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RESEARCH

Diagnostics of *Xylella fastidiosa* vectors: how biometric traits in nymphs and adults play a role in identifying *Philaenus italosignus* and *Philaenus spumarius* in Tuscany (Italy)

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Abstract
The accurate identification of *Philaenus* Stål, 1964 species is essential for effective monitoring of *Xylella fastidiosa* Wells et al., 1987 (*Xf*) vectors in Europe. Although *Philaenus spumarius* (Linnaeus, 1753) is the primary vector of *X. fastidiosa* subsp. *pauca* ST53 in Apulia (Italy), the Mediterranean-restricted *Philaenus italosignus* Drosopoulos & Remane, 2000 also exhibits transmission capability, though its role in field epidemiology remains poorly defined. To date, discrimination between *P. italosignus* and *P. spumarius* has relied exclusively on adult male genitalia examination or molecular analyses. Here, we investigated biometric features of both adults and nymphs from sympatric populations in southern Tuscany to evaluate their potential use as practical diagnostic characters. Our study shows that adult female body length is clearly different between species: females of *P. italosignus* were consistently longer than 7.0 mm, while those of *P. spumarius* never exceeded this threshold. Nymphal head capsule width also differed significantly between species across instars. In fact, *P. italosignus* nymphs collected on *Asphodelus ramosus* Linnaeus, 1753 were consistently larger than those of *P. spumarius* from other host plants. The integration of size-based criteria into monitoring programs may enhance vector surveillance and support early measures against *Xf* spread in areas where the two species coexist.

Keywords Olive Quick Decline Syndrome · Spittlebugs · Xylem Sap-feeders · Quarantine Pest Surveillance

Introduction

The transmission of the xylem-inhabiting phytopathogenic bacterium *Xylella fastidiosa* Wells et al., 1987 (*Xf*), causal agent of Olive Quick Decline Syndrome, as well as other plant diseases, is mediated by xylem sap–feeding insects (Redak et al. 2004). In Europe, three Aphrophoridae (Hemiptera) species – the meadow spittlebug (*Philaenus spumarius* Linnaeus, 1753), *Philaenus italosignus* Drosopoulos & Remane, 2000, and *Neophilaenus campestris* (Fallén, 1805) – are currently recognized as vectors of *Xf* (Cornara et al. 2017a; Cavalieri et al. 2018, 2019). Following the first record of *X. fastidiosa* subsp. *pauca* ST53 in Apulia (Italy) in 2013 (Saponari et al. 2013), epidemiological studies have demonstrated that *P. spumarius* is the primary vector of the bacterium, whereas the roles of *N. campestris* and *P. italosignus* appear limited within this pathosystem (Ben Moussa et al. 2016; Cornara et al. 2017b). Nevertheless, *P. italosignus* and *N. campestris* resulted abundant in other natural areas (Cornara et al. 2021), where their potential involvement in the dissemination of other *Xf* subspecies and genotypes remains largely unknown (Panzavolta et al. 2019; EPPO 2020; Cornara et al. 2021; Nencioni et al. 2024; Falsini et al. 2025).

The genus *Philaenus* Stål, 1964 comprises ten recognized species, traditionally arranged into two taxonomic groups: the '*spumarius*' and the '*signatus*' groups (Drosopoulos and Remane 2000; Remane and Drosopoulos 2001; Tishechkin 2013). Among them, *P. spumarius* is the most widespread

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species, occurring across the Holarctic region and its host range is arguably the widest among phytophagous insects as it is confirmed to develop on more than 1,300 plants (Thompson et al. 2023). In contrast, members of the *'signatus'* group, including *P. italosignus*, have a localized distribution range and show a marked host-plant specialization during the nymphal stage, with the majority of them exclusively feeding on the lily *Asphodelus ramosus* Linnaeus, 1753 (Drosopoulos 2003; Maryańska-Nadachowska et al. 2010, 2012).

In Italy, the genus *Philaenus* includes the widespread *P. spumarius*, *P. italosignus*, and *Philaenus philopotamos* Bückle & Guglielmino, 2025. *Philaenus italosignus* is distributed mainly across Central and Southern Italy and locally associated with patches of *A. ramosus*, although detailed information on its chorology remaining scarce (Drosopoulos and Remane 2000; Guglielmino et al. 2005; Maryańska-Nadachowska et al. 2008, 2010, 2012; Panzavolta et al. 2019; Bückle and Guglielmino 2025). More recently, *P. philopotamos* was described from northeastern Italy, especially riparian alpine habitats, with juvenile stages developing on *Myricaria germanica* (L.) Desv. 1825 (Bückle and Guglielmino 2025).

European *Xf* vectors, which differ in geographical range and ecological preferences, may coexist within Mediterranean ecosystems, complicating surveillance efforts to discriminate among species in field collections (Holzinger et al. 2003; Maryańska-Nadachowska et al. 2010; Villa et al. 2020; EPPO 2020; Cornara et al. 2021; Gallego et al. 2023). Species discrimination has traditionally relied on anatomical characters such as fore-wing venation, frons plate, hind tibia, and male genitalia. However, the high degree of dorsal colour polymorphism in *Philaenus* further hampers morphological identification: all six colour morphs reported for *P. italosignus* also occur in *P. spumarius* (Drosopoulos and Remane 2000; Lahbib et al. 2022). In practice, while adult male genitalia examination is a reliable method for separating these two species, adult females and immature stages, based on available literature, can be distinguished only using molecular analysis (EPPO 2020; Rizzo et al. 2022).

