Asia [12] (electronic supplementary material, S1 and S2).

3. Results

(a) CF females are widespread in *Eurema mandarina* on Tanegashima Island

PCR and sequencing showed that all of the 33 wild-caught *E. mandarina* females were infected with *wCI* [6,7]. Among them, 23 females were also infected with *wFem* [6,7] (table 1). Offspring obtained from eight CF females were all females (281 females in total), while five C females produced both males and females (156 and 149 in total; figure 1).

(b) Divergence of mtDNA between C and CF females in *E. mandarina*

Among 30 wild-caught females of *E. mandarina* (22 CF and eight C females), Z-linked *Tpi* (430 bp) was polymorphic in 31 nucleotide sites, constituting 24 haplotypes. Because the Z is driven out of Z0 females [2], *Tpi* is always a marker of paternal origin in CF females. The maximum-likelihood (ML) tree based on *Tpi* did not show any signs of divergence between CF and C lineages (figure 2a), indicating that CF and C lineages share a common gene pool through males.

By contrast, ML trees based on mtDNA exhibited a clear distinction between CF and C (figure 2b). Among 25 *E. mandarina* females (19 CF and six C females), concatenated sequence of *COI* and *COIII* (2032 bp) was polymorphic in 16 nucleotide sites, constituting four haplotypes; one haplotype (corresponding to haplotype 1 in electronic supplementary material, S2) was possessed only by C females, while three haplotypes (corresponding to haplotypes 2, 3 and 4 in electronic supplementary material, S2) were possessed only by CF females. It is apparent that *COI* and *COIII* did not undergo recombination (electronic supplementary material, S3).

(c) Phylogenetic analysis of *Eurema mandarina* and *Eurema hecabe* based on mtDNA

Mitochondrial haplotypes of *E. mandarina* collected on Tanegashima Island were compared with the previously published mitochondrial haplotypes of *E. mandarina* and *E. hecabe*, which were collected in other populations in Japan and Southeast Asia [12] (figure 2c,d).

On the haplotype spanning network based on the concatenated sequences of *COI* and *COIII* (1777 bp in total), haplotypes of *E. hecabe*, irrespective of the infection status, were indistinguishable from the haplotypes of *E. mandarina* C females, except for an enigmatic Hong Kong-derived sample (CN2-a; haplotype 31 in figure 2c) [12], but were different from CF females of *E. mandarina* (figure 2c,d). Unique haplotypes of *E. mandarina* CF females compared

