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Ecophysiologival characterization and molecular differentiation of *Culex pipiens* forms (Diptera: Culicidae) in Tunisia

Marwa Beji¹, Adel Rhim¹, David Roiz² and Ali Bouattour¹*

**Abstract**

**Background:** The *Culex pipiens* complex (Diptera: Culicidae) includes the most widespread mosquito species in the world. Members of this complex are the primary enzootic and epidemic vectors of the West Nile virus (genus *Flavivirus*) in several countries. The two recognized forms of *C. pipiens* (Linnaeus, 1758) - *pipiens* and *molestus* - exhibit behavioral and physiological differences. Natural populations of *C. pipiens* were investigated in several sites in Tunisia to evaluate the ecophysiologival and molecular characteristics of their forms.

**Results:** The analysis showed the sympatric presence of *C. pipiens* forms and hybrids in all studied sites. Of all the tested larvae of *C. pipiens*, 33.5% were identified as *pipiens*, 30.8% were identified as *molestus*, and 35.6% were identified as hybrids. The *molestus* and hybrid forms were positively correlated with urban habitats and belowground sites while the *pipiens* form was positively correlated with rural habitats and aboveground sites. Autogeny was expressed in all types of habitats and breeding sites. By contrast with the microsatellite CQ11, the two molecular markers, *ace-2* and *cytb*, did not allow differentiation between the *C. pipiens* forms.

**Conclusions:** Our study shows the ubiquitous distribution and the plasticity of the different forms of *C. pipiens* in a wide range of ecological conditions. It suggests that the behavioral traits assigned to the forms of *C. pipiens* seem to be more flexible than previously assumed. Our analysis also proves that the microsatellite CQ11 remains an efficient tool for distinguishing between *C. pipiens* forms.

**Keywords:** *Culex pipiens*, Form *molestus*, Form *pipiens*, Hybrid, Tunisia, Ecology, Autogeny, Microsatellite CQ11, Genetic diversity

**Background**

The epidemic and zoonotic potential of mosquito-borne diseases make mosquitoes an important threat to public health [1]. Mosquitoes of the *Culex pipiens* complex, the most widespread species, are among the principal vectors of diseases including the Rift Valley fever virus (RVFV) and West Nile virus (WNV) [2].

In Tunisia, favorable environmental conditions created by rapid urbanization and changing agriculture practices [3, 4] are contributing to the widespread proliferation of *Culex pipiens* mosquitoes and their abundant presence in urban and rural areas. This in turn is leading to the spread of WNV [5, 6], as several recent studies have shown, which has become the most important arboviral disease in Tunisia. WNV is a flavivirus maintained in an enzootic cycle (bird-mosquito-bird transmission), that can lead to encephalitis/meningitis in humans and horses [7]. In Tunisia, three large outbreaks of WNV meningoencephalitis (1997, 2003 and 2012) have led to several deaths [8–11].

The *C. pipiens* complex includes six members: *Cx. quinquefasciatus* Say, *Cx. pipiens pallens* Coquillet, *Cx. australicus* Dobrotworsky & Drummond, *Cx. globosciutus* Dobrotworsky and the nominal species, *Cx. pipiens* Linnaeus, comprising two forms: *Culex pipiens f. pipiens* and *Culex pipiens f. molestus* [2, 12]. The difficulty in distinguishing among these forms has made the taxonomy and phylogeny of the *C. pipiens* complex controversial [13]. Molecular assays have been developed to

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differentiate the species and forms and to detect hybridization events [14]. Several studies using molecular tools have led to the description of the two forms of *Cx. p. molestus* in several parts of the world, particularly in North Africa, and have provided evidence of various ecological features. The *pipiens* form is eurygamous (mates in open spaces), anautogenous (requires a blood meal for egg development) and heterodynamic (goes into diapause during the winter). By contrast, the *molestus* form is stenogamous (mates in confined spaces), autogenous (can lay its first batch of eggs without a blood meal) and homodynamic (does not enter diapause) [5, 13, 15–18].