In entomological diagnostics, the integration of morphological, molecular, and ecological approaches has become increasingly important to strengthen monitoring strategies for insect vectors. Molecular assays undoubtedly offer the highest accuracy for taxonomic identification, but their routine use in large-scale surveys is limited by cost, laboratory requirements, and processing time. Moreover, specific molecular tests can fail to detect meaningful changes in specimen, due to limitations in the test's sensitivity, pre-analytical errors, the presence of previously unknown DNA variations possibly due to incomplete population structure analysis, or an insufficient amount of the target mutation in the sample (Junqueira et al. 2024). This limitation is exemplified by the End-Point PCR protocol targeting the COI gene described in PM7/141(1) (EPPO 2020), which is reported as specific for the *P. spumarius*/*P. italosignus*/*P. tesselatus* Melichar, 1899 complex but does not discriminate among the three species (Lantero et al. 2018; EPPO 2020).

Conversely, morphological identification is fast and accessible but often constrained by colour polymorphism, intraspecific variability, and the need of high taxonomic skills by operators. Biometric traits such as body length and head capsule width represent a promising middle ground: they are straightforward to measure, reproducible across observers, and can be assessed in both laboratory and field settings without dissecting specimens. Moreover, when combined with ecological information, such as host plant specialization and biological cycle traits, biometric characters have the potential to greatly enhance diagnostic reliability.

Despite both this promise and the confirmed status of *P. italosignus* as a potential vector of *Xf* (Cavalieri et al. 2018), no detailed information is currently available on the external morphology of this species. Although previous studies have noted that '*signatus*' group species tend to be larger than *P. spumarius* (Drosopoulos and Remane 2000; Maryańska-Nadachowska et al. 2010; EPPO 2020), body size and other biometric traits have never been systematically explored as practical characters for species identification, neither for adult nor nymphal specimens. More specifically, data concerning *P. italosignus* adults are exceedingly limited, based on a paucity of specimens, and no corresponding information is available for the nymphal stages. Thus, establishing clear morphometric thresholds for *P. italosignus* and *P. spumarius* not only addresses a taxonomic gap for Italian spittlebugs, but also provides a practical tool that could be readily implemented in national phytosanitary surveillance protocols. Specifically, in areas where populations of the less common *P. italosignus* occur together with those of *P. spumarius*, having additional morphological criteria could significantly enhance the efficiency of vector monitoring programs for *Xf*. A better understanding of biometric variation in adults and immature stages may therefore provide valuable, non-destructive tools to support species discrimination and improve surveillance strategies.

Materials and methods

Study areas and sampling

Two sites in southern Tuscany (Italy), where both *P. spumarius* and *P. italosignus* are known to be present, were chosen for the study: the Maremma Park (42.67003° N, 11.08671° E) and Ansedonia (42.41657° N, 11.29905° E), respectively in the municipalities of Grosseto and Orbetello. These sites are particularly of interest since they are located in a coastal

area relatively close to Monte Argentario, southern Tuscany, where *X. fastidiosa* subsp. *multiplex* ST87 was also reported in 2018 (Marchi et al. 2018; Saponari et al. 2019). The vegetation in both areas is constituted by olive, *Olea europaea* L., 1753 and holm oak trees, *Quercus ilex* L., 1753, as well as several herbaceous plants, including the exclusive host for *P. italosignus* nymphal stages, *A. ramosus*.

Adult specimens and nymphal stages of *Philaenus* spp. were collected during 2020–2022 in both sites selected for the study. Based on information from a previous study carried out in the same area (Panzavolta et al. 2019), adult specimens were collected on different host plant categories: tree crowns (*O. europaea* and *Q. ilex*), herbaceous vegetation, and *A. ramosus*. A sweep net was used to catch specimens on herbaceous vegetation and tree crowns, while a mouth aspirator was additionally used on *A. ramosus* leaves. Sampling was conducted from May to November of all years. Adult spittlebugs were preserved in ethanol, separated according to location, host plant category, and sampling date. Finally, they were transported to the laboratory for further analysis.

In the same sites, herbaceous host plants, including *A. ramosus*, were checked for nymphs from January until the end of preimaginal development of both species. While the lily *A. ramosus* was a necessary choice to ensure the inclusion of *P. italosignus* nymphs in the sample, the other host plants were selected randomly based on the frequency of spittlebugs on each sampling. Collected nymphs were kept separate according to location, host plant species, and sampling date. They were stored in ethanol until they could be processed in the laboratory. In addition, to diversify the adult sample, more specimens were collected on herbaceous vegetation with a sweep net, in three additional sites in Tuscany, where *A. ramosus* was absent: Capraia F.na (43.74359° N, 11.01035° E), in the Province of Florence, Florence (43.78295° N, 11.21662° E), and Sitorni, in the Province of Arezzo (43.50917° N, 11.86572° E).

Morphological and molecular identification of Philaenus specimens

All adult males were identified at the species level based on the shape and number of apical projections on the aedeagus, which are the key features to distinguish *P. spumarius* and *P. italosignus* (Drosopoulos and Remane 2000; Holzinger et al. 2003). DNA extraction was performed on all adult females, 10% of adult males, and 10% of the nymphs collected from the various host plants. All DNAs were initially screened using the End-Point PCR protocol targeting the COI gene described in the PM7/141(1) as specific for *P. spumarius*/*P. italosignus*/*P. tesselatus* (Lantero et al. 2018; EPPO 2020). Then, all DNAs from which an amplicon of the expected size (128 bp) was produced, were further characterized using the *P. italosignus* specific Real-Time PCR protocol targeting the EF-1α gene (Rizzo et al. 2022). All specimens that tested positive for the former assay but not to the latter, were presumptively assigned to *P. spumarius*/*P. tesselatus*.