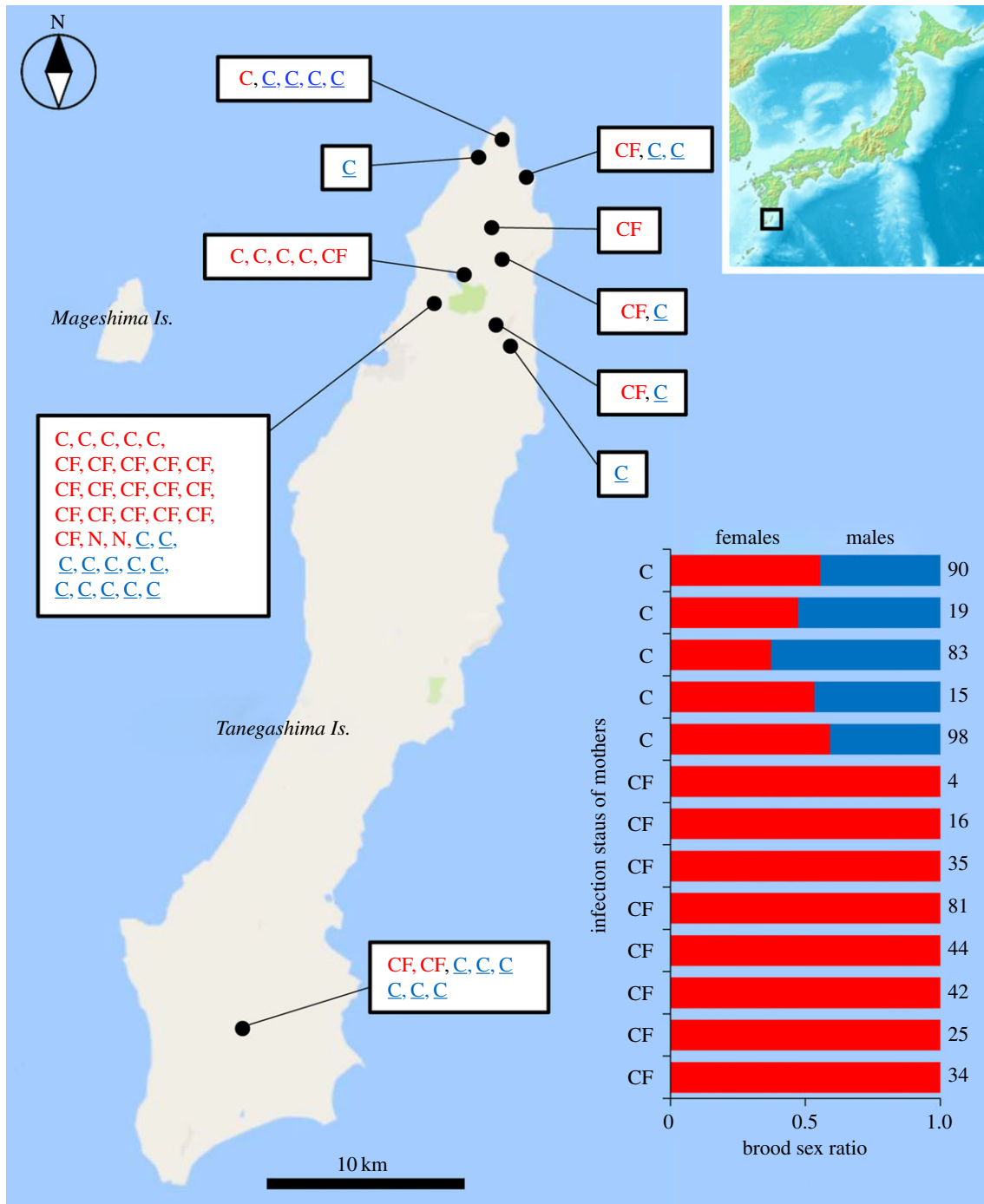


Figure 1. Ten sampling sites of *E. mandarina* on Tanegashima Island. Red, females; blue and underlined, males; CF, doubly infected with wCl and wFem; C, singly infected with wCl. The bar graph represents the brood sex ratio produced from 13 females (five C and eight CF females) collected in this study. Brood size is given on the right. Original map images were from Google Maps (Google Inc.).

with others may suggest a different evolutionary history of *E. mandarina* CF matriline.

4. Discussion

This study suggested that *Wolbachia*-induced MD is widespread among *E. mandarina* on Tanegashima Island. Considering the fact that the sex ratios of collected butterflies are usually male-biased (probably due to the behavioural differences between males and females) [13], the nearly 1:1 sex ratio (33 females and 29 males) of the collected butterflies probably reflects a female-biased primary sex ratio. Assuming that the ratio of CF and C females in this population is 23/10 and that equal numbers of offspring are produced by these females, the sex

Table 1. Number of *E. mandarina* adults collected in Tanegashima Island in 2015. C, singly infected with wCl; CF, doubly infected with wCl and wFem; n.a., unknown (samples lost).

sexual phenotype	C	CF	n.a.	total
females	10	23	2	35
males	29	0	0	29

ratio of the subsequent generation would be 5.6 females per male. Because the coexistence of all-female-producing CF females and normal C females is unstable under simple model formulations, some unknown factors might be involved