The transmission of WNV is greatly influenced by the ecology, competence, and feeding behavior of the mosquito vectors: *C. p. molestus* is ornithophilic, feeding mainly on birds, while *C. p. pipiens* is anthropophilic, feeding mainly on mammals, especially humans [19]. Hybrids of the *pipiens* and *molestus* forms have an intermediate host preference that makes them "bridge vectors" for WNV transmission from birds to mammals [18, 19]. The recently reported detection of hybrids of the two forms in several countries presents a complex scenario regarding the hypothesis of a clear behavioral separation among the forms of *C. pipiens* [20–23].

Taxonomic studies of mosquito vectors, their ecology and their physiology are therefore needed to understand the epidemiology of the diseases that they transmit and to establish surveillance and control programs. Indeed, the unresolved debate about the status of the physiological, ecological and genetic characteristics of the *C. pipiens* complex makes their ecology, biology and taxonomic status important subjects of study and discussion.

This study used molecular methods to investigate the occurrence and distribution of both forms of *C. p. pipiens* and their hybrids to characterize different populations, to determine their expression and rate of autogeny in different environments in Tunisia. These traits are known to have obvious implications for the vectorial capacity of this mosquito.

**Methods**

**Mosquito collection and identification**

From 2013 to 2015, mosquito larvae were collected by dipper sampling from 22 sites covering seven bioclimatic zones of Tunisia in both urban and rural habitats and in above- and belowground breeding sites (Table 1). Live larvae were brought to the insectary of the Pasteur Institute of Tunis for identification according to the identification key of Mediterranean Africa mosquitoes [24].

| ID | Bioclimatic zone       | Locality       | Collection date | Latitude | Longitude | Habitat | Breeding site       | No. of specimens analyzed |
|----|------------------------|----------------|-----------------|----------|-----------|---------|---------------------|--------------------------|
| 1  | Humid                  | Cap serrat     | August 2015     | 37°20′14″ | 10°46′14″ | rural   | aboveground         | 20                       |
| 2  | Sub-humid              | Skhira         | October 2015    | 37°03′10″ | 10°20′31″ | urban   | aboveground         | 10                       |
| 3  | Sub-humid              | Utique         | December 2014   | 37°04′17″ | 10°00′42″ | urban   | aboveground         | 17                       |
| 4  | Higher semi-arid       | Manar          | April 2015      | 37°01′77″ | 09°52′20″ | rural   | aboveground         | 20                       |
| 5  | Zaarour                | October 2015   | 37°06′86.8″     | 09°44′37.8″ | 10°11′31.5″ | rural   | aboveground         | 20                       |
| 6  | Beja oued              | December 2013  | 36°43′08.1″     | 09°11′31.5″ | 10°11′31.5″ | urban   | aboveground         | 20                       |
| 7  | Cité nozha             | June 2015      | 36°52′00.8″     | 10°11′78.0″ | 10°11′31.5″ | rural   | aboveground         | 20                       |
| 8  | Chotrina               | July 2015      | 36°54′11.1″     | 10°13′10″ | 10°11′31.5″ | urban   | aboveground         | 20                       |
| 9  | Cave 1                 | October 2015   | 36°48′10.7″     | 10°10′45.2″ | 10°11′31.5″ | urban   | aboveground         | 20                       |
| 10 | Cave 2                 | October 2015   | 36°48′08.6″     | 10°10′44.4″ | 10°11′31.5″ | urban   | aboveground         | 20                       |
| 11 | Cité olympique         | September 2015 | 36°50′36.4″     | 10°11′69.0″ | 10°11′31.5″ | urban   | aboveground         | 20                       |
| 12 | Korba                  | June 2014      | 36°34′43.0″     | 10°51′53.1″ | 10°11′31.5″ | urban   | aboveground         | 20                       |
| 13 | Tastour                | May 2015       | 36°32′41.8″     | 09°24′16.7″ | 10°11′31.5″ | rural   | aboveground         | 20                       |
| 14 | Middle semi-arid       | Cité el Arayes | July 2013       | 36°24′26.0″ | 10°08′13.1″ | urban   | aboveground         | 20                       |
| 15 | Higher arid            | Kairouan       | July 2014       | 35°39′85.1″ | 10°06′41.9″ | urban   | aboveground         | 20                       |
| 16 | Cité bassatin          | December 2014  | 35°10′20.0″     | 08°49′42.2″ | 10°06′41.9″ | urban   | aboveground         | 20                       |
| 17 | Sidi bouzid            | May 2015       | 34°39′10.0″     | 09°35′18.3″ | 10°06′41.9″ | urban   | aboveground         | 20                       |
| 18 | Lower arid             | Gafsa          | December 2014   | 34°26′43.0″ | 08°38′15.4″ | rural   | aboveground         | 20                       |
| 19 | Route d’el Ain         | August 2014    | 34°44′52.5″     | 10°45′16.4″ | 10°06′41.9″ | urban   | aboveground         | 20                       |
| 20 | Teboulbou              | June 2013      | 33°50′28.2″     | 10°07′52.5″ | 10°06′41.9″ | urban   | aboveground         | 20                       |
| 21 | Saharan                | April 2015     | 33°57′21.1″     | 08°11′04.1″ | 10°06′41.9″ | rural   | aboveground         | 20                       |
| 22 | Douz                   | December 2013  | 33°25′90.8″     | 09°00′55.2″ | 10°06′41.9″ | urban   | aboveground         | 8                        |
A pool of *Cx. pipiens* larvae was taken from each site (*n* = 22) and stored in 70% alcohol in preparation for the molecular characterization and genetic analysis of *Cx. pipiens* forms. Other larvae pools taken from seven breeding sites representing different combinations of habitat (rural/urban) and breeding site (above/belowground) were reared to adults under laboratory conditions, in order to evaluate their autogenic behavior.