Adult and nymph measurements

Total body length was recorded for every adult specimen using an eyepiece micrometre mounted on a stereo microscope, with a resolution of 0.05 mm. Each insect was oriented ventrally (with the underside facing upward) to ensure consistent measurements. The micrometre scale was aligned with the frontal margin of the head and the posterior margin of the wings. Sex was also recorded for each specimen by external morphology. In adult *Philaenus* spittlebugs, the terminal abdominal segments offer reliable characters for sex identification and can be examined quickly under a stereomicroscope or even with a hand lens. In females, the terminal sternites extend in a broad, rounded contour when viewed laterally, while in ventral view the last visible sternites form a gently tapering outline that frames the ovipositor. In contrast, males show a more angular termination of the abdomen, with the last tergites forming a short, truncate profile in lateral view. From a ventral perspective, the male terminalia appear compact, with the pygofer margins enclosing the anal tube. The anal tube itself is short, tubular, and projects slightly dorsally, providing a diagnostic feature when specimens are viewed from behind or in lateral aspect. These external differences, allow for a rapid and non-invasive separation of the sexes in both *P. spumarius* and *P. italosignus*, without the need for dissection.

The total width of the head capsule was recorded for every nymphal specimen by using an eyepiece micrometre mounted on a stereo microscope, with a resolution of 0.025 mm. The micrometre scale was aligned with the exterior margin of the right eye to the exterior margin of the left eye. We assigned each specimen to one of the five nymphal instars (I–V) using established morphological criteria for *Philaenus* species detailed in the literature (e.g. Weaver and King 1954; Yurtsever 2000). Considering that specific morphological data for *P. italosignus* instars are currently lacking and given the close systematic relationship between *P. italosignus* and *P. spumarius*, we hypothesized that the same morphological features could be reliably used to distinguish the five nymphal instars in both species. This primary assumption was validated by carefully observing the specimens collected from *A. ramosus* (which were later subject to

molecular analysis) to ensure the described morphological features held true for the *P. italosignus* nymphs.

Statistical analysis

Before statistical analysis, assumptions for parametric analyses were evaluated. For ANOVA models, normality of residuals and homogeneity of variances were assessed using Shapiro–Wilk and Levene tests, respectively. For linear discriminant analyses (LDA), normality of the discriminating variable within each species group and homogeneity of within-group variances were checked; the presence of extreme outliers was also inspected. As the assumptions were met, parametric analyses were applied. Differences in length between adults were compared using the Two-way analysis of variance (ANOVA) with sex, insect species, and their interaction as fixed factors. Estimated marginal means were used to compare species within sex, with Bonferroni adjustment for multiple comparisons. To assess whether body length could be used to discriminate between *P. italosignus* and *P. spumarius* adults, we performed LDA, with equal prior probabilities, separately for females and males, given the significant effect of sex on body size. In each analysis, species was treated as the grouping variable and body length as the discriminating variable. The performance of the discriminant functions was evaluated based on both the original classification and leave-one-out cross-validation. Variation in nymphal head capsule width was first analysed using a Two-way ANOVA with head width (mm) as the dependent variable, and instar and species as fixed factors, including their interaction. Estimated marginal means were used to compare species within each instar, with Bonferroni adjustment for multiple comparisons. To address the main biological question of species discrimination, we then performed LDA using head width as the predictor and species as the grouping variable. Because head width changes markedly across instars, discriminant analyses were conducted separately for each instar, and classification performance was assessed by leave-one-out cross-validation with equal prior probabilities. Finally, to exclude host plant effect on head width within the species associated with multiple host plants (*P. spumarius*), we fitted a Two-way ANOVA for this species with head width (mm) as the dependent variable, and instar, host plant, and their interaction as fixed effects. Nymphs from a host plant species were considered separately if all instars were found on that plant species; otherwise, specimens from more plant species were grouped. Statistical significance was set at $\alpha = 0.05$. All statistical analyses were performed using IBM SPSS (Statistical Package for the Social Sciences) Statistics (Version 29.0.2.0, Armonk, NY, USA).

Results

Adult measurements and DNA analyses

A total of 474 adults (290 in the Maremma Park, 62 in Ansedonia, 58 in Sitorni, 21 in Capraia F.na, and 43 in Florence) were collected, identified, and measured. This sample included 208 *P. spumarius* (75 males and 133 females) and 266 *P. italosignus* (174 males and 92 females), ideally representative of the Tuscan population. According to PCR screening using both a species-specific qPCR assay for *P. italosignus* and a conventional PCR targeting *Philaenus* spp., of the 225 females, 41% could be confidently identified as *P. italosignus*, meanwhile the remaining 59% as *P. spumarius*/*P. tesselatus*. The same PCR analyses when conducted on a subsample of 25 males — of which, 72% belonged to *P. italosignus* and 28% to *P. spumarius* based on genitalia examination —always confirmed the assigned taxonomic position (Fig. 1). We assigned all specimens not identified as *P. italosignus* to *P. spumarius*. This assignment was based on the absence, to our knowledge, of published records of *P. tesselatus* from Italy, together with our morphological examination of males, in which we never observed male genitalia consistent with *P. tesselatus*. The same issue did not arise when discriminating *P. italosignus* from the *P. spumarius*/*P. tesselatus* complex, since *P. italosignus* can be reliably separated using molecular approaches based on mitochondrial and nuclear markers (Maryańska-Nadachowska et al. 2008, 2010; Rizzo et al. 2022) as well as male genital morphology (EPPO 2020).