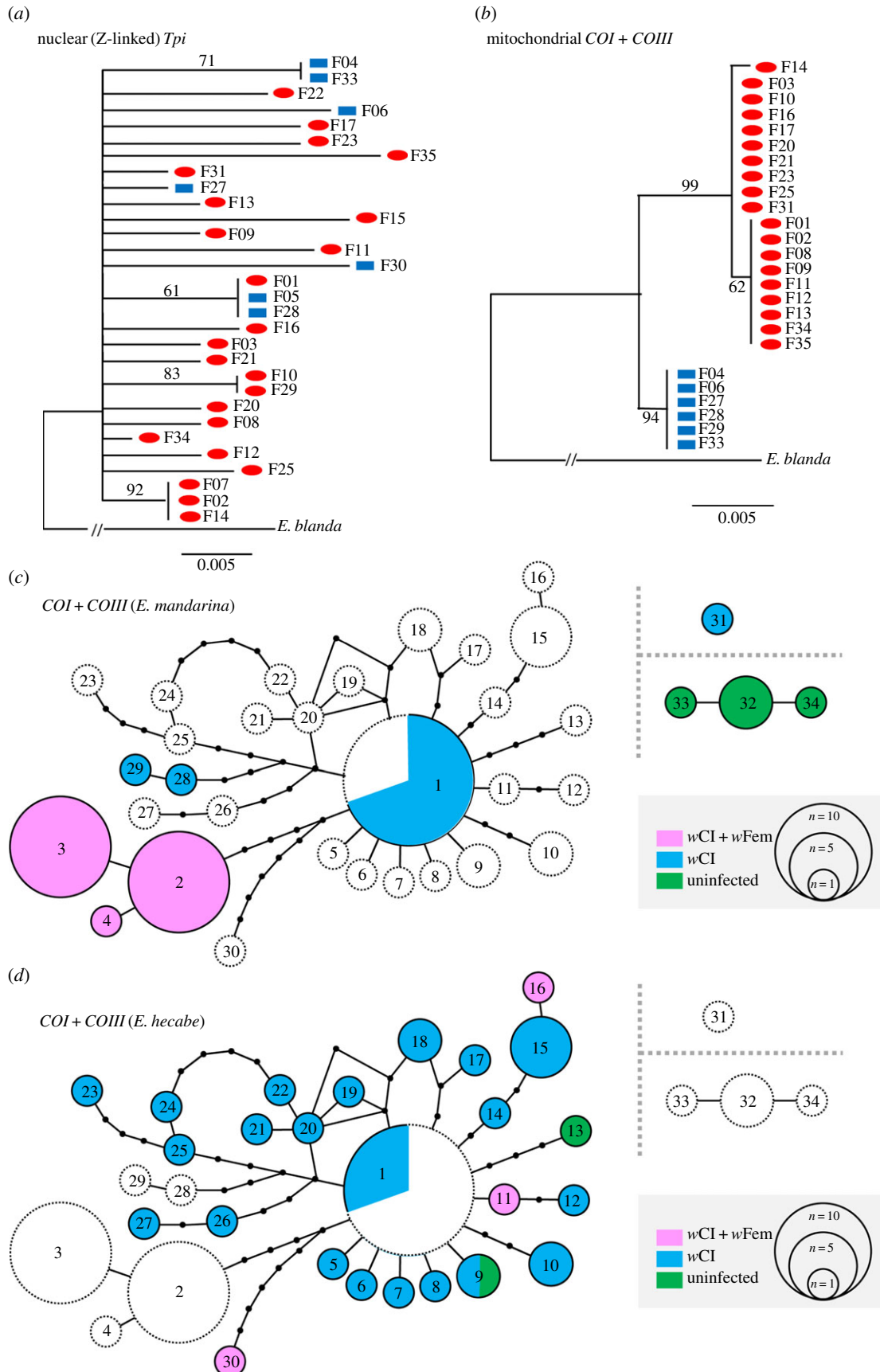


Figure 2. Molecular phylogenetic analyses of *Eurema* butterflies. ML trees of *E. mandarina* collected on Tanegashima Island based on nuclear *Tpi* (a) and concatenated sequences of mitochondrial *COI* and *COIII* (b). Red ovals, CF females; blue rectangles, C females. A haplotype spanning network of *E. mandarina* and *E. hecabe* based on concatenated sequences of *COI* and *COIII* (c,d). Haplotypes of *E. mandarina* (c) and *E. hecabe* (d) are highlighted. Each circle represents a single haplotype with an area proportional to the sample size. Dots: missing haplotypes. Circles and dots are connected by single-nucleotide substitutions. Pink, CF females; blue, C females; green, uninfected females. Dotted circles and dotted parts of circles represent haplotypes that are absent in this particular species but are present in the sister species.