**Molecular identification of *Cx. pipiens* mosquitoes**

DNA from individual *Culex pipiens* larvae and adults from each breeding site (Table 1) were extracted using the Cetyltrimethylammonium bromide (CTAB) protocol [25]. Isolated DNA from each sample was stored at -20 °C.

The CQ11 polymorphic microsatellite marker of *Culex pipiens* complex was used to distinguish between forms *pipiens* and form *molestus*. The amplification of the CQ11 microsatellite was carried out using sets of primers CQ11F2, molCQ11R and pipCQ11R. The PCR reactions were performed in 20 μl of reaction mix using the cycling conditions listed in Bahnck & Fonseca [26]. Amplified fragments were visualized on a 2% agarose gel. The *pipiens* and *molestus* forms presented a PCR product of 200 bp and 250 bp, respectively. Hybrids exhibited both amplicons (200 bp/250 bp) [26].

A second PCR was subsequently used to detect polymorphism in the nucleotide sequence of the *ace-2* gene of the different forms of *Cx. pipiens* and to test its usefulness as a nuclear marker for form identification. Sequences of sections of exons 2 and 3 and the entire intron 2 in the *ace-2* gene (the ACE locus) were obtained using the oligonucleotide primers, specific for *Cx. pipiens* (s.s.), F1457 and B1246 as described by Bourquet et al. [27]. PCR products were run on a 1.5% agarose gel and showed a band of 714 bp specific of *Cx. pipiens*.

In addition, samples were analyzed by PCR targeting the *cytb* gene that was used in species identification [28–30] to detect any polymorphism in the nucleotide sequence of *Cx. pipiens* forms. Amplification of the *cytb* gene was carried out using the primers *cytb*-F and *cytb*-R [30]. Polymerase chain reaction products were run on a 1% agarose gel and displayed a band of 853 bp specific of *Cx. pipiens*.