Philaenus italosignus adults were collected on *A. ramosus* (200 specimens), other herbaceous vegetation (48), *O. europaea* (15), and *Q. ilex* (3). *Philaenus spumarius* adults were collected on *A. ramosus* (34), other herbaceous vegetation (173), and *O. europaea* (1). Body length differed significantly between species and sexes, with a non-significant species × sex interaction (ANOVA, species: $F(1, 470) = 1211.912$, $p < 0.001$; sex: $F(1, 470) = 101.967$, $p < 0.001$; species × sex: $F(1, 470) = 1.975$, $p = 0.161$). Overall, *P. italosignus* was larger than *P. spumarius*, and females were larger than males in both species (Fig. 2a). Bonferroni-adjusted pairwise comparisons of estimated marginal means showed that *P. italosignus* was significantly larger than *P. spumarius* in both females (mean difference = 1.102 mm, $p < 0.001$) and males (mean difference = 1.017 mm, $p < 0.001$). Females were also significantly larger than males in both *P. italosignus* (mean difference = 0.350 mm, $p < 0.001$) and *P. spumarius* (mean difference = 0.265 mm, $p < 0.001$).

Discriminant analyses performed separately for females and males showed that body length provided strong

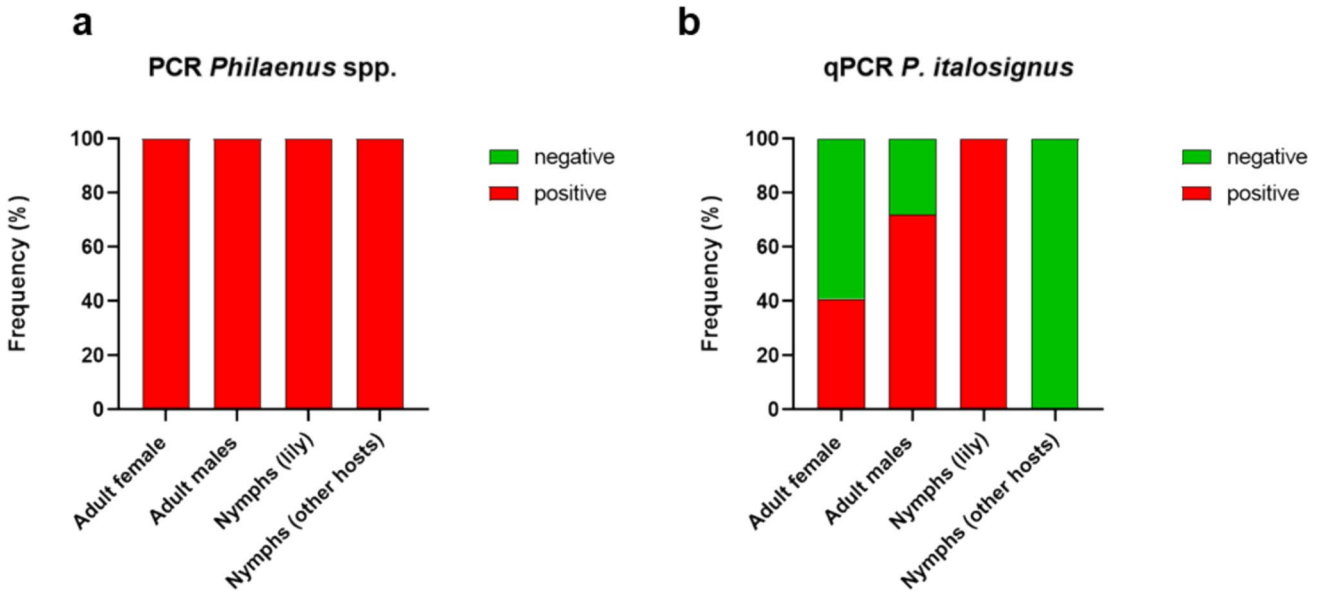

Fig. 1 Results of the molecular analyses performed on DNA extracted from all adult females and from a subset of adult males (10%) and nymphs (10%) collected from different host plants (*A. ramosus* and other plant hosts) in Tuscany (Italy). **a** Frequency of positive and negative specimens tested using the End-Point PCR protocol targeting the COI gene, provided by EPPO as specific for *P. spumarius, P. italosignus,* and *P. tesselatus* (Lantero et al. 2018; EPPO 2020). **b** Frequency of positive and negative samples tested using the *P. italosignus* specific Real-Time PCR protocol (qPCR) targeting the EF-1α gene (Rizzo et al. 2022)