in selection for or against CF females in a frequency-dependent manner. Alternatively, this polymorphism may be transient, leading to the extinction or fixation of CF matriline (the latter

would lead to a population collapse). Either way, our study highlights that *Wolbachia*-induced MD has a significant impact on the ecology and evolution of *E. mandarina*.

This study also reveals distinct nucleotide divergence in mitochondrial lineages in a population of *E. mandarina* according to *Wolbachia* infection status (i.e. C versus CF). This can be explained by distinct selective forces acting on the two types of matrilines, one showing a 1:1 sex ratio and the other all females. The reason for the clear distinction of mtDNA despite the potentially incomplete vertical transmission of *wFem* [10] can be considered as follows. Failure in *Wolbachia* vertical transmission in a CF matriline would result in all-male production [2,6,8], and hence the extinction of the particular mtDNA haplotype of the CF matriline. In typical cases of all-female production caused by male-killing *Wolbachia*, however, removal of *Wolbachia* would result in the production of females that produce 1:1 sex-ratio offspring. Therefore, divergence in mtDNA may be purged, to some extent, by the matrilines that lost *Wolbachia*. Contrastingly, C and CF females of *E. mandarina* would lead to an irreversible divergence of mtDNA. Although empirical evidence is lacking, this would also be the case in *Ostrinia* moths, wherein *Wolbachia* causes male-killing, but the elimination of *Wolbachia* results in the production of all-male offspring [14,15].

By comparison with the previously published sequences of *E. hecabe* and *E. mandarina*, we found that the mtDNA haplotypes of CF females formed a unique clade (figure 2d). Although these haplotypes apparently originated from *E. hecabe*, the distinctiveness from those of *E. mandarina* C females suggests that the C and CF females may have independently experienced hybrid introgression from *E. hecabe*, possibly representing consecutive mitochondrial sweep, firstly by *wCI* and then by *wFem*. In the cherry fruit fly,

Rhagoletis cerasi, consecutive mtDNA sweeps were suggested due to multiple invasions of host populations by CI-inducing *Wolbachia* strains [16]. The fact that the mtDNA haplotypes possessed by *E. mandarina* CF females were not found in *E. hecabe* may imply that the introgression of CF mtDNA occurred a long time ago, so that considerable nucleotide substitution in mtDNA may have made these haplotypes extremely rare or extinct in *E. hecabe*. However, alternative possibilities still remain such as mitochondrial introgression together with *wFem* from another yet unknown *Eurema* species or horizontal transmission of *Wolbachia* multiple times in *E. hecabe* but only once in *E. mandarina*. Unlike the male-killing cases, the appearance of a suppressor against MD in the host would result in all males [2,8], leading to the elimination of *Wolbachia* as well as the accompanying mtDNA from the population.

Ethics. Collection of *E. mandarina*, a non-endangered butterfly, made in prefectural roadsides on Tanegashima Island, does not violate any laws.

Data accessibility. Sequences of *Tpi*, *COI* and *COIII* were submitted to DDBJ/EMBL/GenBank (electronic supplementary material, S2). Information for butterfly samples was deposited in Dryad (<http://dx.doi.org/10.5061/dryad.7qf5f>) [17].

Authors' contributions. M.M., M.N. and D.K. designed the study. M.M., T.K., K.Y. and D.K. collected and analysed the data. M.M., M.N., K.Y. and D.K. wrote the manuscript with input from T.K. All authors approved the final manuscript and are accountable for its content.

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