**Sequencing**

Some PCR products obtained by targeting the CQ11, *ace-2* and *cytb* were randomly chosen and sequenced to confirm the PCR results and to determine whether nucleotide polymorphisms were informative to distinguish between *Cx. pipiens* forms. PCR products were purified using the ExoSAP cleanup procedure (Amersham Biosciences, Piscataway, NJ, USA). Cycle sequencing was performed using BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed using a capillary automated sequencer 3500 Genetic Analyzer (Ruo. Hitachi, Foster City, CA, USA). Sequences were aligned using BioEdit 7.1.9 [31] and identified by comparison with sequences deposited in the GenBank database.

**Determination of autogeny**

To evaluate the expression of autogeny according to the type of habitat and breeding site, *Cx. pipiens* larvae from ID3, 4, 6, 9, 11 and 13 sites (Table 1) were raised in the insectary under controlled conditions (25 ± 2 °C; 70 ± 10% relative humidity, and a 12:12 h light:dark photocyte). Larvae were fed fish flakes and brewer's yeast. Emerging males and females of *Cx. pipiens* housed in cages (20 × 20 × 20 cm) were given access to a cotton pad soaked in a 10% sugar solution and an oviposition small tray containing deionized water that was inspected daily for 30 days for the presence of egg-rafts. We subsequently calculated the number of fertile egg-rafts (which produce larvae) to estimate the percentage of autogenous females.

In a second test evaluating the expression of autogeny by form of *Cx. pipiens*, two types of breeding sites (aboveground ID8 and belowground ID11) were chosen. Pupae were separated individually in glass tubes of distilled water until adults emerged. The adults were isolated by couples (one male and one female) in cups covered with a mesh screen with access to a honey solution and an oviposition tray. The presence of egg-rafs was recorded daily for 30 days. During this time, females that laid eggs without blood-feeding were considered to be autogenous. This test was replicated by visiting the two sites three times (once a month). We started our experiment with 60 couples from ID8 and 57 from ID11 but we used molecular analysis only for the survived females to determine the form.

**Data analysis**

The relationship between the form of *Cx. pipiens* and bioclimatic area, breeding site, habitat and autogenic behavior was analyzed using a Generalized Linear Model (GLM) with Poisson distribution (as the data were over-dispersed). Statistical analyses and figures were carried out in R 3.2.2.

**Nucleotide sequence accession numbers**

Sequence data were deposited in the GenBank database under the accession numbers KY744191–KY744222.

**Results**

During our study, 1517 mosquito larvae were collected from 22 sites in Tunisia’s seven bioclimatic zones (Table 1) and identified as *Cx. pipiens* (*n* = 989), *Cx. theileri* (*n* = 404), *Cx. perexiguus* (*n* = 11), *Cx. impudicus* (*n* = 9), and
Ochlerotatus caspius \((n = 16)\), O. detritus \((n = 10)\), Anopheles labranchiae \((n = 28)\), Culexita longiareolata \((n = 48)\), Orthopodomyia pulchripalpis \((n = 1)\) and Uranotaenia unguiculata \((n = 1)\).

Among the collected larvae, 415 larvae of *Cx. pipiens* were molecularly typed using CQ11, ace-2 and cytb PCR at the form level. Furthermore, approximately 574 larvae were raised to obtain adults to determine their expression of autogeny.

### Occurrence and distribution of *Cx. pipiens* forms

Amplification of the CQ11 microsatellite showed different frequencies of the *Cx. pipiens* forms in all 22 sites (Fig. 1). Of the 415 larvae that were analyzed, 139 (33.50%) specimens were *pipiens* form, 128 (30.84%) were *molestus* form, and 148 (35.66%) were hybrids (Additional file 1: Table S1). A statistical analysis (using GLM with Poisson distribution) showed no significant differences in the frequencies of forms according to bioclimatic zones (Additional file 2: Table S2). Of the 22 sites, 19 (86.36%) were characterized by a sympatric presence of the two *Cx. pipiens* forms with their hybrids; two sites [ID21 and ID22; 2/22 (9.1%)] shared *pipiens* form and hybrids, and one site [ID3; 1/22 (4.55%)] shared *molestus* form and hybrids. No pure sites (only *pipiens* or *molestus*) were observed.