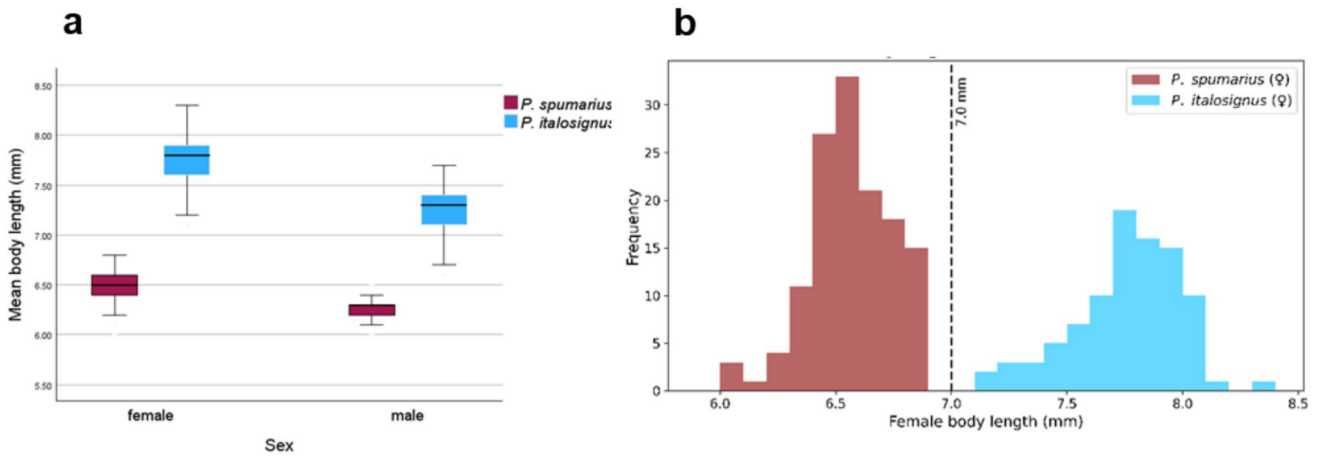

Fig. 2 **a** Mean body length of *Philaenus spumarius* and *P. italosignus* adults (both sexes). Bars represent standard error. **b** Female adult body length distribution with the 7.0 mm threshold (dashed line). All specimens were collected in Tuscany during the study

discrimination between the two species in both sexes (Table 1). In females, the discriminant function was highly significant and correctly classified 100% of individuals, both in the original and cross-validated analyses. In fact, *P. spumarius* females in our sample never reached 7.0 mm, while all *P. italosignus* females were longer than 7.0 mm (Fig. 2b). In males, the discriminant function was also highly significant and correctly classified 98.8% of individuals, with the same accuracy in cross-validation. While the majority of *P. spumarius* males never exceeded 6.5 mm in length, one male measured 6.7 mm, overlapping with the three smallest *P. italosignus* males (1.72% of their total), which ranged from 6.6 to 6.7 mm. Thus, only a very small proportion of adult males (1.61% of the total) could not be unambiguously identified on the basis of body length alone.

Nymph morphology and measurements

A total of 993 *Philaenus* nymphs (852 in the Maremma Park, 141 in Ansedonia) were collected from various host

Table 1 Size range and average size (±SD) for both sexes of *Philaenus spumarius* and *P. italosignus* collected in Tuscany during the study

Instar	Body Length						LDA results			
	P. spumarius			*P. italosignus*						
	Min (mm)	Max (mm)	Mean (±SD)	Min (mm)	Max (mm)	Mean (±SD)	Wilks' λ	χ^2	Classification accuracy (%)	***p***
Female	6.0	6.8	6.5 ± 0.18	7.1	8.3	7.7 ± 0.23	0.255	200.02	100	< 0.001
Male	5.9	6.7	6.4 ± 0.15	6.6	7.7	7.3 ± 0.22	0.162	449.42	98.8	< 0.001

plants in different years. These hosts were primarily the lily *A. ramosus* (526 nymphs), *Foeniculum vulgare* Mill., 1768 (171 nymphs), and *Medicago sativa* L., 1753 (18 nymphs). All five nymphal instars of *Philaenus* ended up being collected on each of these hosts. An additional set of host plants provided 278 specimens, but each additional host provided only some of the five nymphal instars. These hosts included mainly *Cistus monspeliensis* L., 1753 and six more plant genera (*Euphorbia* L., *Geranium* Tourn. ex L., *Hordeum* L., *Sonchus* L., *Scabiosa* L., and *Vicia* L.). Samples from these additional plants were grouped in a fourth category for the dataset regarding morphological observations.

To gather evidence regarding the taxonomic identity of the nymphs, 100 specimens collected from different plant-hosts were analysed using the two PCR assays. According to these analytical methods, all 46 specimens collected from *A. ramosus* tested positive in the qPCR assay for *P. italosignus,* meanwhile the remaining 54 specimens, all collected from host plants other than *A. ramosus*, tested negative in the qPCR assay but positive to *Philaenus* PCR (Fig. 1). Based on these findings, we generalize that the nymphs collected from *A. ramosus* in this study are attributable to *P. italosignus*, whereas those from *F. vulgare*, *M. sativa*, and other host plants are likely consistent with *P. spumarius.* This is supported by the observation of 194 callow adults on *A. ramosus* in spring, all identified as *P. italosignus,* and by the earlier development of this species compared with *P. spumarius.* Indeed, when the first *P. italosignus* adults emerged on *A. ramosus, P. spumarius* was still exclusively at the nymphal stage. Nymphs that were first positively identified as *P. italosignus* using species-specific PCR allowed for a direct comparison of their morphological characters across nymphal instars. These observations confirmed that *P. italosignus* nymphs share the same diagnostic morphological features described for *P. spumarius* in the works by Weaver and King (1954) and Yurtsever (2000). Crucially, our study confirms that coloration is an unreliable feature for instar differentiation, consistent with the two cited papers. Despite this unreliability in pigmentation, other specific morphological characters, like wing pads and legs, proved sufficient to easily and reliably distinguish the various immature instars (Fig. 3).

Head width differed significantly according to instar, species, and their interaction in the overall ANOVA model. Specifically, instar had a very strong effect on head size, $F(4, 983) = 17{,}595.44$, $p < 0.001$, partial $\eta^2 = 0.986$, and

Fig. 3 Nymphal instars I to V (from left to right) of *Philaenus italosignus*. Scale bars 0.5 mm

species also had a significant effect, $F(1, 983) = 2276.37$, $p < 0.001$, partial $\eta^2 = 0.698$. In addition, the instar × species interaction was significant, $F(4, 983) = 98.48$, $p < 0.001$, partial $\eta^2 = 0.286$, indicating that the magnitude of the species difference varied across developmental stages. Species differed significantly within each instar: instar I, $F(1, 983) = 70.23$, $p < 0.001$; instar II, $F(1, 983) = 166.14$, $p < 0.001$; instar III, $F(1, 983) = 453.52$, $p < 0.001$; instar IV, $F(1, 983) = 1132.72$, $p < 0.001$; instar V, $F(1, 983) = 1626.83$, $p < 0.001$. Specifically, nymphs collected from *A. ramosus* (*P. italosignus*) were found to have significantly larger head widths than those of *P. spumarius,* collected from the other three sets of host plants (Fig. 4). Within the nymphs collected from *F. vulgare*, *M. sativa*, and other host plants, head capsule size was further analysed as a function of instar, host plant, and the instar × host plant interaction. Instar remained highly significant, $F(4, 511) = 3741.94$, $p < 0.001$, whereas neither host plant, $F(2, 511) = 0.64$, $p = 0.53$, nor the instar × host plant interaction, $F(8, 511) = 1.31$, $p = 0.23$, were significant. These results indicate that, within the species associated with multiple host plants (*P. spumarius*), head capsule size varied strongly across development but did not show a consistent overall host-plant effect. In each instar, the discriminant function (LDA) was highly significant (Table 2), and classification success was high, particularly from instar II onward. In fact, instars II-V showed both a less evident range overlapping and lower frequency of overlapping measurements between the two *Philaenus* species (Fig. 5). Only the first instar showed an overlap closer to the median value of both species. The overlap visible in Fig. 5 between two consecutive nymphal instars (different colours) across species is irrelevant as each nymphal instar has different morphological features.

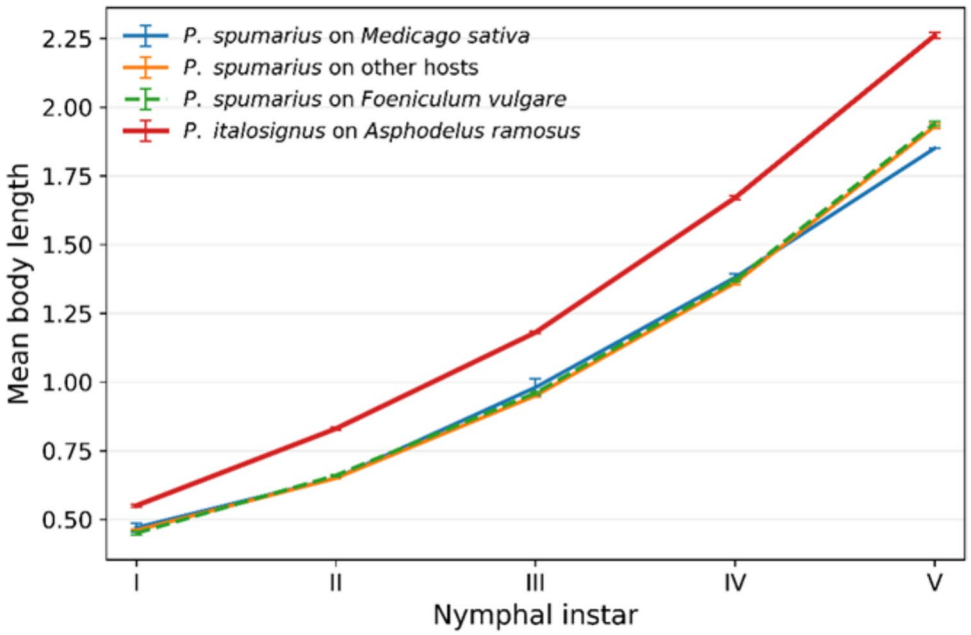

Fig. 4 Mean head capsule width of *Philaenus* nymphs collected in the Maremma Park and Ansedonia (Tuscany—Italy) during the three-year study. Bars represent standard error

Table 2 Head width ranges for each nymphal instar in *Philaenus spumarius* and *P. italosignus*, with the results of LDA analysis. *N* is referred to the number of specimens measured per each instar and each species