Regarding habitat type (Fig. 2a), statistical analysis showed that the frequency of *Cx. pipiens* f. *pipiens* was significantly higher in rural locations than in urban locations; that *Cx. pipiens* f. *molestus* was significantly more abundant in urban areas than in rural areas and that the frequency of hybrids was significantly higher in urban sites than in rural sites (see Additional file 3: Table S3).

### Sequencing and genetic analyses

To clarify the taxonomic status of the *Cx. pipiens* forms determined by PCR, we sequenced 12 randomly chosen amplicons obtained by targeting CQ11, ace-2 and cytb genes. The results allowed us to compare three available molecular methods to distinguish the *Cx. pipiens* forms.

#### CQ11 microsatellite variability

Eight PCR products of *pipiens* \((n = 4)\) and *molestus* \((n = 4)\) forms were sequenced (GenBank: KY744215–KY744222). A BLAST analysis of these sequences confirmed the results of the PCR but revealed some variability among available sequences in GenBank. The four sequences of *pipiens* form (GenBank: KY744215–KY744218) showed significant similarity (98–99%) with sequences of *Cx. p. molestus* described in the UK (Table 2).
Ace-2 gene variability
The DNA of larvae samples including those previously sequenced for CQ11 locus (n = 4 pipiens; n = 4 molestus) and hybrid samples (n = 4), were amplified and sequenced targeting the ace-2 gene (714 bp) (GenBank: KY744203–KY744214). A BLAST analysis of these sequences (n = 12) showed a 99–100% similarity with a sequence of Cx. pipiens previously described in the USA (AY196910.1) [32].

Multiple alignments of our sequences (n = 12) showed that variable sites were mainly in intron 2 (non-coding region from 118 bp to 477 bp), which is characterized by a higher mutation rate [33].

Cytb gene variability
The same DNA samples (n = 12) previously sequenced for the nuclear gene (ace-2) were amplified and sequenced for the mitochondrial gene (cytb) (GenBank: KY744191–KY744202).

Following the BLAST analysis, 4 of the 12 analyzed DNA sequences were 100% identical to the sequence of Cx. p. pipiens from Turkey and shared a 99% similarity with Cx. p. pipiens previously described in Tunisia (Table 2). The remaining 8 sequences were 100% similar to the sequence of Cx. p. pipiens from Tunisia available on GenBank.

Multiple alignments of sequences showed no variability among Cx. pipiens forms as identified by the CQ11 microsatellite.

Autogeny
To determine the autogenic expression of the field-collected mosquitoes, adults (males and females) from six breeding sites (ID3, 4, 6, 9, 11 and 13) were reared in six cages in the insectary. Females that produced fertile eggs without access to a blood meal were considered autogenous. The results of this test are represented in Fig. 3 and Additional file 4: Table S4. Statistical analysis shows that the highest proportion of autogenous mosquitoes were found in belowground breeding sites (Fig. 3a; Additional file 5: Table S5) and in urban habitats (Fig. 3b; Additional file 5: Table S5).

In a second test, we evaluated the Cx. pipiens form versus autogeny in two types of breeding sites (ID8: aboveground; ID11: belowground) by placing couples from each site in cups and following them for 30 days for the presence of egg-rafts. These two sites were visited three times to replicate the test. From 117 tested couples (60 couples for ID8 and 57 for ID11), survived females (n = 90) were subsequently identified molecularly at the form level targeting the CQ11 microsatellite.

The CQ11 assay of autogenous females collected from ID8 (aboveground) showed that 50% (11/22) of the samples were Cx. p. molestus, 36.36% (8/22) hybrids, and 13.64% (3/41) were Cx. p. pipiens. From the belowground ID11 site, 52.78% (19/36) of the samples belonged to the molestus form, 44.44% (16/36) were hybrids and the remaining 2.78% (1/36) corresponded to the pipiens form (Additional file 6: Table S6; Fig. 4).