Instar	Head width range						LDA results			
	P. spumarius			*P. italosignus*						
	Min (mm)	Max (mm)	*N*	Min (mm)	Max (mm)	*N*	Wilks' λ	χ^2	Classification accuracy (%)	***p***
I	0.40	0.50	77	0.45	0.65	84	0.378	261.27	76.2	< 0.001
II	0.60	0.70	106	0.75	0.90	36	0.111	306.33	100.0	< 0.001
III	0.85	1.05	92	1.05	1.30	84	0.109	383.89	99.4	< 0.001
IV	1.25	1.50	125	1.45	1.85	104	0.132	458.14	97.4	< 0.001
V	1.75	2.10	126	2.00	2.55	159	0.298	341.89	95.1	< 0.001

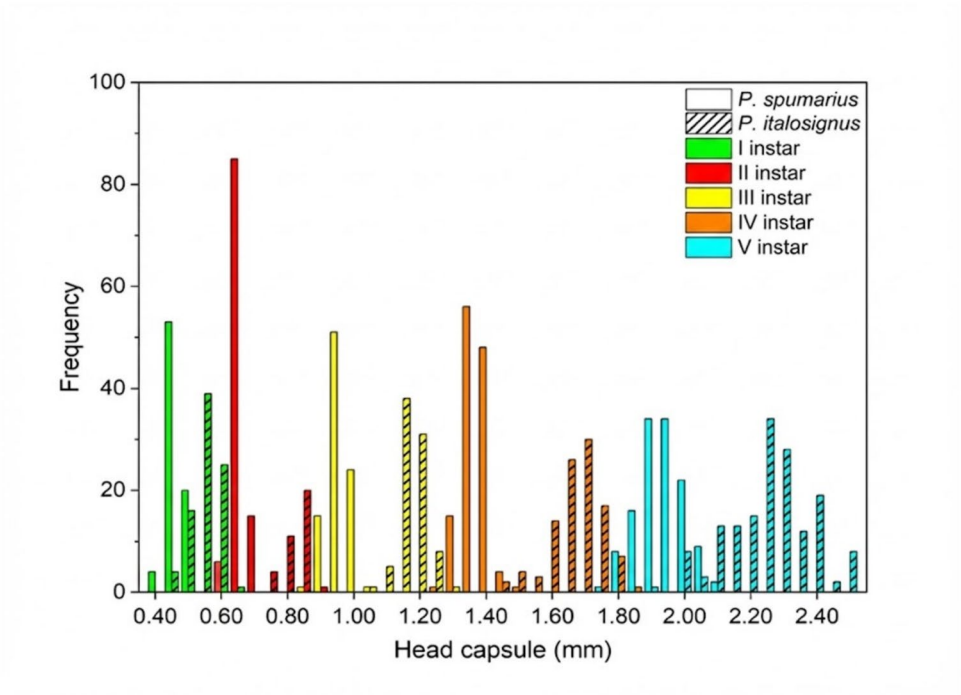

Fig. 5 Frequency of the head capsule size in the five nymphal instars of both *Philaenus* species collected in Maremma Park and Ansedonia (Tuscany—Italy) during the three-year study period

Discussion

This study brings strong evidence that adult body size may represent a useful morphological trait for supporting the diagnosis of *Xf* vectors. Specifically, a 7 mm length serves as a robust discriminatory benchmark for adult females: in our samples, individuals below this size were reliably identified as *P. spumarius*, whereas those exceeding it indicated *P. italosignus*. This diagnostic threshold is supported by existing literature; previous research across Europe and Lebanon consistently reports a maximum length of 6.88 mm for *P. spumarius* females (Drosopoulos and Asche 1991; Abdul-Nour and Lahoud 1995; Drosopoulos and Remane 2000; Thompson et al. 2023; Bückle and Guglielmino 2025). Although bibliographic data for *P. italosignus* are limited, available evidence (Drosopoulos and Remane 2000; Thompson et al. 2023; Bückle and Guglielmino 2025) also aligns with our observations, confirming that females of this species consistently exceed 7 mm, as seen in our Tuscan samples. Such collective evidence establishes size as a practical criterion for field-based identification; for instance, a simple digital caliper could be employed. Furthermore, these findings likely extend to the entire range of *P. italosignus*, though broader external validation remains necessary.

For adult males, the morphological distinction based on size presents a more nuanced situation. Our study suggests that male specimens measuring up to 6.5 mm can be reliably identified as *P. spumarius*, whereas those measuring 6.8 mm or larger are indicative of *P. italosignus*. However, a "grey area" in the 6.6–6.7 mm range was observed within our sample, encompassing a small percentage (1.61% of the total 474 spittlebugs sampled) of male specimens from both species. Existing literature further highlights this overlap; while *P. spumarius* males are generally smaller, some populations, specifically those from Lebanon, have been reported to reach lengths of up to 6.9 mm (Abdul-Nour and Lahoud 1995). Conversely, *P. italosignus* males, typically larger, have been recorded at sizes as small as 6.3 mm in other studies (Bückle and Guglielmino 2025). Considering both our data and the available bibliography, a clear size-based distinction for males is possible at the extremes: individuals measuring 7 mm or larger can be confidently identified as *P. italosignus*, and those under 6.3 mm are likely *P. spumarius*. In conclusion, given the high reliability of male genitalia identification, it is advisable, as suggested by other authors (Picciotti et al. 2021), to maintain this technique, particularly for males falling within the intermediate size range of 6.3 mm to 6.9 mm. However, in contrast to these authors (Picciotti et al. 2021), we found adult size a useful character for distinguishing between females of the two species, otherwise discernible only by molecular identification.