Anautogenous females from the ID8 site were 42.11% (8/19) hybrids, 31.58% (6/19) Cx. p. pipiens and 26.32% (5/19) Cx. p. molestus. From the ID11 site, 61.54% (8/13) of anautogenous females were the molestus form, 38.46% (5/13) hybrids and 0% were the pipiens form (Additional file 6: Table S6; Fig. 4).
| ID | CQ11 PCR | CQ11 sequences | ace-2 sequences | cytb sequences |
|----|----------|----------------|-----------------|----------------|
|    | Form     | GenBank ID     | Reference sequence | Similarity (%) | Form     | GenBank ID     | Reference sequence | Similarity (%) |
| 19 | P         | KY744215       | DQ470145.1       | 99             | Cx. pipiens | KY744203       | AY196910.1          | 99             |
| 13 | P         | KY744216       | DQ470145.1       | 99             | Cx. pipiens | KY744204       | AY196910.1          | 99             |
| 5  | P         | KY744217       | DQ470148.1       | 98             | Cx. pipiens | KY744205       | AY196910.1          | 100            |
| 16 | P         | KY744218       | DQ470142.1       | 99             | Cx. pipiens | KY744206       | AY196910.1          | 100            |
| 13 | M         | KY744219       | DQ470150.1       | 100            | Cx. pipiens | KY744207       | AY196910.1          | 99             |
| 15 | M         | KY744220       | DQ470150.1       | 100            | Cx. pipiens | KY744208       | AY196910.1          | 99             |
| 3  | M         | KY744221       | DQ470149.1       | 99             | Cx. pipiens | KY744209       | AY196910.1          | 100            |
| 5  | M         | KY744222       | DQ470149.1       | 99             | Cx. pipiens | KY744210       | AY196910.1          | 100            |
| 15 | H         | –              | –                | –              | Cx. pipiens | KY744211       | AY196910.1          | 99             |
| 21 | H         | –              | –                | –              | Cx. pipiens | KY744212       | AY196910.1          | 99             |
| 7  | H         | –              | –                | –              | Cx. pipiens | KY744213       | AY196910.1          | 99             |
| 13 | H         | –              | –                | –              | Cx. pipiens | KY744214       | AY196910.1          | 99             |

Abbreviations: P, Cx. pipiens f. pipiens; M, Cx. pipiens f. molestus; H, hybrid
*Details in Table 1
Statistical analyses showed that *Cx. p. molestus* was the most autogenous form in the two types of breeding sites (50% in ID8; 52.78% in ID11) and that autogeny was negatively related to the *p ipiens* form. Statistical analyses also demonstrated that differences between *Cx. p. molestus* and hybrids concerning the rate of autogeny in the aboveground and belowground site were not significant (Additional file 7: Table S7).

**Discussion**

Of the 1517 mosquito larvae collected from 22 breeding sites distributed in seven different climatic zones of Tunisia, *Culex pipiens* was the most abundant (65%). This mosquito species occurs throughout temperate latitudes and is involved in the transmission of West Nile virus in Tunisia [5, 34].

In this study, we investigated the physiological, ecological, and genetic characteristics of the *Cx. pipiens* populations that we collected. The screening of 415 *Cx. pipiens* larvae by CQ11 microsatellite showed the presence of two *Cx. pipiens* forms (*pipiens* and *molestus*) and their hybrids. All 22 breeding sites contained both *Cx. pipiens* forms and hybrids with varying frequencies. A previous study in Tunisia has already identified *pipiens* and *molestus* and their hybrids occurring in sympatry in different aboveground collection sites, but found no *pipiens* form in belowground sites [16]. Previous studies had shown that the different forms of *Cx. pipiens* were separated primarily on the basis of their ecological and physiological characteristics and that they occupied distinct habitats [35–38]. By contrast, our results showed the co-occurrence of both *Cx. pipiens* forms and their hybrids in different breeding sites, matching other studies conducted in Algeria [39, 40], Morocco [5], several European countries, i.e. Portugal [21, 41], the Netherlands [22] and Italy [23], and in the USA [20]. Whereas the *molestus* form was previously considered to be strictly anthropophilic and limited to belowground and confined breeding sites, we found that it can occur naturally in open and aboveground habitats. Similar observations were reported in other studies in Chicago and New York (USA) and in Algeria [39, 42].