In contrast to the thoroughly described adult morphology, accurate descriptions of the nymphal stages of *P. italosignus* have been lacking in the literature to date. Given the close morphological similarity observed between *P. italosignus* and *P. spumarius* adults, it could be expected that their nymphal instars would also share similar features. However, no previous scientific work has definitively documented this information. This gap highlights the need for a detailed characterization of *P. italosignus* nymphs to fully discriminate

them from those of its closely related congener. Our results confirm that, like adults, nymphs of *P. italosignus* and *P. spumarius* share the same fundamental morphological characters across all immature stages, with one notable distinction: nymphs of all instars of *P. italosignus* are notably larger than those of *P. spumarius*, particularly regarding the head capsule width.

The limited overlap in the upper and lower dimensional limits across nymphal instars, and the high classification accuracy emerged from LDA, suggest that head width could be used to distinguish between *P. italosignus* and *P. spumarius*. In fact, while there is more overlap at the first and fifth instars, median values for each instar (and thus the most frequently recorded values) are distinct between the two species, offering an additional diagnostic tool for these two *Philaenus* spittlebugs based on morphology alone, particularly when the host plant is unknown. Furthermore, this morphological analysis should be integrated with phenological data, as *P. italosignus* nymphs were observed to emerge notably earlier than those of *P. spumarius* (unpublished data). Therefore, for *Philaenus* nymphs whose head width falls within the overlapping range, the collection date can be used as a supplementary reference to rule out the species less likely to be present at that specific nymphal instar. This integration of morphometrics and seasonal timing enhances the overall accuracy of nymphal identification.

Consistent with previous literature, *P. spumarius* exhibited polyphagy across developmental stages, while *P. italosignus* displayed strict juvenile monophagy on *A. ramosus*. Interestingly, while *P. spumarius* nymphs were abundant on diverse herbaceous hosts; they were absent from *A. ramosus,* as the qPCR analysis and callow adults confirmed. We may hypothesize that this segregation is driven by phenological mismatch; *A. ramosus* may decline in quality or availability before *P. spumarius* nymphs complete their development, unlike the earlier-developing *P. italosignus.* This could be true in the Tuscan environment during the study period; however, it cannot be excluded that in other years or in different geographical areas, for example, in southern Italian regions, *P. spumarius* nymphs could also be found on *A. ramosus*. In any case, this restriction does not apply to the adult stage, as *P. spumarius* adults were observed feeding on *A. ramosus*, during autumn, confirming it as a viable food source for the imago.

The relevance of our data, following further validation in new areas, may also be applied in other Mediterranean contexts. This would export our methodology outside of Italy for the systematic description and confirmation of the biometric traits of key '*signatus'* group members, such as *Philaenus signatus* Melichar, 1896, *Philaenus maghresignus* Drospoulos & Remane, 2000, and *Philaenus tarifa* Remane & Drosopoulos, 2001. The recently described species, *Philaenus elbursianus* Tishechkin, 2012 and *P. philopotamos*, seem less suitable candidates for this analysis, as they are reported to be smaller in size (Tishechkin 2013; Bückle and Guglielmino 2025).

Conclusions

In conclusion, our study contributes to the current knowledge of the putative *Xf* vector, *P. italosignus*, by integrating morphological and molecular data across all life stages. Based on the Tuscan specimens examined here, adult *P. italosignus* are consistently larger than *P. spumarius*, and females body size, particularly the 7 mm threshold, provide a useful preliminary screening criterion for distinguish the two species in this regional context. While adult males exhibit dimensional overlap, necessitating reliance on genitalia morphology or molecular analysis for reliable identification, the female size threshold provides an immediate diagnostic advantage. The 7 mm size threshold for adult *P. italosignus* females is particularly vital as it provides a first-level diagnostic tool, given that, unlike males, no clear alternative morphological features, such as genitalia, are available to distinguish them from *P. spumarius* other than costly and time-consuming molecular analysis.

We also provided the first morphological data on the nymphal instars of *P. italosignus*, confirming their overall similarity to *P. spumarius* across the five instars, while highlighting their significantly larger size. Although we found *P. italosignus* nymphs exclusively on *A. ramosus*, whereas *P. spumarius* nymphs were never observed on this plant, relying solely on host plant association to distinguish between the two species can be misleading. In fact, it cannot be ruled out that in southern Italian regions, *P. spumarius* might also exploit *A. ramosus* for immature development. Consequently, our method of identification based on head capsule size proves useful. This holds true despite an overlap between the two species in the first instar. In fact, since the median values for each instar remain clearly distinct, reliable differentiation at the population level is achievable through representative sampling.

The biometric thresholds proposed here have important implications for applied entomology and plant health protection. A tiered diagnostic framework could be envisaged, where preliminary size-based screening is performed on field-collected samples, while only ambiguous specimens are subjected to dissection (males) or molecular analysis. Furthermore, the integration of biometric data with ecological parameters such as host plant use and phenology could lead to the development of multifactorial identification keys, providing a flexible and accessible diagnostic system. Nevertheless, the applicability of these thresholds may be influenced by environmental and ecological factors, including host plant quality, local climatic conditions, altitude,

phenology, and population-level variation. Although published data support these findings, external validation was not performed in this study. Therefore, further testing across the few known additional Italian populations of *P. italosignus*, other members of the '*signatus*' group, and different Mediterranean environments is needed before these biometric criteria can be incorporated into broadly applicable identification keys or monitoring protocols.

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Declarations

Competing interests The authors declare no competing interests.

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