The sympatric occurrence thus favors mating between the two forms and the emergence of hybrid populations. Indeed, hybrids were found in all breeding sites shared by the two parental forms. Interestingly, our results revealed that hybrids share the same ecological preferences of the *molestus* form, which may have increased the transmission of WNV to humans. The significant role played by hybrids in transmitting pathogens is well established; their opportunistic feeding behavior acts as a bridge vector for WNV transmission between birds and humans [4, 19, 20, 43, 44].

These findings confirm that *Cx. pipiens* forms can share the same site regardless of breeding site or habitat, without competitive exclusion. They also point to the
The adaptive capacity of *Cx. pipiens* forms to various environments and support the species’ ecological and physiological adaptability to urbanization [4, 45]. Man-made artificial habitats including canals, storage lakes, swimming pools, gardens and stormwater drainage systems, act as new breeding sites that primarily favor *Cx. pipiens*. Changes in climate may also influence mosquito morphology and ecology. Rises in temperature are known to influence adult flight activity, the digestion of blood meals, and egg development [46, 47]. Indeed, exposure to high temperatures can cause genetic mutations such as DNA methylation, which seems to play a role in facilitating plasticity in response to environmental stress [48, 49].

Insofar as the CQ11 microsatellite may overestimate the rate of hybrids when compared with full microsatellite analysis [42], we chose to compare the CQ11 amplification and sequencing results with the ace-2 and cytb genes to evaluate their utility for discriminating *Cx. p. pipiens* forms.

The sequencing of the CQ11 PCR product confirmed the presence of the *pipiens* and *molestus* forms in the sites studied, and confirms the results of other, similar studies. It constitutes a valuable tool for characterizing the *Cx. p. pipiens* forms in Tunisia and remains the most appropriate tool of confirmation, especially given the evolved ecological differences.

The amplification and sequencing of the PCR products targeting the ace-2 and cytb did not show any specific differences in sequences and did not allow the recognition of the different forms. Even though, when comparing two available sequences of ace-2 gene in GenBank [from Iran (*pipiens*) and from Japan (*molestus*)], the result did show differences in two nucleotide positions (Additional file 8: Table S8). In fact, our results showed that the two forms of *Cx. p. pipiens* are genetically too close to permit their discrimination using a nuclear (ace-2) [32] or mitochondrial (cytb) genes. Indeed, previous research comparing different mitochondrial genes (cox1, nad4 and 12S) confirmed their limited utility for the intraspecific differentiation of *Cx. p. pipiens* [50]. Thus, to date the molecular analyses seeking to differentiate the forms of *Cx. p. pipiens* indicate that the CQ11 locus remains the most promising diagnostic marker [21, 41] as it makes it possible to differentiate the two forms of *Cx. p. pipiens* and their hybrids.

This study shows the simultaneous occurrence of the two forms of *Cx. p. pipiens* with their hybrids in the same breeding sites. It is still necessary to determine whether they are also autogenous, a character always related to the *molestus* form that occur in urban belowground sites [13]. Our results demonstrated that autogeny was expressed in the collected females from above- and belowground sites, but that it was significantly higher in the latter. This could be due to the fact that subterranean mosquitoes adapt to habitats where potential blood meals are scarce by developing autogeny [51]. This suggests that *Cx. p. pipiens* has a capacity to adapt to the absence of nutrition by carrying over reserves from the larval stage to produce eggs. In aboveground sites, the low percentage of autogeny in tested females corroborated studies conducted in North Africa [16, 52], East Asia [53] and Portugal [54].

Autogeny was expressed more in urban than in rural habitats, suggesting that environmental factors such as limited access to a breeding site, larval nutrition and photoperiod, would affect it. Its expression may also be influenced by the non-availability of hosts for a blood meal and limited space for mating [35]. This high expression of autogeny may be related to the high proportion of *molestus* form observed in this habitat, which supports previous studies conducted in Australia and Italy [23, 51]. Our findings also demonstrate that a low proportion of *pipiens* form can also lay eggs without blood meals, a rare observation that corroborates a study in Portugal [21] and further confirms the ecological and physiological flexibility of the *Cx. p. pipiens* mosquito. We also observed that some *molestus* females can be anautogenous. Poor adaptation to insectary conditions may cause gonotrophic dissociation, which could explain the absence of oviposition in families that might otherwise be autogenous [21].

**Conclusions**

Our study shows the ubiquitous distribution of *Cx. p. pipiens* in Tunisia and provides evidence for the sympatric occurrence of *Cx. p. molestus*, *Cx. p. pipiens* and their hybrids. We also demonstrated the great plasticity of this complex of mosquitoes to a wide range of ecological conditions. The results suggest that the behavioral traits assigned to the forms of *Cx. p. pipiens* seem to be more flexible than previously assumed, especially the dispersion of *molestus* and hybrids forms. Our observations also highlight the abundance of autogeny, which is expressed in *molestus* and hybrids in belowground and aboveground sites. Our analysis proved that CQ11 microsatellite continues to be an appropriate molecular tool for the identification of the *Cx. p. pipiens* forms and their hybrids. However, further studies are needed to develop additional molecular markers given the genetic complexity of *Cx. p. pipiens* and the limitation of the use of a single molecular marker.

**Additional files**

A **Table S1.** Frequencies of *Cx. p. pipiens* forms determined by PCR targeting the CQ11 microsatellite. (PDF 89 kb)

A **Table S2.** Relationship between the bioclimatic region and the proportion of *Cx. p. pipiens* forms based on a Generalized Linear Model (GLM) with Poisson distribution. (PDF 98 kb)
Additional file 3: Table S3. Relationship between the different types of habitat and breeding site and the proportion of Cx. pipiens based on a Generalized Linear Model (GLM) with Poisson distribution. (PDF 98 kb)

Additional file 4: Table S4. Autogenic expression in field female mosquitoes. (PDF 168 kb)

Additional file 5: Table S5. Results of the relationship between habitat type and breeding site type and the percentage of autogeny of Cx. pipiens mosquitoes, based on a Generalized Linear Model with Poisson distribution. (PDF 88 kb)

Additional file 6: Table S6. Autogeny according to Cx. pipiens form. (PDF 89 kb)

Additional file 7: Table S7. Results of the relationship between percentage of autogeny of Cx. pipiens forms and the type of breeding site, based on a Generalized Linear Model with Poisson distribution. (PDF 313 kb)

Additional file 8: Table S8. Nucleotide variants in 714 bp of the ace-2 gene in Cx. pipiens (PDF 160 kb)

Abbreviations
Ace: Acetylcholinesterase; CTAB: Cetyltrimethylammonium bromide; cyt b: cytochrome b; Exo: Exonuclease I; GLM: Generalized linear model; ID: Identification number; RVFV: Rift Valley fever virus; SAP: Shrimp alcaline phosphatase; WNV: West Nile virus

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Availability of data and materials
Sequence data were deposited in the GenBank database under the accession numbers KY744191–KY744202 (cyt b gene); KY744203–KY744214 (ace-2 gene) and KY744215–KY744222 (CQ11 microsatellite).

Authors’ contributions
BM sampled mosquitoes, conducted the laboratory examination of samples and sequences analysis and submission, interpreted data and wrote the paper. RA sampled mosquitoes and was involved in the experience of autogeny. RD and sequences analysis and submission, interpreted data and wrote the paper. BA designed and supervised the study, revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study did not affect any endangered or protected species.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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